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CHROMBIO. 085

MASS FRAGMENTOGRAPHIC DETERMINATION OF VANILMANDELIC ACID, HOMOVANILLIC ACID AND ISOHOMOVANILLIC ACID IN HUMAN BODY FLUIDS

SHICHIRO TAKAHASHI, MASANORI YOSHIOKA, SHOHEI YOSHIUE and ZENZO TAMURA

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113 (Japan)

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SUMMARY

Vanilmandelic, homovanillic and isohomovanillic acids in body fluids were efficiently isolated by liquid chromatography on an Amberlite XAD-4 column and by organic extraction in a special apparatus. The purified metabolites were converted into their trifluoroacetylhexafluoroisopropanol esters and analyzed by mass fragmentography. The working curves of the metabolites were linear from 0.5 to 5 ng injected. The minimum detectable concentrations of all the metabolites were 2 ng/ml for plasma and cerebrospinal fluid, and 120 ng/ml for urine. The metabolite concentrations in plasma, cerebrospinal fluid and urine of normal persons and patients were determined.

INTRODUCTION

Vanilmandelic acid (VMA), homovanillic acid (HVA) and isohomovanillic acid (iso-HVA) are the main metabolic end-products of catecholamines. Determination of these metabolites in human body fluids is essential to the diagnosis of, for example, neuroblastoma, phaeochromocytoma and Parkinson's disease.

The quantitative analyses of the metabolites in body fluids are usually carried out by fluorimetry [1], thin-layer chromatography [2, 3], gas chromatography (GC) [4—9] and high-speed liquid chromatography (HSLC) [10—12]. However, they are susceptible to interference of many other constituents in human body fluids, and a highly selective and sensitive analysis is desired. Recently, Sjöquist et al. reported a determination of VMA [13] or HVA [14] in human body fluids by mass fragmentography (MF). Karoum et al. [15] also determined VMA and HVA in spinal fluids and brain tissues by MF.

In previous work, we reported briefly a determination method of the metab-

olites by GC [16]. In this paper, we describe the development of a simultaneous determination method for VMA, HVA and iso-HVA in body fluids by MF.

EXPERIMENTAL

Materials

VMA, HVA, trifluoroacetic anhydride (TFAA) and hexafluoroisopropanol (HFIP) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Iso-HVA was kindly supplied by Dr. I. Kuruma of Nippon Roche (Tokyo, Japan). 3-Methoxy-4-hydroxyphenylethanol (HMPE), obtained from Aldrich (Milwaukee, Wis., U.S.A.), was dissolved in dioxane and stored at −20° until used as an internal standard. *n*-Hexane for absorption spectrometry was obtained from Wako Junyaku Kogyo (Tokyo, Japan). Bovine albumin fraction V and rabbit γ-globulins fraction II were obtained from Sigma (St. Louis, Mo., U.S.A.) and Miles Labs., (Kankakee, Ill., U.S.A.), respectively. Ethyl acetate and 6 *M* hydrochloric acid were used after distillation. All the other chemicals used were of reagent grade purity.

Samples

Venous blood was added with heparin (Novo, Copenhagen, Denmark) and centrifuged to obtain plasma. Three ml of plasma or cerebrospinal fluid (CSF), which was acidified with 180 μl of 6 *M* hydrochloric acid and stored at −20°, was added with 1.6 μg of HMPE.

Urine was collected in a polyethylene bottle containing about 30 ml of 6 *M* hydrochloric acid during 24 h and stored at −20°. Seventy-eight μg of HMPE, 2 ml of water and 1.5 g of sodium chloride were added to 3 ml of the urine to make a sample solution.

Preparation of Amberlite XAD-4 column

Amberlite XAD-4 (Rohm and Haas, Philadelphia, Pa., U.S.A.) was ground by a rotary grinder in water and the particles of 60—150 mesh were collected and further fractionated by sieves in water. The particles were washed with water, 1 *M* sodium hydroxide, water, 1 *M* hydrochloric acid, water, methanol acetone, *n*-hexane, acetone, methanol and water, successively. The adsorbent was stored in water together with sodium azide until used. The particles were transferred with water into a glass tube of 0.45 cm (I.D.) to make columns of various heights.

Clean-up and derivatization of the metabolites in samples

Three ml of the sample solution from plasma or CSF were poured onto Amberlite XAD-4 columns of 6 or 10 cm height and different meshes. Each column was washed with 15 ml of 0.1 *M* formic acid and eluted with 5 ml of 50% methanol and 5 ml of 100% methanol. The two eluates were mixed and concentrated to dryness in vacuo. The residue was dissolved in 5 ml of water, and 50 μl of 6 *M* hydrochloric acid and 1.5 g of sodium chloride were added.

The solution thus obtained or the sample solution from urine was extracted

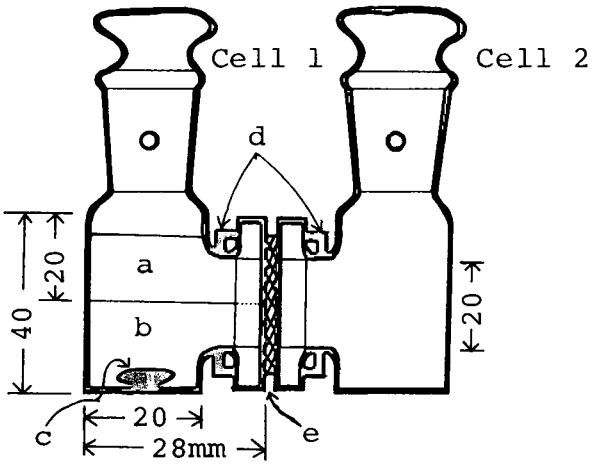

Fig.1. Solvent extraction apparatus. A PTFE membrane filter (extra coarse, 30—60 μm, Chemplast, Wayne, N.J., U.S.A.) was clamped between the twin glass cells. Cell 2 was stoppered, and 5 ml of the aqueous solution and 3 ml of ethyl acetate were poured into cell 1. Then cell 1 was stoppered and stirred for 5 min. By turning off the stoppers, the organic phase penetrated through the filter into cell 2. a = Ethyl acetate phase; b = aqueous phase; c = magnetic stirring rod; d = clamp; e = PTFE filter.

twice with 3 ml of ethyl acetate with an apparatus* shown in Fig. 1. All the volume for plasma and CSF or, for urine, 100 μl of the organic phase was collected into a 10-ml test tube (13 × 1.3 cm I.D.) with a glass stopper and evaporated in vacuo. Next, the residue was dried over phosphorus pentoxide in vacuo for 15 min.

To the dried residue 300 μl of TFAA and 150 μl of HFIP were added. The tube was stoppered and heated in an oil-bath at 75° for 1 h. The tube was kept in ice and the excess of the reagents were removed under a stream of dried nitrogen with a moisture-protecting attachment. Two hundred μl of *n*-hexane were added immediately to the residue, and 1 or 2 μl of the solution was injected into a gas chromatograph [16] or a gas chromatograph—mass spectrometer.

Mass fragmentography

A gas chromatograph—mass spectrometer Hitachi Model-52 fitted with a multiple-ion detection system was used at the Central Research Laboratory, Mitsubishi Chemical Industry Co. (Kanagawa-ken, Japan) under the following conditions. A 1 m × 3 mm (I.D.) glass tube was packed with 2% OV-1 on Chromosorb W (AW-DMCS). The column inlet pressure of the carrier gas (helium) was 1.0 kg/cm^2 (40 ml/min). The temperatures of column, column injector and ion source were 120°, 200° and 200°, respectively. The ionizing potential was 20eV.

RESULTS

As shown in Table I, a column of Amberlite XAD-4, 10 cm high and 80—

*The apparatus was a modification of that of T. Igarashi and S. Tamura [17].

TABLE I

THE EFFECTS OF THE COLUMN HEIGHT AND THE PARTICLE SIZE OF AMBERLITE XAD-4 ON THE RECOVERY PERCENTAGE OF THE METABOLITES BY GC

Metabolite	60—80 mesh		80—115 mesh		115—150 mesh
	6 cm	10 cm	6 cm	10 cm	10 cm
VMA in water	70	100	99	102	—
HVA in water	98	96	100	98	—
iso-HVA in water	97	103	105	100	—
VMA in plasma	44	80	70	103	96
HVA in plasma	107	106	96	105	103
iso-HVA in plasma	103	105	100	98	105

150 mesh, was found to be suitable for purification of protein-containing body fluids such as plasma. By the use of the column, even VMA, the weakest adsorbing metabolite, was quantitatively adsorbed. More than 95% of proteins were removed by washing the column with 0.1 *M* formic acid as shown in Fig. 2; otherwise the proteins would disturb the next extraction step with an organic solvent by emulsification. As shown in Fig. 2 and Table I, the elution of the metabolistes and the internal standard from the column was completed with 50 and 100% methanol. On the other hand, the ordinary deproteinization with perchloric acid or acetone gave less recovery of the metabolites. For urine, which contained the metabolites at higher concentrations and no protein, the chromatographic step was unnecessary (Fig. 2 and Table I).

The next step, extraction with ethyl acetate of the acidified and salted sample solution [6], was effective in removing from the samples traces of proteins, electrolytes, uric acid and urea, which would disturb the GC of the metabolites. As an example, 5 ml solution of 1.5 g sodium chloride in 0.06 *M*

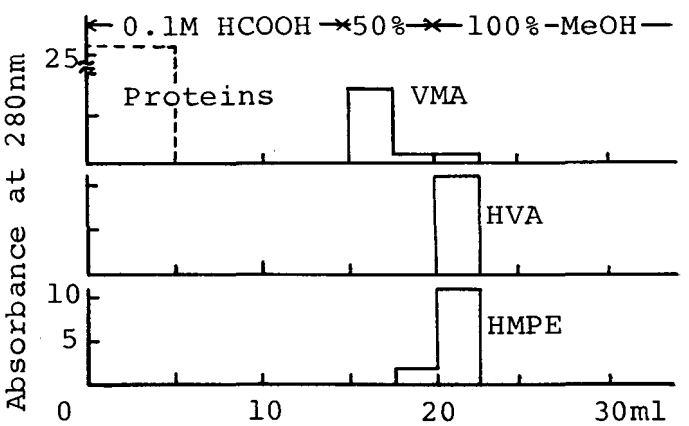

Fig.2. Elution profile of VMA, HVA, HMPE and proteins from Amberlite XAD-4. Three ml of 3.5 m*M* aqueous solutions of the metabolites or 3 ml solution of 180 mg of albumin and 30 mg of γ-globulin in 0.01 *M* phosphate—0.15 *M* sodium chloride (pH 7.4) were applied to the XAD-4 column (10 × 0.45 cm I.D.).

hydrochloric acid (45.8 mS/cm) was extracted with 3 ml of ethyl acetate twice, the organic phase was evaporated to dryness and the residue was dissolved in 5 ml of water. The conductivity was much reduced to 0.54 μS/cm. In the case of a urine of 6.3 mS/cm, treated similarly, the conductivity of the final solution became 50 μS/cm. The absorbance at 280 nm of the residue dissolved in 5 ml of water was 0.012 when 5 ml of a solution saturated with uric acid of 0.531 absorbance was extracted. Further, 0.5 *M* urea in water at the normal urinary concentration was also removed by the extraction, as judged from the disappearance of the GC peak of the derivative, which would overlap with the VMA peak.

On the other hand, *p*-hydroxyphenylacetic acid and hippuric acid in urine were not removed, and they interfered with the following derivatization. The interference was neglected when 100 μl of the organic phase were analyzed. The recoveries of 8 μg each of VMA, HVA and iso-HVA added to the sample solution of normal urine were within 95—105% by MF.

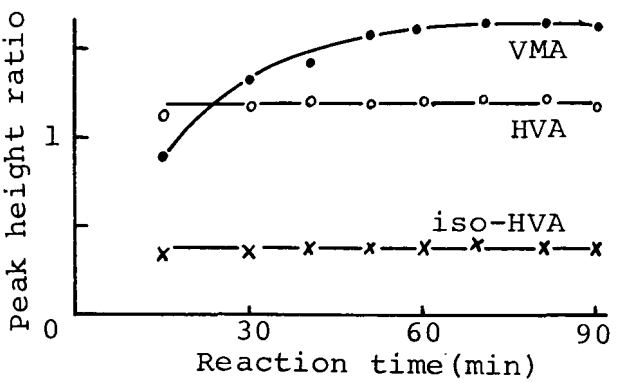

Fig.3. Rate of formation of the trifluoroacetyl-hexafluoroisopropanol ester derivatives of the metabolites. A mixture of 10 μg of VMA, 7.5 μg of HVA, 17 μg of iso-HVA and 78 μg of HMPE, dried over phosphorus pentoxide, was reacted with the reagents under the conditions described in the text then analyzed by GC [16].

An hour was enough for the derivatization, as shown in Fig. 3. The derivatized metabolites were identified by their mass spectra as shown in Fig. 4. The major ions of 345, 428, 428 and 360 *m/e*, respectively from the spectra of VMA, HVA, iso-HVA and HMPE derivatives were used for the MF.

The MF patterns of the metabolites from the authentic mixture and normal plasma are shown in Fig. 5. The working curves for the metabolites obtained by the mass fragmentographs were linear from 0.5 to 5 ng injected as shown in Fig. 6. The minimum detectable concentrations of all the metabolites were 2 ng/ml for plasma and CSF, and 120 ng/ml for urine. In ordinary body fluids, the content of endogenous HMPE was negligible compared with the amount of the internal standard, so that the correction was unnecessary. The contents of the metabolites in body fluids were measured and are summarized in Table II.

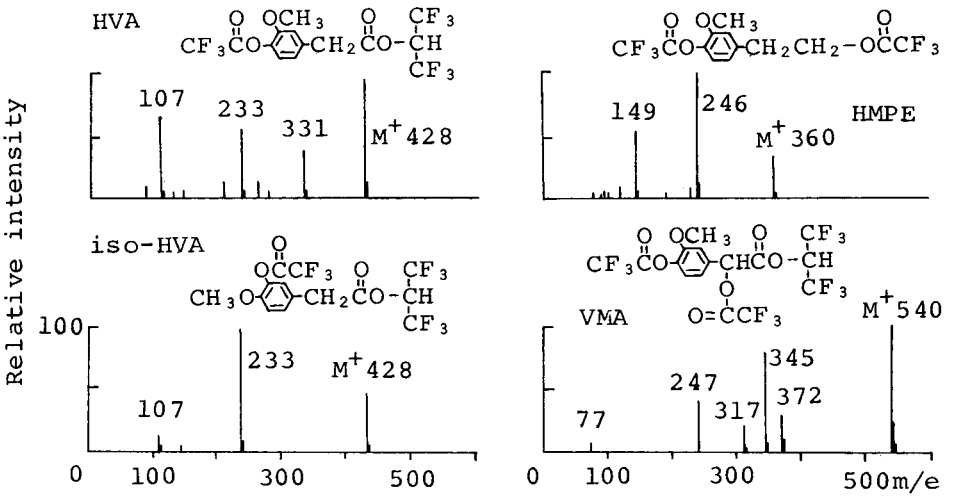

Fig.4. Mass spectra of the derivatives.

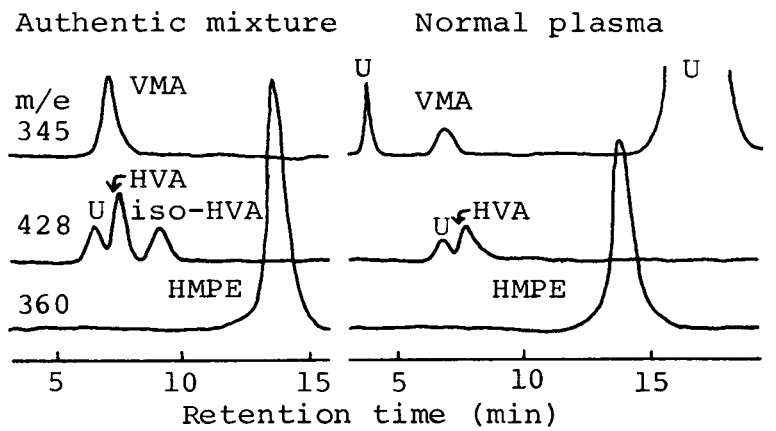

Fig.5. Mass fragmentographic patterns of the metabolites from the authentic mixture and normal plasma. U, unknown peaks.

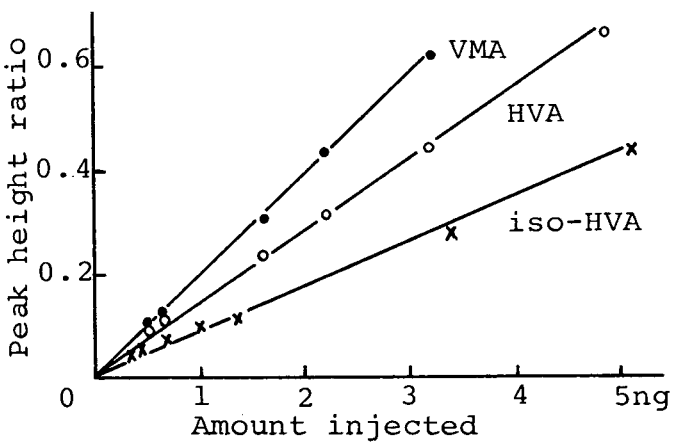

Fig.6. Working curves of the metabolites. Fifteen ng of HMPE were injected.

TABLE II

CONCENTRATION OF THE METABOLITES IN HUMAN BODY FLUIDS BY MF

Subject	Sex	Age (years)	Concentration						
			Plasma* (ng/ml)		CSF* (ng/ml)		Urine (mg/day)		
			VMA	HVA	VMA	HVA	VMA	HVA	iso-HVA
Normal	M	27	20	9	—	—	1.6	4.2	0.1
	M	35	15	11	—	—	1.3	3.9	0.1
	M	25	17	19	—	—	—	—	—
	M	30	10	8	—	—	—	—	—
	F	52	14	4	12	40	—	—	—
Parkinson's**	M	16	18	16	6	<2	1.5	4.5	0.1
Neuroblastoma	M	3	—	—	—	—	2.0	15	0.4

*Iso-HVA was not detected in plasma and CSF.

**Administration of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) as a medicine was stopped f several days before sampling. Lack of influence of L-DOPA was confirmed by the observati that the excretion of VMA and HVA in urine analyzed by HSLC [10, 11] was in the norm range.

DISCUSSION

We have developed a reliable method for the simultaneous determination of VMA, HVA and iso-HVA in body fluids, using convenient and efficient clean-up techniques and highly selective and sensitive MF. MF determinations of these metabolites in body fluids have been reported, but a simultaneous determination of the metabolites from a normal person has not been reported. The comparable data are summarized in Table III. Sjöquist et al. [13, 14] purified the metabolites from plasma by the use of an Amberlite XAD-2 column, although, according to our experiments, the recovery of VMA from the column was not so good. On the contrary, quantitative adsorption and sharp elution of the metabolites on the Amberlite XAD-4 used in this method were carried out as shown in Fig. 2 and Table I. As shown in Fig. 1, with the solvent extraction apparatus using a PTFE membrane we have been able to separate completely the organic and water phases without standing or nervous watching. The derivatization of the metabolites by past methods consisted of two steps of methylation and acylation, whereas the present method offers one step of derivatization.

ACKNOWLEDGEMENTS

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TABLE III

COMPARATIVE DATA FOR METABOLITE CONCENTRATIONS OBTAINED BY MF

Author	N	Concentration					
		Plasma (pmole/ml)		CSF (pmole/ml)		Urine (nmole/ml)	
		VMA	HVA	VMA	HVA	VMA	HVA
Present authors	*	51—100	22—100	60	220	6.6—8.1	21—29
Sjöquist [13]	10	31—71	—	14—25**	—	11.3±0.21	—
Sjöquist et al. [14]	1	—	48	—	420	—	20
Gordon et al. [18, 19]	6	25—130	55—110	—	—	—	—
Karoum et al. [15]	5	—	—	2.3±0.9***	280±44***	—	—
Jimerson et al. [20]	34	—	—	5.2±0.9***	150±18***	—	—
Narasimhachari et al. [21]	17	—	27—450 §	—	0.02—0.38 §	—	—

*Plasma (N=5), CSF (N=1) and urine (N=2).
**Neurological patients.
***Psychiatric patients.
§ Free plus conjugates.

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Biomedical Applications

CHROMBIO. 075

RAPID PROCEDURE FOR CHROMATOGRAPHIC ISOLATION OF DOPA, DOPAC, EPINEPHRINE, NOREPINEPHRINE AND DOPAMINE FROM A SINGLE URINARY SAMPLE AT ENDOGENOUS LEVELS

Y. DALMAZ and L. PEYRIN

Laboratoire de Physiologie, UER Médicale Grange-Blanche, Université Claude Bernard Lyon I, 8 Avenue Rockefeller, 69373 Lyon Cedex 2 (France)

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SUMMARY

A three-step procedure has been investigated to extract 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), epinephrine (E), norepinephrine (NE) and dopamine (DA) from a single urinary sample with the object of obtaining extracts pure enough for specific fluorimetric assay. The procedure described in this paper results from the combination of urine purification on an aluminum oxide column, separation by ion-exchange chromatography of the DOPA—DOPAC fraction from catecholamines, and ether isolation of DOPAC from DOPA. The whole procedure is rapid and easily performed in one work day. Extraction recoveries were 72.4 ± 3.5%, 76 ± 2%, 85.7 ± 3.3%, 85.6 ± 1.4% and 92.4 ± 5.5% for DOPA, DOPAC, E, NE and DA respectively (n= 6).

The lowest amounts of the five catechols that could be detected in urinary samples by a combination of this extraction procedure and the methods of assay used in our laboratory were 15 ng for DOPA, 40 ng for NE, 20 ng for E, 152 ng for DA and 2.95 μg for DOPAC. Urinary volumes convenient for accurate estimation of each compound were 20 ml for healthy human subjects. For pathological or pharmacological purposes, 5 ml of human urine were sufficient.

The daily excretion of DOPA, DOPAC, E, NE and DA found by this procedure agrees with data obtained by other authors in healthy subjects. In pathological samples, our three-step procedure led to lower amounts than methods using alumina purification only. The discrepancies between the two methods are discussed in terms of development of internal standards, relative specificity of fluorimetric assays, values of blank eluates, and the possibility of interference from unknown abnormal body metabolites or pharmacological drugs not eliminated by a single-step alumina purification.

INTRODUCTION

A number of procedures have been previously reported for determination of catecholamines, epinephrine (E), norepinephrine (NE), dopamine (DA), (3,4-dihydroxyphenylalanine (DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in urine or tissues. Most of them enable isolation of one, two, three or four catechols [1—26].

None of these methods can be used for specific extraction and assay of DOPA, DOPAC, E, NE and DA from a single urinary sample. The only procedures suitable for this purpose are those of Sroka et al. [27], Sourkes et al. [28] and Routh et al. [29], but they lack sensitivity for accurate estimation of the five catechols and are suitable only for pathological studies.

Specific isolation of DOPA, DOPAC and catecholamines from one another is necessary because of the spectral similarities of these compounds and the small amounts to be detected at endogenous levels. Thus, DOPA interferes in the fluorimetric assay of DA [30] and NE [31] and in the colorimetric assay of DOPAC [32] ; on the other hand, DOPAC must be separated from catecholamines before its colorimetric determination [32].

In this paper, a procedure for isolating DOPA, DOPAC, E, NE and DA from a single urinary sample is described. Great care has been taken to obtain each compound in conditions convenient for its subsequent specific assay without any interference of DOPA or DOPAC on one another or on catecholamines. Furthermore, the final extracts may be used for flow diagram analysis. Our method involves initial purification of urine on aluminum oxide followed by the fractionation of DOPA, DOPAC and catecholamines on Amberlite CG-50 (NH_4^+). Complete separation of DOPA and DOPAC is achieved by diethyl ether extraction.

MATERIALS

The following materials were used:

Pyrex glass columns (1 cm diameter), with long-fibre glass wool (Corning, Corning, N.Y., U.S.A) for alumina adsorption, and a porous glass plate for Amberlite use, at the bottom of the stem; A pH meter Metrohm E 510; a rotating vertical mechanical shaker (home-made); and a Technicon AutoAnalyzer (for fluorimetric and colorimetric assays).

Reagents were: 0.2 and 0.5 *M* sodium acetate buffer containing 0.01% of disodium EDTA (buffer A and buffer B, pH 8.40); 0.2 *M* ammonium acetate buffer, pH 6.10 (buffer C); 0.4 *M* ammonium acetate buffer, pH 5.0 (buffer D). Ascorbic acid (0.3 and 2%) in water, 0.01 *M* citric acid, hydrochloric acid (R.P. d = 1.19), diethyl ether (peroxide free), 10% (w/v) EDTA (disodium salt) in water, 20% (w/v) EDTA (disodium salt) adjusted to pH 8.6 with 1 *M* sodium hydroxide, and 0.1 *M* Tris buffer (pH 5).

All chemicals were analytical grade and purchased from Prolabo(Rhône-Poulenc, France) or Merck, (Darmstadt, G.F.R.).

Aluminum oxide (activity II—III), standardized for Brockmann chromatographic adsorption, from Merck, and activated as previously described [33] .

Amberlite CG-50 (H^+; 200—400 mesh) from BDH (Poole, Great Britain) is used in NH_4^+ form as described by Hirs et al [34] and Kirshner and Goodall [22] . The pH of the resin was stabilized to 6.10 by five successive washes (30 min each) in buffer C. At the end of urinary analysis, Amberlite may be reused after numerous washings with buffer D and buffer C.

Standard solutions of DOPA (Fluka, Buchs, Switzerland), DOPAC (Fluka), DA hydrochloride (Fluka), L-(NE) bitartrate (Fluka), L-(E) (Sigma, St. Louis, Mo., U.S.A.); stock solutions of catechols (100 μg/ml) were prepared by dis-

solution in 50 ml of 0.01 *M* hydrochloric acid containing 5 mg of DOPA, 5 mg of DOPAC, 6.2 mg of DA, 9.95 mg of NE and 5 mg of E respectively; these solutions were stored at 4° and kept for no more than three weeks. For specificity studies, the following compounds were used: epinine hydrochloride (Regis, Morton Grove, Ill., U.S.A.), 3-O-methyldopamine (3-MT) hydrochloride (Sigma), 3,4-dihydroxymandelic acid (DOMA) (Sigma), 3,4-dihydroxybenzoic acid (DOBA) (Sigma), 3,4-dihydroxycinnamic acid (DOCI) (Merck), 3,4-dihydroxyphenylglycol (DHPG) (Regis), tyrosine hydrochloride (Sigma).

The following radioactive compounds were obtained from Commissariat Energie Atomique, CEA, Saclay, France: DOPA-3-^{14}C (specific activity 58 mCi/mmole), DOPA-2-^{14}C (25 mCi/mmole), DA-2-^{14}C (58 mCi/mmole), DOMA-2-^{14}C (47 mCi/mmole), DHPG-7-^{14}C (45 mCi/mmole), DL-NE-7-^{14}C (48 mCi/mmole), L-tyrosine-3-5-^{3}H (54 Ci/mmole), E-^{14}C (38.6 mCi/mmole).

METHODS

Urines were collected, stored and hydrolyzed as described elsewhere [33]. The successive steps of the method are explained in Fig. 1.

Alumina adsorption

Hydrolyzed human urine (25 ml) or rat urine (2 ml) were diluted to 50 or 20 ml, respectively, with distilled water; 1 ml of 10% EDTA and 0.5 ml of 0.3% ascorbic acid were added, and the pH was adjusted to 8.4 by successive use of 5 *M*, 2.5 *M*, 0.2 *M* sodium hydroxide. Activated aluminum oxide (2 g) was poured into the glass columns and washed just before use with 20 ml of buffer A. After the urinary sample had slowly percolated (10 drops per min) through the column, the alumina was washed with 10 ml of buffer B and 100 ml of 0.01% aqueous disodium EDTA. The elution of all catecholic compounds was performed at a very slow rate (5 drops per min) by 7 ml of 1 *M* hydrochloric acid.

Fractionation of alumina extract on Amberlite CG-50

Amberlite CG-50 columns (10 × 1 cm) were prepared in buffer C and washed with 20 ml of this buffer just before use. 100 µl of 20% EDTA and 100 µl of 2% ascorbic acid were added to the alumina eluate, whose pH was then brought to 6.10. The volume of the extract was made up to 10 ml with buffer C, centrifuged at 6000 *g* at 20° for 10 min and poured carefully on the top of the Amberlite column. As soon as it had been completely adsorbed, buffer D was poured on to the column as eluant. Five fractionated eluates were collected (Fig. 2): fractions A (containing urinary pigment) and C (following neutral and acidic catechols) were discarded; fraction B contained DOPA and DOPAC and fractions D1 and D2 contained E, NE and DA. Eluates D1 and D2 were used without further treatment for the specific assay of E, NE and DA.

Separation of DOPA from DOPAC

In eluate B, a further separation of DOPA and DOPAC was achieved by ether extraction: 2.5 ml of fraction B were brought to pH 2 and extracted for

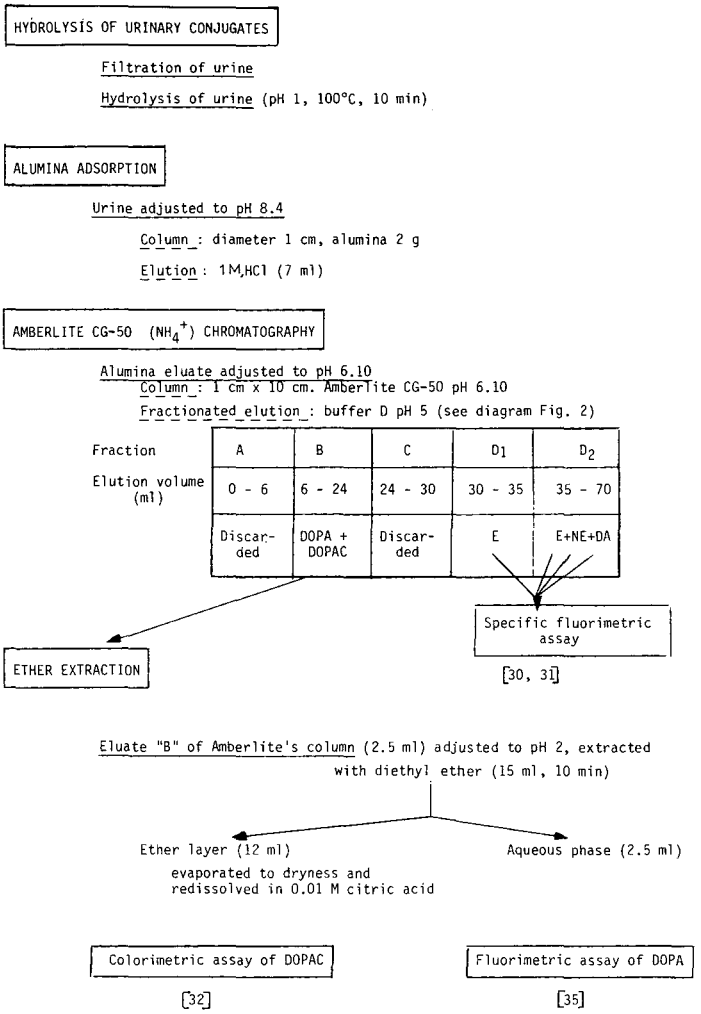

Fig. 1. Main steps of the general procedure used for the extraction of DOPA, DOPAC, E, NE and DA from a single urinary sample. For details, see text.

10 min with 6 volumes of diethyl ether (15 ml) on a rotating vertical shaker. 12 ml of the ether layer were evaporated to dryness at 40° under atmospheric pressure. The dry residue was dissolved by vigorous shaking (5 min) in 5 ml of 0.01 *M* citric acid. DOPAC was estimated in this "final DOPAC extract" and DOPA in the aqueous phase saved after ether treatment of fraction 3 ("final DOPA extract").

Assay of the various compounds

DOPAC. The DOPAC estimation was performed in the "final DOPAC extract" as described by Peyrin et al. [32] by using the automated colorimetric assay based on the formation of a red compound under effect of nitromolyb-

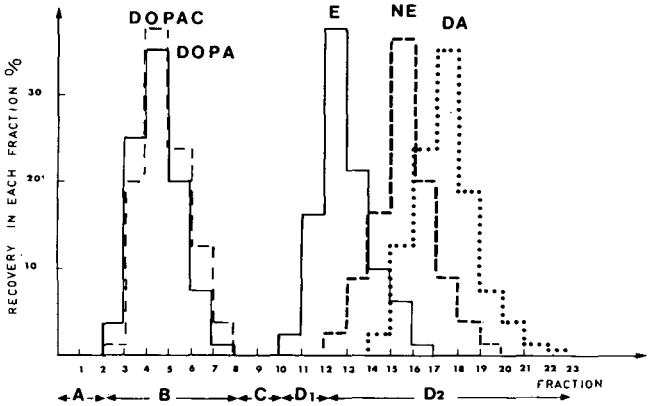

Fig. 2. Elution pattern of exogenous DOPA, DOPAC, E, NE and DA on an Amberlite CG-50 NH_4^+ column (10 × 1 cm) at pH 6.10. The solution applied was the acidic alumina eluate obtained from urine (20 ml). In this experiment stable DOPA (10 μg), E (10 μg), NE (10 μg), DA (10 μg) and radioactive ^{14}C DOPAC (0.25 μCi i.e. 1.6 μg) were added to the alumina eluate just before ion-exchange chromatography. The eluant was buffer D (pH 5.0). Fractions of 3 ml were collected.

dic reagent and sodium hydroxide on DOPAC. External standards of DOPAC (1 μg/ml) were prepared in 0.01 *M* citric acid.

DOPA. The acidic aqueous phase (2.5 ml) saved after DOPAC extraction was adjusted to pH 6 by the addition of an equal volume of 0.38 *M* ammonia. DOPA was then estimated as described by Cottet-Emard and Peyrin [35] using automated fluorimetric assay based on the formation of a 5,6-dihydroxyindole derivative after ferricyanide oxidation of DOPA. Internal standards of DOPA were prepared by mixing equal parts of "DOPA eluate" with standard DOPA solutions (100 ng/ml for human urines, 50 ng/ml for rat urines) in 0.2 *M* ammonium acetate of pH 6.

Epinephrine. E was estimated in fractions D1 and D2 as described by Peyrin and Cottet-Emard [31] using the automated fluorimetric assay of 3,5,6-trihydroxyindole derivative, resulting from specific ferricyanide oxidation of E. Internal standards of E were prepared by mixing equal parts of eluates D1 or D2 with standard E solutions (50 ng/ml for human urines, 10 ng/ml for rat urines) in 0.2 *M* ammonium acetate of pH 5.2.

Norepinephrine. NE was estimated in Amberlite fraction D2 by applying the automated specific fluorimetric assay of NE trihydroxyindole derivative [31]. Internal standards of NE were prepared by mixing equal parts of eluate D2 with standard NE solutions (200 ng/ml for human urines, 50 ng/ml for rat urines) in 0.2 *M* ammonium acetate of pH 5.2.

Dopamine.The determination of DA was made in Amberlite fraction D2 by fluorimetric assay of the 5,6-dihydroxyindole derivative resulting from iodine oxidation of DA. The procedure was the same as for 3-MT [36] except that 0.2 *M* ammonium acetate buffer (pH 5.2) was used for preparation of standards. Internal standards of DA were prepared by mixing equal parts of eluate D2 with standard DA solutions (100 ng/ml for human urines, 50 ng/ml for rat urines) in 0.2 *M* ammonium acetate of pH 5.2.

RESULTS

Stability of each compound to heating in the course of acidic hydrolysis

Table I shows that the five compounds studied were not destroyed by boiling at pH 1 for 10 min.

Pattern of elution from amberlite CG-50

The ion-exchange chromatographic analysis of the alumina eluate on Amberlite CG-50 (NH_4^+) led to efficient separation of catechol compounds into acidic or neutral (fraction B) and basic fractions (D1 and D2) (Fig. 2). 5—10% of E were present in fraction D1, the remainder being eluted together with NE and DA in fraction D2. Fraction D1 may be useful to control any displacement of the elution pattern; however, for routine use, fractions D1 and D2 were mixed for E assay. The methods used for E, NE and DA assay were sufficiently specific so that a more effective separation of these amines is not necessary.

After the column has been prepared, the resin has to be washed again with 20 ml of buffer C to avoid the release in the eluates of disturbing fluorescent materials from Amberlite CG-50 resin [7, 10, 12]. Fluorescence blanks from resin were then very low (Table IV).

Extraction recoveries for each compound

Satisfactory recoveries ranging from about 72 to 92% were obtained throughout the whole procedure for the five catechols (Table II).

Reproducibility of the procedure

Results obtained from a urinary sample were satisfactorily reproducible, whatever its catechol content (Table III).

Sensitivity of the method for each catechol

Taking into account, for each compound, extraction recoveries, elution or recuperation volumes and assay sensitivity, the least amounts of catechols which must be present in the sample to be adsorbed on alumina are 15 ng for DOPA, 40 ng for NE, 20 ng for E, 152 ng for DA and 2.95 μg for DOPAC. The smallest volume of urine to be extracted is calculated from the sensitivity

TABLE I

STABILITY OF CATECHOLS TO THE ACIDIC HYDROLYSIS

Exogenous ^{14}C compound added to urinary sample (0.25 μCi)	Recovery from aluminum oxide	
	Compound boiled with urine 10 min at 100° (%)	Compound added at the end of acidic hydrolysis of urine (%)
DOPA ^{14}C	86	83
DOPAC ^{14}C	78.8	79
E ^{14}C	92	90
NE ^{14}C	90	93
DA ^{14}C	92.5	94

TABLE II

EXTRACTION RECOVERIES FOR EACH CATECHOL AFTER ALUMINA ADSORPTION, ION-EXCHANGE CHROMATOGRAPHY ON AMBERLITE CG-50 OR ETHER EXTRACTION

Exogenous ^{14}C compound (0.2 μCi)	Extraction recoveries after			Whole extraction recovery (%)
	Alumina adsorption* (%) (n = 6)	Amberlite CG-50** chromatography (%) (n = 6)	Ether extraction*** (%) (n = 6)	
DOPA	94 ± 6	80 ± 2	0 ± 0.9	72.4 ± 3.5
DOPAC	86.4 ± 4	90 ± 2	98 ± 1.9	76 ± 2
E	91 ± 1.8	96 ± 2		85.7 ± 3.3
NE	95 ± 1	93 ± 2		85.6 ± 1.4
DA	94 ± 2	98 ± 3		92.4 ± 5.5

*Urinary samples were added with a single radioactive compound and adsorbed on alumina as described in Methods. Recoveries were calculated on the 1 M HCl eluate (7 ml).
**Alumina eluates free of radioactive compound were added with one radioactive catechol and chromatographed on Amberlite CG-50 as described in Methods. Recoveries were calculated in the corresponding Amberlite eluates.
***Amberlite eluates free of radioactive traces were added with radioactive DOPA or DOPAC just before ether extraction as described in Methods. Recoveries were calculated in the aqueous and ether layers.

TABLE III

REPRODUCIBILITY OF THE PROCEDURE EXPRESSED AS VALUES (MEAN ± S.E.) OF TOTAL URINARY DOPA, DOPAC, E, NE AND DA OBTAINED FROM THREE DETERMINATIONS ON THE SAME URINARY SAMPLE

Urinary sample	Volume of urine extracted	Concentration (μg/l)				
		DOPA	DOPAC	E	NE	DA
Adults	25 ml	31±3.2	1547±74	18.2±1.1	138±9.5	701±8.4
Children	25 ml	44.3±2.6	1290±65	3.7±0.34	29.8±3.4	1016±8.3
Children with neuroblastoma	5 ml	93.3±12.9	1643±3.3	16.5±1.67	96.5±6.3	1788±73.5
Mean standard error %		9.9±2.3	3.9±0.96	8.4±1.2	8.2±1.6	2.0±1

procedure. Accordingly, urinary volumes of 25 ml for healthy human subjects or 2 ml for human patients are recommended.

Specificity of the extraction

Related compounds that interfere in the colorimetric or fluorimetric assay were tested as to their extraction properties, recoveries and occurrence in the final eluates (Fig. 3). Over-all interference is summarized as follows.

Monophenols (octopamine, tyramine), acidic or alcoholic phenols (homovanillic acid, vanillomandelic acid, 3-methoxy-4-hydroxyphenylglycol and methoxylated amines (3-MT, normetanephrine, metanephrine) are removed in the alumina step. Thus, the interference of 3-MT on DA iodine assay is completely eliminated.

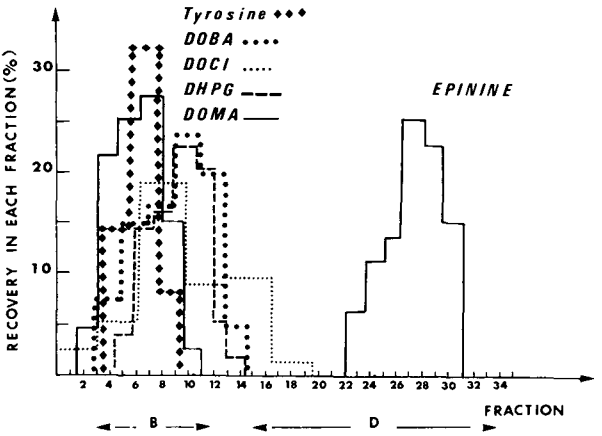

Fig. 3. Elution pattern of other related compounds on an Amberlite CG-50 NH_4^+ column (10 × 1 cm) at pH 6.10.
Compounds were dissolved in 0.9% NaCl (7 ml), mixed with 20% EDTA (pH 8.60) (100 µl) and 2% ascorbic acid (50 µl), adjusted to pH 6.10 and brought up to 10 ml before ion-exchange chromatography. The eluant was buffer D (pH 5.0). Fractions of 2 ml were collected. The positions of the fractions usually collected are noted under the figure.
The whole interference (per cent) of related compounds in the determination of DOPA, DOPAC, E, NE and DA is calculated on the basis of three parameters: final recoveries after alumina, Amberlite and diethyl ether extractions; position in the elution pattern of Amberlite CG-50 (i.e. occurrence in the same eluate as the studied catechols).
Interference in the fluorimetric assay of DOPA [35], E, NE [31], DA [30] and colorimetric assay of DOPAC [32]. Each compound was dissolved in 25 ml of 0.9% NaCl. Alumina adsorption, Amberlite CG-50 chromatography and ether extraction are described in Methods. Radioactive ^{14}C DHPG was used for recoveries.

Compound	Relative interference in assay procedure of		Extraction recoveries			Final recoveries in fractions of		Whole interference on	
(a)	DOPA	DOPAC	Alumina	Amberlite	Ether	DOPA	DOPAC	DOPA	DOPAC
DOMA	0	15.7	76	96.7	27	53.4	20	0	3
DOBA	0.11	83	— 88	—	39	38.7	25	0.04	20
DOCI	1.2	41	— 64	—	54	20	22	0.24	9
DHPG	1	78	70	85.6	12	49	6.7	0.49	5
(b)	E NE DA					E + NE + DA		E NE DA	
Epinine	0 3.8 0.5		62	96	—	59.5		0 2.2 0.30	

Most catechols are extracted by the double-step procedure (alumina, Amberlite); their location in the elution pattern of Amberlite CG-50 is shown in Fig. 3. Epinine was extracted in the same fraction as catecholamines (E, NE, DA): its final interference was 2.2, 0.3 and 0% on NE, DA and E assays respectively. The catechol compounds DOBA, DOMA, DOCI, DHPG were eluted together with DOPA and DOPAC; their final interference was respectively 21, 3, 9 and 5% on DOPAC determination, and 0.04, 0, 0.24 and 0.49% on DOPA assay. These interferences are acceptable for our purpose. Epinine, DOBA and DOCI have never been found in urinary samples [32];

the amounts of DOMA present in urine are low compared with DOPAC concentrations and their interference may be neglected. The possible interference of tyrosine has been investigated in more detail because the high concentration of this monophenol in urine (the range varied for total tyrosine from 41 111 mg per day) [37, 38] and because of the possibility of hydroxylation of tyrosine to DOPA during the alumina step as suggested by Lindqvist et al. [39].

For this purpose, tritiated tyrosine (1 μCi in 25 ml of buffer A) was first purified from its radioactive DOPA content by alumina adsorption. Tyrosine present in the alumina filtrate was used immediately. Only 0.13% of the initial tyrosine was present in the DOPA fraction from Amberlite (Fig. 3). When the interference of tyrosine on the DOPA assay (0.02%) is taken into account, the whole interference of tyrosine is of 0.000024%. No detectable interference on DOPA assay may be expected from the great amounts of tyrosine normally present in urine. Furthermore, no added fluorescence in DOPA determination was found when purified tyrosine (20 mg/l) was added to urinary samples before extraction. However, the final interference of tyrosine may be increased to 0.05% when the alumina columns, after urine percolation, are left to stand overnight before being washed. This effect may be due to the hypothetical transformation of tyrosine into DOPA, previously suggested by Lindqvist et al. [39]. A consequence of these observations is that the alumina step must be completed in reasonable time (no longer than 4 h).

Biological applications

Our final method enabled us to estimate DOPA, DOPAC, E, NE and DA in urinary samples from healthy human subjects or from patients with diseases correlated with adrenergic dysfunction and in urine of laboratory animals. Daily excretion in 34 healthy adults was 2.85±0.3 mg for DOPAC, 385±84 μg for DA, 89±11 μg for NE, 81±8 μg for DOPA and 19±3 μg for E. except for NE and E, only few data on total daily excretion from men at physiological levels are available in the literature. Our E, NE, DA and DOPAC values agree with data published earlier by other authors for hydrolyzed urines [14, 15, 40, 41]. No comparison was possible for DOPA results because all the values reported up to now in the literature came from analyses of unhydrolyzed urines [26, 42, 43].

Comparison of the data obtained by our procedure or by the use of alumina purification only will be discussed below.

The method allows separate estimation of the five compounds even in urines of patients with excessive amounts of DOPA and catecholamines, i.e. children with sympathetic tumors, adult subjects with chromaffin tumors or parkinsonian patients under treatment with L-DOPA. Even in these cases, no overlap was observed of DOPA on DOPAC nor from neither of them on catecholamines.

DISCUSSION

Choice of extraction procedure

Whatever the method used to extract and separate the urinary catechols

(neutral, acidic or aminated) specific isolation of DOPA and DOPAC is necessary because of the interference of DOPAC on DOPA, E, NE and DA [32] and of DOPA on the three catecholamines [35]. On the other hand, rigorous separation is not necessary for E, NE or DA since the last does not interfere in E and NE assay and specific assay of E and NE may be performed [31].

It is now well known that all catechols may be adsorbed on alumina and eluted together by an acidic agent [5, 8, 9, 15–17, 26–28]. To estimate the relative amounts of DOPA, DOPAC, E, NE and DA, an alternative method consists in applying specific assay procedures of each compound to the mixed eluate [9, 17]. However, the spectral properties of these catechols are too similar for complete elimination of interference from one another. Accordingly, the specific estimation of these five compounds may be expected only after their true separation has been achieved by a convenient isolation procedure.

Separation of catechols contained in the alumina eluate has been achieved by paper or thin-layer chromatography [15, 20]. Some authors have also suggested the separation of catecholamines (E, NE, DA) from DOPA or DOPAC by a graduated acidic elution of catechols adsorbed on alumina [19, 24]. Cationic exchange resins have been used to elute the three catecholamines together [6, 14, 29] or to separate them partially [1, 7, 9, 13, 18, 23] or completely from one another [12, 22, 24, 44, 45].

The problem of specific isolation of DOPAC from DOPA has not been satisfactorily resolved by earlier methods based on the use of organic solvents, coupled with chromatographic analysis [2, 3, 41, 46–48].

Most of the published methods either lack specificity [2] or are not convenient for catechol determinations at physiological levels.

The aim of our work has been not only to extract the above five compounds from a single sample, but also to attain: rigorous isolation so as to overcome spectral interference inherent in assay procedures; reliable recoveries for each compound; and short experimental duration for the method to be used in serial analysis.

The method described in this paper results from a combination of the most convenient steps of previously published procedures for either amine [5, 22, 24]. The method is based on the association of adsorption of catechols on alumina, separation of acidic (DOPAC) or neutral (DOPA) catechol compounds from catecholamines on Amberlite CG-50 at pH 6.10 and selective extraction of DOPAC with ether. Several steps have been studied to optimize isolation and recoveries.

Critical study of alumina adsorption

Adsorption pH as a function of the nature of the saline content. pH values lower than 8.5 have been used to adsorb pure catechol compounds on alumina [19, 24]. To investigate the effect of adsorption pH and nature of the saline content on the alumina procedure, NE and DOPA solutions were prepared either in 0.2 *M* sodium acetate or in 0.2 *M* sodium phosphate and adsorbed on alumina columns (as described above) at two different pH values 6.10 and 8.40). When adsorption was carried out at pH 8.40 recoveries were good for both DOPA and NE and higher when 0.2 *M* sodium acetate (96 and 95% respectively) instead of 0.2 *M* sodium phosphate (85% for DOPA, 89% for NE) was

used as a diluent to dissolve these compounds before alumina adsorption. At pH 6.10, NE and DOPA in phosphate buffer were not retained on alumina; when catechols were in acetate buffer at pH 6.10, DOPA was strongly fixed (recovery 93%), whilst NE was found in part in the water washes applied just before acidic elution (recovery in alumina eluate 42%). Owing to the presence of great amounts of phosphate ions in most urinary samples, it seems necessary to perform alumina adsorption at pH 8.40.

Molarity of the washing solutions. To ensure the highest recoveries for NE, it was important to use washing solutions (sodium acetate) with molarities greater than that of the initial mixture placed on the column. On the other hand, the molarity of the washings did not modify DOPA recoveries. The same conclusions were obtained by Drell [19].

Attempts to separate catecholamines, DOPA and DOPAC by a double-step elution of alumina. Fractionated elution of catechols retained on alumina has been applied by some authors [5, 19, 24, 41]. However, none of these procedures was completely satisfactory. We therefore studied the eluting properties of orthophosphoric acid, hydrochloric acid, acetic acid and ammonium acetate buffer at different molarities. All the eluents studied were able to elute catecholamines but recoveries greater than 75% were obtained only for eluants with pH values at or below 4 and molarities of 0.05 *M* or more.

On the other hand, ammonium acetate (pH 4) with molarity greater than 0.05 *M* did not elute DOPAC, but did elute 32% of the DOPA. The complete recovery of DOPA and DOPAC needed the use of acids with strong molarities, which, moreover, also eluted catecholamines. An alternative method of separating the two groups of catechols may consist in the use of a calculated volume of 0.05 *M* ammonium acetate (pH 4) to elute catecholamines, followed by a strong acid (0.2 *M* phosphoric acid) to achieve complete elution of DOPA and DOPAC. In these conditions, a satisfactory separation of pure E, NE and DA

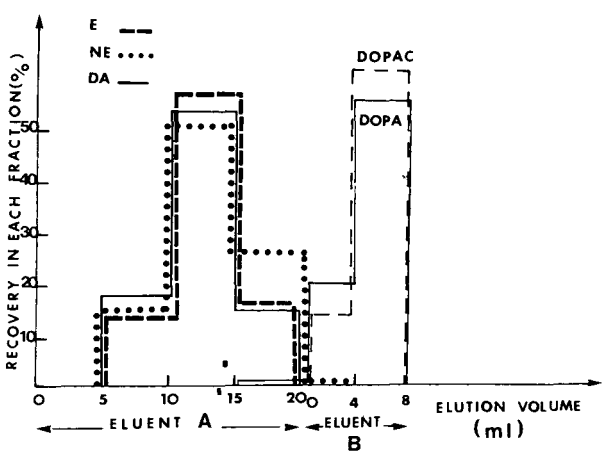

Fig. 4. Separation of catecholamines from DOPA and DOPAC by a single alumina adsorption. Solutions applied were catechols diluted in 0.2 *M* sodium acetate buffer (pH 8.40) and added with 10% EDTA (1 ml) and 0.3% ascorbic acid (0.5 ml). Alumina adsorption was performed as described in Methods but eluant A and B were used instead of 1 *N* HCl. Eluant A: 0.05 *M* ammonium acetate buffer (pH 4.0) (20 ml). Eluant B: 0.2 *M* phosphoric acid (8 ml).

from DOPA and DOPAC was obtained (Fig. 4); however, results from urinary samples were not reproducible and a variable overlap (2–30%) of DOPA and DOPAC was observed on catecholamine fractions. By the use of a similar procedure, Drell [19] reported an overlap of 10% between aminated and acidic catechols. For these reasons, the double-step alumina elution was not further used.

Critical study of Amberlite CG-50 procedure

pH and ionic form of the resin. The pH value of 6.10 previously used by other authors [6, 22, 24] has been found convenient for separation of acidic or neutral catechols from catecholamines. Higher recoveries were obtained when the resin was used in the NH_4^+ form, suggested by Kirshner and Goodall [22] instead of the Na^+ form used by Fleming et al. [6] and Lishajko [13].

Volume of the extract to be deposited and height of the column. Changing the extract volume (from 7 to 10 ml) — but neither the amount of catechol nor the column height (7.5 cm) — delayed the elution of neutral and acidic catechols and increased the eluting volumes of the catecholamine fractions. A column height of 10 cm improved the separation — with a parallel increase of elution volumes by only 20%.

Effect of the urinary saline content. Because of the disturbing effect of salts previously observed on the elution pattern from Amberlite CG-50 [30] the position of peaks and recoveries for each compound were studied on four urinary samples with different initial saline contents (creatinine from 160 to 1500 mg/l). All of them resulted in high recoveries of added catechols and in stability of the fractionation pattern.

Effect of catechol concentration in the alumina eluate. The amounts of catechols to be passed through Amberlite may be fivefold to tenfold the normal human daily excretion without great modifications in recoveries, except for DA whose recovery fell to 80% at very high concentrations (20 mg/l). The eluting pattern was constant over a wide range of concentrations for either compound; however 5% overlap was seen between fractions B and D, when 50 μg of DOPA, E, NE and DA (2 mg/l) or 500 μg of DOPAC (20 mg/l) had been deposited on the column. Such amounts may be found only in highly pathological samples, or in urines from L-DOPA-treated patients. In such cases, it would be advisable to use small volumes of urinary samples (2–5 ml) instead of the 25 ml suggested for normal subjects.

Critical study of the ether separation of DOPAC and DOPA

Diethyl ether [5] or ethyl acetate [2, 3, 9, 41, 46, 47] have been used to extract DOPAC from biological samples. Although similar recoveries were found with both of these solvents, we have preferred diethyl ether for routine use because of easy evaporation at low temperature to avoid oxidative destruction of DOPAC. The prior purification of diethyl ether on alumina, suggested by Spano and Neff [5], has not improved DOPAC recoveries.

Comparative studies with eluates at pH 1, 2 or 3 treated by ether in amounts from 2 to 6 volumes, showed that recoveries were improved from 67 to 98% when an eluate at pH 2 was extracted by 6 volumes of ether. The best recoveries were obtained after evaporation of the ether layer and dissolution of the

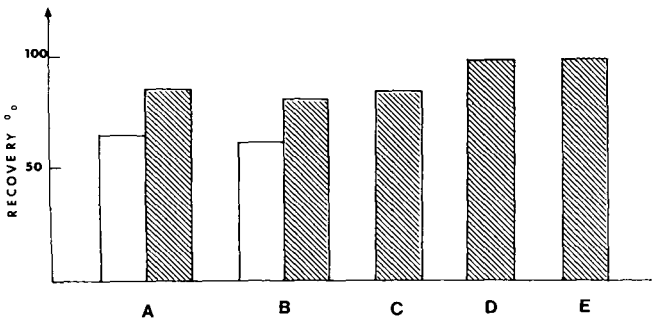

Fig. 5. Transfer of DOPAC from ether to aqueous layer under various buffers.
^{14}C DOPAC was diluted in 2.5 ml of 0.4 *M* ammonium acetate at pH 5.0, adjusted to pH 2.0 and dissolved in 6 volumes of diethyl ether. After shaking two methods were used: open areas, direct transfer of the ether layer in two volumes of buffer; hatched areas, evaporation of the ether layer and redissolution of the dry residue in 5 ml of buffer A, B, C, D, E. A: 0.1 *M* tris (pH 8.0), B: 0.25 *M* disodium phosphate (pH 7.0), C: 0.4 *M* ammonium acetate (pH 5.70), D: 0.2 *M* ammonium acetate (pH 5.70), E: 0.01 *M* citric acid.)

dry residue in 0.01 *M* citric acid (5 ml) (Fig. 5). Furthermore, this diluent is the most convenient for development of color from DOPAC [32].

Purity of final eluates

The extracts obtained by our procedure are clear and suitable for fluorimetric assay. This was demonstrated by comparing the absorbance at 310 nm, of alumina eluates or Amberlite fractions at 0.44 ± 0.07% (range 0.2 to 0.8) and 0.06 ± 0.01% (range 0 to 0.09) respectively. The high degree of purification of Amberlite extracts was further proved by the low blank values obtained in the fluorimetric assay of each compound (Table IV).

The final DOPAC extract obtained by diethyl ether treatment of Amberlite eluates was completely free from pigments (absorbance at 310 nm = 0.03%) and much clearer than if directly obtained from an alumina eluate as suggested by other authors [5, 9, 40, 41].

TABLE IV

FLUORESCENCE COMING FROM THE ALUMINUM OXIDE AND AMBERLITE CG-50 RESIN

Blank values are expressed as ng of each compound in the whole fraction collected. 20 ml of 0.2 *M* sodium acetate buffer (pH 8.40) were adsorbed on alumina as described in Methods and eluted either by 0.25 *M* acetic acid (10 ml) or by 1 *M* HCl (7 ml). The HCl eluate was chromatographed on Amberlite CG-50, and fractions B and D were collected as described in Methods.

Extraxt	DOPA	E	NE	DA
Alumina eluate 0.25 *M* acetic acid	70	10	0	200
Amberlite CG-50 fractions 0.4 *M* ammonium acetate (pH 5)	10	0	0	10

TABLE V

COMPARISON OF THE ALUMINA EXTRACTION PROCEDURE ALONE AND OF THE DOUBLE—STEP METHOD

Two aliquots of the same urine were extracted simultaneously on alumina only (eluted with 10 ml of 0.25 *M* acetic acid) and alumina + Amberlite CG-50 as described in Methods. Fluorimetric assays were performed in the alumina eluate and Amberlite CG-50 fractions. Results are uncorrected for extraction losses.

	Alumina procedure			Double steps alumina and amberlite CG-50			Ratio alumina/amberlite		
	DA	NE	E	DA	NE	E	DA	NE	E
Healthy subjects μg/24 h	328±56 (n= 18)	90±21 (n=25)	20±4 (n=25)	385±84 (n = 18)	89.1±10.8 (n = 25)	19.2±0.3 (n = 25)	0.85	1.01	1.04
Recovery of added amines (%)* (n = 3)	89.3 ±8	80±8.4	89.3±0.6	95.5±1.4	82.2±2	91.6±8			
Patients									
Hypertensive μg/24 h (n= 3)	5348±1980	481±33.6	123.2±17.2	1642±834	155.6±82.5	39.3±10.1	3.26	3.09	3.13
Pheochromocytoma μg/24 h (n = 1)	18116	4480	224	7644	4340	117.9	2.37	1.03	1.90
Development of internal standard (% of external standard) (n = 4)	110±3.5	78±4.5	95±0.7	96.3±3.7	113.7±4.9	103.2±0.3			
Relative interference (%) of each amine in pure solutions									
a/E	1	0	100	0.1	0	100			
b/NE	2	100	3.4	0.7	100	2.2			
c/DA	100	2.9	0	100	2.1	0			

*No differences in recoveries were observed between urinary samples from healthy or ill subjects.

Final recoveries

The final recoveries were satisfactory for the five compounds studied (Table II). Our recoveries obtained for E, NE and DA were in the same range for E and NE as in refs. 9, 12, 24 and for DA as in refs. 9, 12, 19 and 24 or higher for E and NE [15] and for DA [16]. Recoveries of DOPA and DOPAC agreed for DOPA [1, 9] and for DOPAC [5, 48] or were better for DOPA [26] and for DOPAC [5, 48] than those reported by other authors. However, slightly higher recoveries for DOPA were mentioned by some authors [19, 29].

Biological applications

DA, NE and E values obtained by applying our final procedure to urinary samples of healthy subjects correlated satisfactorily with results obtained by the use of alumina extraction alone [31] (Table V).

By contrast, in the same kind of comparison on pathological samples from severe hypertensive patients (n = 3) and from one subject with pheochromocytoma, higher amounts of the three amines were found when alumina purification only was used (Table V).

The discrepancies between the results obtained with the two methods might be explained by differences in the development of internal standard fluorescence, or in the specificity of the amine assay in the two elution buffers (0.25 M acetic acid for alumina and 0.4 M ammonium acetate for the Amberlite procedure). Table V shows that the differences in NE values resulted primarily from higher inhibition of the NE internal standard in alumina extracts. For DA, fluorescence factors (internal standard and assay specificity) accounted for differences averaging 20% between the two methods, but were not sufficient to explain the great variations observed in urinary samples. The fluorescence data are inadequate to account for 40% differences in E amounts obtained after the two methods were applied to pathological samples.

Since the greatest discrepancies between the results were observed in pathological samples, it may be reasonably assumed that interferences arise either from therapeuric drugs or from abnormal metabolites not eliminated by a single-step alumina procedure. The formation of catechols as intermediate metabolites of N-alkyl amphetamines has been recently suggested by Coutts et al. [49]. The possibility of such interference demonstrates the need for high purification. Our final procedure supplies this need.

General comments

No previous published procedure enables one to extract DOPA, DOPAC, E, NE and DA from a single urinary sample, and to obtain these five compounds with a high degree of purification allowing their specific assay.

The extraction procedure described in this paper consists in three steps: (i) purification of hydrolyzed urines on alumina at pH 8.40; (ii) separation of catecholamines from catecholacides on Amberlite CG-50; and (iii) ether separation of DOPA and DOPAC.

The step sequence is well adapted to urine analysis for two reasons. The aluminum oxide purification eliminates from urine most salts, proteins and pigments that would disturb the subsequent ion-exchange separation of the five compounds and their fluorimetric assay. The Amberlite step leads to high-

ly effective separation of DOPA plus DOPAC and catecholamines. Furthermore, the three steps are necessary to ensure a high extraction specificity for the five compounds. DOPAC is extracted by ether and separated from DOPA which is completely retained in the aqueous phase. This step eliminates the high interference of DOPA (44%) on the colorimetric assay of DOPAC. DOPA, which interferes in the E, NE and DA assay, is always separated from these amines without any overlap. E, NE and DA are removed from Amberlite in the same fraction but the low interference of NE (0.7%) and E (0.1%) on the DA fluorimetric assay [30] and the specificity of E and NE assay [31] do not require a better separation between the three catecholamines. The interference of related compounds is either completely eliminated or greatly reduced by the whole procedure. This point has not been especially studied by most other authors.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institute of Medical Research (INSERM), France (ATP 71, No. 1.408); from the Centre National de la Recherche Scientifique (E.R. No. 96, "Catecholamines"); from the Foundation of Industry for Pharmaceutical Research, France; from the Department of Biology of CEA (Saclay, France); and from the UER of Human Biology, University Claude Bernard (Lyon, France). We are indebted to Dr. Pichat from CEA (Saclay) for preparing the required labeled compounds studied in this paper, and to Mrs. Jacqueline Pequignot, CNRS, technician, for her technical assistance.

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Biomedical Applications

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DETERMINATION OF DI-AND POLYAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF THEIR 5-DIMETHYLAMINONAPHTHALENE-1-SULFONYL DERIVATIVES

NIKOLAUS SEILER*, BERND KNÖDGEN

Max-Planck-Institute for Brain Research, Frankfurt/M (G.F.R.)

and

FRIEDHELM EISENBEISS

E. Merck, Darmstadt (G.F.R.)

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SUMMARY

Using a Lichrosorb RP-8 reversed-phase column and a methanol—water gradient elution program, it is possible to separate within 40 min and to determine routinely in picomole quantities the natural di- and polyamines. The precision of the method is comparable to the thin-layer chromatographic procedures, the separations are most efficient, and the method can be fully automated. A modified gradient enables the repeated assay of spermidine and spermine within 20 min. The method is suited for polyamine analyses in tissues and body fluids.

INTRODUCTION

Widespread interest in the natural di- and polyamines [1, 2] has led to the development of a number of methods for their rapid and sensitive assay. Most of these methods have been summarized recently [3—6]. Separation of the amines by ion-exchange column chromatography with automated instruments [7—17] is the favored method for routine assay of polyamines in urine and body fluids. Reaction of amines with fluorescamine either before or after their separation by thin-layer or high-performance liquid chromatography (HPLC) has been published [18—21]. However, no practical application of these methods has been reported. Radio-immunological methods [22, 23] for polyamine determinations in clinical screening programs seem to be most

*Correspondence should be addressed to: N. Seiler, Centre de Recherche Merrell International, 16 rue d'Ankara, 67084 Strasbourg, France.

promising. If the radio-immunoassay be disregarded, the most sensitive method is still the estimation of polyamines as their 5-dimethylaminonaphthalene-1-sulfonyl (Dns) derivatives [3, 24—29], especially if high-performance thin-layer chromatography (HPTLC) is used for the separation [30]. To combine the sensitivity of the dansylation procedure with a method suited for automation, HPLC was suggested for the separation of the Dns derivatives of polyamines. Two systems have been described in the literature, using mixtures of organic solvents for elution [31, 32]. During recent years our laboratory has made several attempts to develop a routine procedure of polyamine determination on the basis of the dansylation reaction, using reversed-phase columns with lipophil molecules bound to silica cores. Separations were adequately rapid and reproducible in successive runs with reference samples. However, difficulties were encountered with putrescine determinations in tissues and body fluids containing only low concentrations (10—20 nmoles/g) of putrescine. These difficulties were overcome by a purification step between the dansylation reaction and the HPLC separation. This procedure and a routine HPLC system are described in the present work.

MATERIALS

Chemicals

Chemicals were of A grade (E. Merck, Darmstadt, G.F.R.). The methanol for the preparation of the gradient was distilled before use. 5-Dimethylaminonaphthalene-1-sulfonylchloride (Dns-Cl) and reference samples of the Dns derivatives were prepared in our laboratory according to published procedures [25]. 1,4-Diamino-2-butyne was a gift of Dr. H. Fischer, Max-Planck-Institute for Brain Research, Frankfurt/M., G.F.R.

Equipment

A Varian 8500 high-pressure liquid chromatograph was used in combination with a Perkin-Elmer fluorescence spectrophotometer 204, equipped with an 8-μl flow cell and a Varian two-channel recorder. To minimize the influence of scattered light, a Woods filter was adjusted in the path of the activating, and a 420-nm cut-off filter in the emitted light. (Activation of fluorescence at 360 nm, fluorescence measurement at 510 nm.) Separations were achieved on a 250 × 3 mm Hibar pre-packed column, Lichrosorb RP-8, 7 μm, Merck; based on its capacity, mass-transfer properties and selectivity this support is especially suited for the separation of compounds of medium polarity.

METHODS

Sample preparation

Tissues and cells were homogenized with 0.2 *N* perchloric acid. A known amount of 1,6-diaminohexane · 2 HCl was added to the homogenates as internal standard. The perchloric acid extracts were reacted with Dns-Cl by addition of 3 volumes of a solution of Dns-Cl in acetone (10 mg/ml), saturation with sodium carbonate, and reaction at room temperature overnight. Excess of reagent was removed by the addition of a concentrated solution of proline in water, as described previously [25]. The Dns derivatives were extracted

by shaking with 6 ml of toluene. Five milliliters of the toluene phase were evaporated to dryness. The residue was redissolved in 3 ml of toluene.

Pre-separation of the polyamine derivatives

Disposable 5-ml polypropylene pipettor tips are closed on the constricted end with a cotton-wool plug and filled with 2 g of silica gel 60 (0.06—0.2 mm). The toluene solutions of the Dns derivatives are applied to these silica-gel columns and the columns are first washed twice with 3 ml of toluene, and then with one 5-ml portion of toluene—triethylamine (10 : 1). The eluates are discarded. The Dns-di- and polyamines are eluted together with other Dns-amine derivatives with 4 ml of ethyl acetate. Dns-amino acid derivatives are not eluted under these conditions. The ethyl acetate solutions are evaporated to dryness. The residues are dissolved in 3 volumes of methanol and then 1 volume of water is added. Dns-amine derivatives of liver extracts equivalent to 100 mg of tissue are dissolved in 0.6 ml of methanol—water (3 : 1), cell extracts in appropriately smaller volumes.

HPLC separation

After equilibration of the column at a rate of 60 ml/h with methanol—water (57.5:42.5) 10—50-μl aliquots of the Dns derivative solutions are applied to the Hibar pre-packed column via the stopped-flow injection system. Gradient elution is started with the same methanol concentration, which is then increased linearly by an increment of 0.5% per minute. From 20 to 30 min the methanol increment is 1.5% per minute and it is further increased to 3% per minute after 30 min. Elution with pure methanol is continued for 4 min to elute impurities (see Fig. 1). At 40 min the gradient is switched to the initial methanol—water mixture. Before the next run the column is equilibrated again with 57.5% methanol for 10 min.

When only spermidine and spermine are to be determined, a two-step methanol—water gradient can be used, as indicated in Fig. 2. This allows the repetitive separation of tissue samples within 20 min.

RESULTS

Fig. 1 shows the separation of equivalent amounts of mono-, bis-, tri- and tetra-Dns derivatives of amines under conditions described in the Methods section and in the legend to this figure. Clearly the retention time increased with the number of Dns residues. Only β-phenylethylamine eluted together with the group of bis-Dns derivatives, owing to the unsubstituted benzene ring. Within the groups of mono-, bis-, tri- and tetra-substituted amines, the length of the hydrocarbon chain dictated the retention time — as is to be expected for reversed-phase elution. This elution pattern is favorable for the analysis of di- and polyamines, since ammonia, which occurs at high concentration in tissues, and Dns-dimethylamine, the most abundant side-product of the dansylation reaction [25], were eluted well before the bis-Dns derivatives of the aliphatic diamines.

Another aspect of the method is also visible in Fig. 1: as pointed out pre-

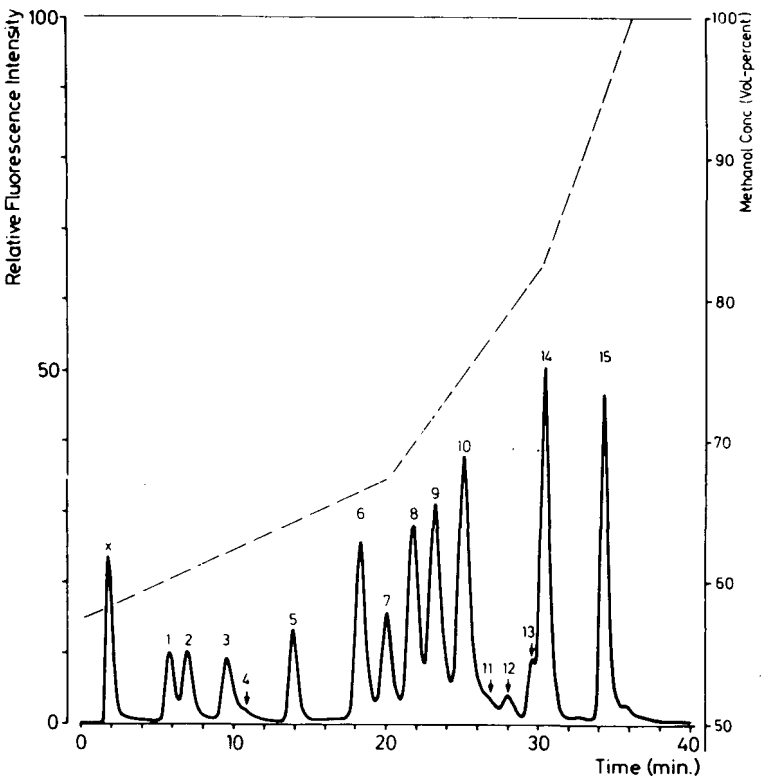

Fig. 1. Separation of Dns-amine derivatives by reversed-phase HPLC. Column: Hibar prepacked column (250 × 3 mm) Lichrosorb RP-8 (7 μm). Chromatograph: Varian 8500. Detector: Perkin-Elmer fluorescence spectrophotometer Model 204, with 8-μl flow cell. Fluorescence activation at 360 nm; fluorescence measurement at 510 nm. Elution: three step water—methanol gradient (60 ml/h). (1) 57.5 to 67.5%, Δmethanol = 0.5%/min (20 min). (2) 67.5% to 82.5%, Δmethanol = 1.5%/min (10 min). (3) 82.5% to 100%, Δmethanol = 3%/min (10 min). Equilibration before the next run for 10 min with 57.5% methanol. X = 5-Dimethylaminonaphthalene-1-sulfonic acid (Dns-OH); 1 = Dns-ammonia; 2 = N-Dns-ethanolamine; 3 = Dns-methylamine; 4 = Dns-2-oxopyrrolidine (reaction product of 4-aminobutyric acid); 5 = Dns-dimethylamine; 6 = bis-Dns-agmatine; 7 = Dns-2-phenylethylamine; 8 = bis-Dns-1,4-diaminobutane (putrescine); 9 = bis-Dns-1,5-diaminopentane (cadaverine); 10 = bis-Dns-1,6-diaminohexane; 11 = bis-Dns-histamine; 12 = O,N-bis-Dns-5-hydroxytryptamine (serotonin); 13 = O,N-bis-Dns-2-(4-hydroxyphenyl)ethylamine (*p*-tyramine); 14 = tris-Dns-spermidine; 15 = tetrakis-Dns-spermine. The derivatives were applied in equimolar amounts (1 nmol) dissolved in 20 μl of methanol—water (3/1, v/v).

viously [25, 33], fluorescence intensity of the Dns derivatives increases with the number of fluorophores attached to the molecule. However, this does not apply to the aryl ethylamine derivatives: even though the dansyl fluorophores are separated by the ethyl side-chain of histamine, serotonin, tyramine, etc., the fluorescence quantum yield of these amines is much lower than that of the corresponding N-mono-Dns derivatives, presumably owing to an intramolecular charge-transfer interaction of the fluorophores. Since the concentrations of

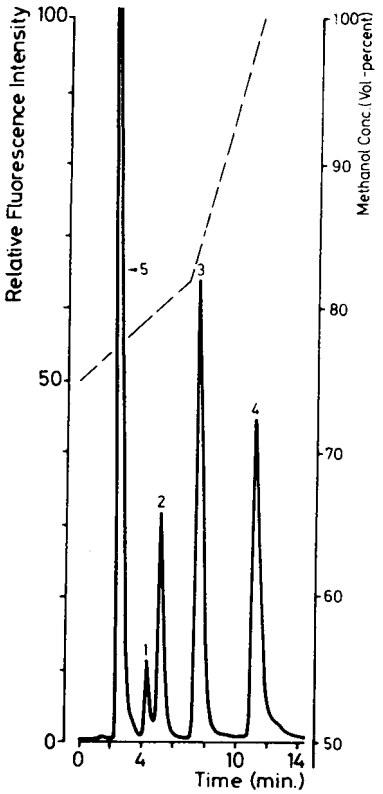

Fig. 2. Separation of the Dns-amine derivatives of a normal mouse liver using an elution program suited for the rapid determination of spermidine and spermine. The sample was identical with that shown in Fig. 4; however, about 0.3 nmoles of bis-Dns-1,4-diaminobutane and 1 nmole of bis-Dns-1,6-diaminohexane were added. The sensitivity of recording was identical with that shown in Fig. 4, dotted line. Elution program: two step water—methanol gradient (75 to 82%, $\Delta_{methanol}$ = 1%/min; 82 to 100%, $\Delta_{methanol}$ = 4%/min. 1 = Bis-Dns-1,4-diaminobutane (putrescine); 2 = bis-Dns-1,6-diaminohexane (internal standard); 3 = tris-Dns-spermidine; 4 = tetrakis-Dns-spermine; 5 = DNS-ammonia.

these amines are normally low in tissues, they do not interfere with the polyamine determinations.

The effectivity of the standard separation procedure and the influence of the steepness of the water—methanol gradient on the separation and peak form is demonstrated in Fig. 3. The separation of the homologous bis-Dns diamines by the standard procedure was not complete (Fig. 3A). Bis-Dns-1,2-diaminoethane, bis-Dns-1,3-diaminopropane, and bis-Dns-1,4-diaminobutane (putrescine) overlapped. Bis-Dns-1,2-diaminoethane and bis-Dns-1,6-diaminohexane are non-natural compounds. They can be used as internal standards. The former compound was suggested for this purpose by Newton et al. [32]. Since bis-Dns-1,6-diaminohexane elutes between bis-Dns-cadaverine and tris-Dns-spermidine, we prefer this compound as standard. (Another reason is that

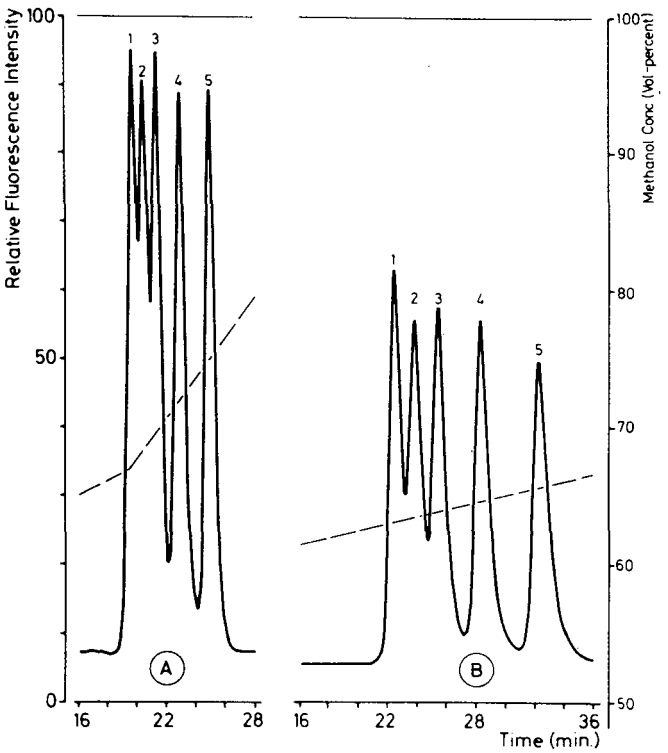

Fig. 3. Separation of the bis-Dns derivatives of the homologous diamines by reversed-phase HPLC. Column, chromatograph and detector as described in the legend to Fig. 1. Elution: water—methanol gradients; 60 ml/h. Two-step gradient (57.5 to 67.5 $\Delta_{methanol}$ = 0.5%/min (20 min); and 67.5% to 82.5%, $\Delta_{methanol}$ = 1.5%/min (10 min). (B) Linear gradient (57.5 to 67.5%, $\Delta_{methanol}$ = 0.25%/min (40 min). 1 = Bis-Dns-1,2-diaminoethane; 2 = bis-Dns-1,3-diaminopropane; 3 = bis-Dns-1,4-diaminobutane (putrescine); 4 = bis-Dns-1,5-diaminopentane (cadaverine); 5 = bis-Dns-1,6-diaminohexane. The amine derivatives were applied in equimolar amounts (1 nmol) dissolved in 20 µl of methanol—water (3:1, v/v). Detector sensitivity is identical in (A) and (B).

the same compound proved to be useful as standard for the mass spectrometric determination of putrescine and cadaverine [34].) Although the peaks of bis-Dns-putrescine and bis-Dns-1,3-diaminopropane overlap, their separation is nevertheless adequate for most purposes. By using a flat gradient, the separation of these overlapping compounds can be somewhat improved at the expense of sensitivity (Fig. 3B).

The method was applied for the determination of putrescine, spermidine and spermine in the livers of normal and 1,4-diamino-2-butyne-treated mice, and in chick embryo fibroblasts treated with the same putrescine analog. (A description of the results of this work will be given elsewhere.) The separation of tissue samples were recorded at two different sensitivities, in order to cover the enormous concentration difference between putrescine and the polyamines spermidine and spermine. As can be seen in Fig. 4, a peak is barely

visible beside the peaks of Dns-ammonia and the Dns derivatives of the polyamines, when recording is performed at reduced (1/20) sensitivity. This shows impressively the quantitative significance of the polyamines in tissues. At a detector sensitivity sufficient to measure less than 20 pmoles of the bis-Dns-diamines, there is still little background noise. It also appears from this figure that 1,3-diaminopropane and 1,5-diaminopentane (cadaverine) are present in this tissue, if at all, only in very low concentrations. Pre-treatment of the animals with 1,4-diamino-2-butyne (100 mg/kg, intraperitoneally) elevated putrescine concentration in liver considerably, probably owing to its inhibitory effect on diamine oxidase [35] (Fig. 5). Eight hours after its administration the compound was still detectable in the liver in amounts of 20 nmoles/g. Putrescine and polyamine concentrations of normal and transformed chick embryo fibroblasts were comparable (Fig. 6). Cadaverine was not detected in these cells.

Peaks were evaluated by measurement of peak height and peak width at

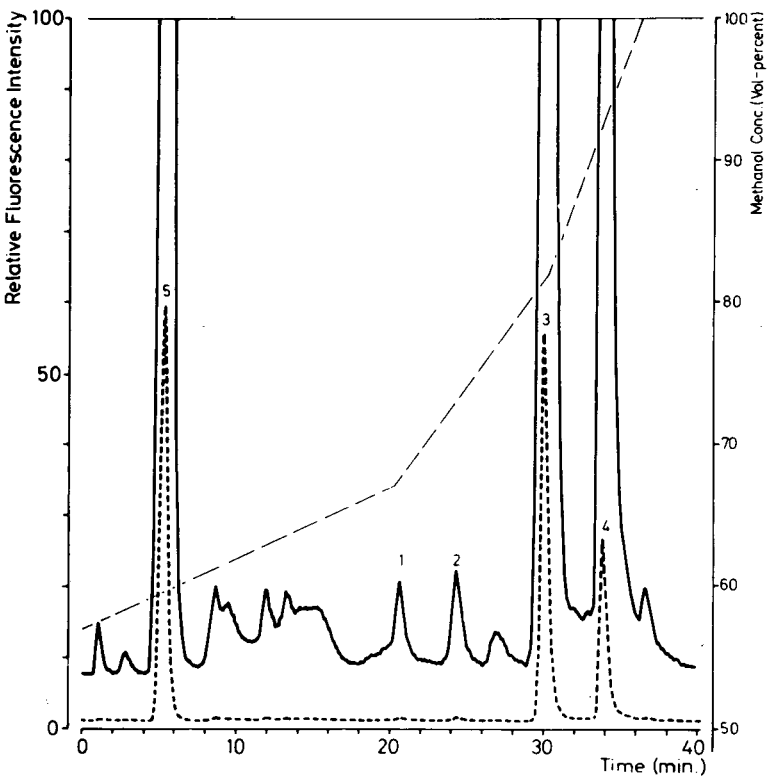

Fig. 4. Separation of the Dns-amine derivatives of a normal mouse liver in the system described in the legend to Fig. 1. 1 = Bis-Dns-1,4-diaminobutane (putrescine); 2 = bis-Dns-1,6-diaminohexane (internal standard, 16.6 pmoles); 3 = tris-Dns-spermidine; 4 = tetrakis-Dns-spermine; 5 = Dns-ammonia. The separated sample corresponded with 2.7 mg of liver. Dotted line: recording at reduced (1/20) sensitivity.

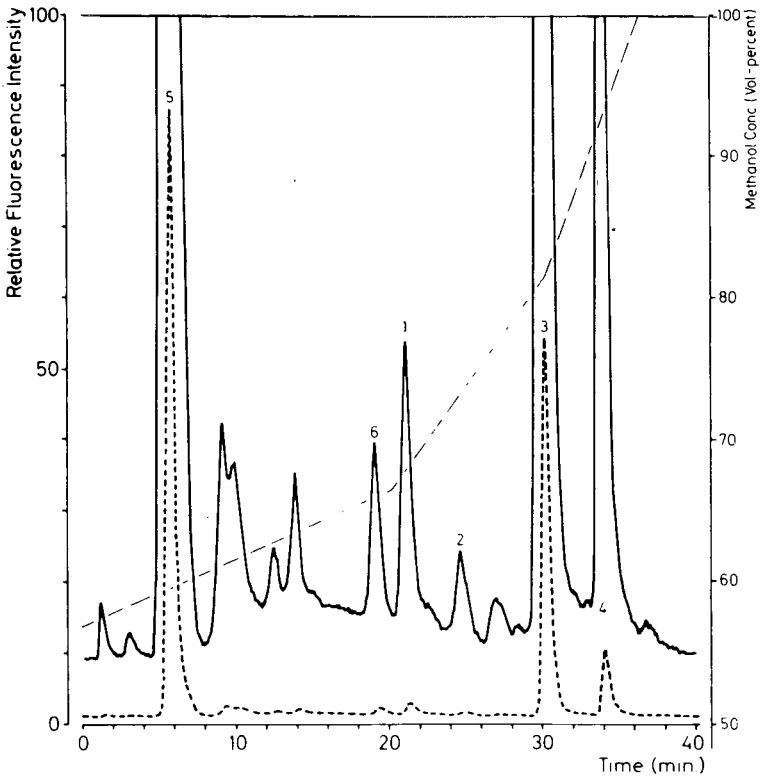

Fig. 5. Separation of the Dns-amine derivatives of mouse liver, 8 h after treatment of the animal with 1,4-diamino-2-butyne at 100 mg/kg. Separation conditions are identical with those in Figs. 1 and 3. Peaks: 1 = bis-Dns-1,4-diaminobutane (putrescine); 2 = bis-Dns-1,6-diaminohexane (internal standard, 16.6 pmoles); 3 = tris-Dns-spermidine; 4 = tetrakis-Dns-spermine; 5 = Dns-ammonia; 6 = bis-Dns-1,4-diamino-2-butyne. The separated sample corresponded with 2.7 mg of liver. Dotted line: recording at reduced (1/20) sensitivity.

half height. Although this method is of limited accuracy, the mean standard deviation (S.D.) of measurements repeated on three different days was nevertheless less than ±7%. Table I summarizes these results. It appears from this table that the S.D. was about the same for the whole concentration range from 20 pmoles to 2 nmoles of amine. The relationship between amount of substance and recorded peak areas was linear over this range (linear regression coefficient > 0.988). The S.D. of the measured values from the calculated regression curve was within ±2% for all amines.

DISCUSSION

Estimation of polyamines as their Dns deviations in tissues and body fluids by thin-layer chromatography has proved its usefulness as a sensitive and reliable method [26, 28, 29]. Using automated application of sample and fluorescence scanning in situ, especially of HPTLC plates [30], makes the

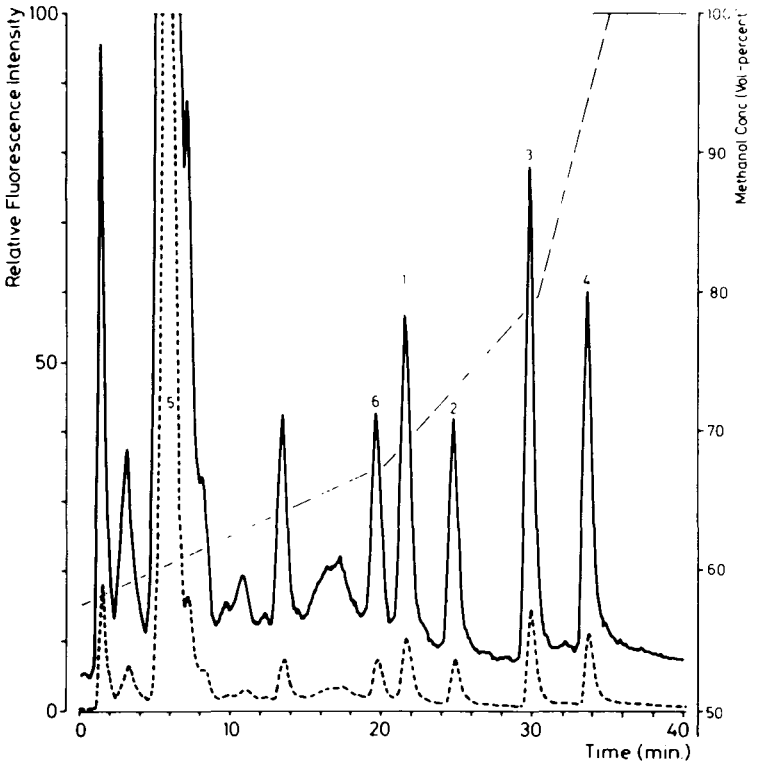

Fig. 6. Separation of the Dns-amine derivatives of chick embryo fibroblasts transformed with Rous sarcoma virus (Schmidt—Ruppin) and treated with 0.1 m*M* 1,4-diamino-2-butyne for 24 h. The separated sample corresponded with 0.044 mg of cell protein. Separation conditions were identical with those described in the legend to Fig. 1. Peaks: 1 = bis-Dns-1,4-diaminobutane (putrescine); 2 = bis-Dns-1,6-diaminohexane (internal standard, 125 pmoles); 3 = tris-Dns-spermidine; 4 = tetrakis-Dns-spermine; 5 = Dns-ammonia; 6 = bis-Dns-1,4-diamino-2-butyne. Dotted line: recording at reduced (1/5) sensitivity.

method very rapid. Many samples can be handled in parallel. From this point of view, sequential separation methods, such as HPLC, are of comparable usefulness only if their superior separatory quality is decisive or if they are automated. For various reasons the method described in this paper is a favorable alternative to TLC. (1) Adequate separations of the homologous diamines and polyamines are achieved within a short time. TLC of these amines is not adequate in a one-dimensional separation. For complete separation a two-dimensional separation is required [25, 36]. (2) The reversed-phase column allows rapid regeneration. Repeated separations are possible within a short time. This is an advantage over the otherwise highly effective ion-exchange column separations with advanced technology [15—17]. (3) The Dns derivatives can be collected from the column outflow and characterized by mass spectrometry and by other suitable methods. (4) Since radioactivity measurements can be achieved on collected fractions, HPLC separations of Dns-amines

TABLE I

REPRODUCIBILITY OF HPLC DETERMINATIONS OF Dns-AMINE DERIVATIVES

Mean values ± S.D. of three determinations; relative fluorescence unit = peak height × peak width at half height of the recorded curves.

Amount (nmoles)	Ammonia	Ethanol-amine	Methyl-amine	Dimethyl-amine	Agmatine	β-Phenyl-ethylamine	Putrescine	Cadaverine	Hexamethylene-diamine	Spermidine	Spermine
2.0	4025 ±385	4617 ±161	5125 ±156	5300 ±229	13125 ±675	6800 ±606	16617 ±176	15625 ±715	15818 ±588	20625 ±1231	20017 ±825
1.0	2321 ±203	2522 ±376	2725 ±220	2500 ±173	6138 ±376	3100 ±241	8104 ±188	7659 ±420	7675 ±435	10358 ±429	9367 ±555
0.5	1099 ±69	1223 ±48	1465 ±131	1297 ±91	3430 ±93	1653 ±134	4227 ±162	4002 ±238	3667 ±510	5360 ±688	4880 ±450
0.075	188 ±15	204 ±32	236 ±10	200 ±11	490 ±14	269 ±10	605 ±16	638 ±24	600 ±48	868 ±25	731 ±61
0.02	50 ±4.6	60 ±6.5	71 ±9.6	57 ±3.0	134 ±6.1	75 ±4.9	182 ±14.9	171 ±5.1	201 ±13.0	228 ±13.3	211 ±4.6

may prove useful in metabolic studies and for quantitation using double-isotope methods [6]. Although the equipment used in the present work was not automated, the separation method is suitable for full automation. The ease with which the separations can be adapted to special requirements may be a further aspect in favor of reversed-phase HPLC as a routine method.

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TOWARDS A DEFINITIVE ASSAY OF CREATININE IN SERUM AND IN URINE: SEPARATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C.K. LIM, W. RICHMOND, D.P. ROBINSON and S.S. BROWN

Division of Clinical Chemistry, Clinical Research Centre, Harrow, Middlesex HA1 3UJ (Great Britain)

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SUMMARY

A fast and sensitive method for the separation of serum and urinary creatinine is described. For the preliminary purification of serum and urine, a cation-exchange column is used to remove protein, anions and neutral compounds prior to isolation of creatinine by high-performance liquid chromatography. A reversed-phase system with 0.01 *M* ammonium acetate solution as the mobile phase can separate creatinine in 7.5 min at a flow-rate of 1 ml/min. The purity of the separated creatinine is proved by derivatization using trifluoroacetic anhydride, followed by gas chromatography and mass spectrometry.

Although this method of purification was designed for incorporation into a definitive assay, the ease and speed of analysis makes it very attractive for routine clinical use.

INTRODUCTION

The concentrations of creatinine in serum and in urine are commonly estimated in clinical laboratories since their ratio for a given patient affords a quantitative index of renal function. The majority of assay methods are based on the Jaffe reaction [1] in which creatinine reacts with alkaline picrate to form an amber-yellow colour. This technique, however, is non-specific, and many compounds are known to react with picric acid or cause interference [2]. Preliminary purification of creatinine by ion-exchange chromatography has been used in efforts to overcome these difficulties [3—8], but it is unlikely that such a method will abolish all interferences.

We are interested in developing a definitive method of assay [9] with high specificity and accuracy, based on stable isotope dilution—mass spectrometry in a similar way to that described for serum calcium [10] and serum phosphate [11]. The development of such a method as for uric acid [12] would necessitate the isolation of pure creatinine and the preparation of a volatile

derivative for gas chromatography and mass spectrometry. Tris(trimethylsilyl)creatinine has been prepared [13] by treating creatinine with N-methyl-N-trimethylsilyltrifluoroacetamide and chromatographed on a 3% OV-1 column. This derivative, however, was found to be extremely sensitive to moisture and was unsuitable for accurate isotope-dilution work as its mass spectrum had too many interfering peaks due to the many naturally occurring isotopes of silicon. A new derivative was therefore required.

In this paper we describe the separation of creatinine from biological fluids by high-performance liquid chromatography (HPLC) and the synthesis of O-trifluoroacetylcreatinine. The HPLC separation is fast, reliable and requires minimal work-up. It can also be applied to the routine analysis of serum and urinary creatinine. The volatile derivative, which has not previously been described, is much more stable than the trimethylsilyl derivative and its mass spectrum displays no interfering peaks since naturally occurring fluorine has only one isotopic form.

EXPERIMENTAL

Materials and reagents

Unless otherwise stated, all reagents were AnalaR grade (BDH, Poole, Great Britain).

Ion-exchange procedure. Cation-exchange resin AG 50W-X12 (H^+), 200—400 mesh (Bio-Rad Labs, Richmond, Calif., U.S.A.).

Adsorption buffer: this buffer (pH 3.0) contained citric acid (40 mmoles/l) and disodium hydrogen orthophosphate (20 mmoles/l).

Elution reagents: sodium acetate, 0.1 and 0.5 mole/l; ammonium acetate, 0.1 and 0.5 mole/l.

Sodium hydroxide solution: 2.5 moles/l.

Lowry protein estimations. Alkaline tartrate reagent: this was prepared by dissolving sodium carbonate (20 g) and sodium or potassium tartrate (0.5 g) in 1l of sodium hydroxide solution (0.1 mole/l).

Copper sulphate solution: 4 mmoles/l.

Working alkaline copper reagent: this was prepared freshly each day by mixing alkaline tartrate reagent (45 ml) with copper sulphate solution (5.0 ml).

Folin—Ciocalteu reagent: Folin and Ciocalteu's phenol reagent (BDH) was diluted 4-fold with distilled water.

Standard protein solution: bovine serum albumin 100, 200, 300, 400, and 500 mg/l in distilled water.

Control serum. Wellcomtrol 3 (Wellcome Reagents, Beckenham, Great Britain) stated to contain 0.71 mmole creatinine per litre was used as control serum.

Creatinine determination by the Jaffe reaction. Sodium hydroxide: 0.5 and 1.5 moles/l.

Saturated picric acid: about 14 g/l.

Standard creatinine solutions: 0.443, 0.885, 1.33 and 1.77 mmoles/l in distilled water.

Instruments. The following instruments were used: a Pye-Unicam SP 1800 ultraviolet—visible spectrophotometer, a Perkin-Elmer Model 577 grating

infrared spectrophotometer, a Pye-Unicam Series 106 gas chromatograph fitted with a flame ionization detector (FID), a Varian MAT-731 high-resolution mass spectrometer and a Waters Assoc. Model ALC - GPC 204 liquid chromatograph comprising of a M6000A solvent delivery system a U6K universal injector and a Model 440 absorbance detector fixed at 254 nm.

Isolation of creatinine from serum and urine by cation-exchange chromatography

Polypropylene pipette tipes (Oxford Macro-set, Boehringer London, Lewes, Great Britain) were plugged with non-absorbant cotton wool and used as semi-micro chromatography columns. Aliquots of the resin (100 mg) were suspended in portions (2.5 ml) of sodium hydroxide solution, transferred to the columns, and allowed to drain briefly. Each column was then washed with water (2.0 ml), followed by adsorption buffer (4.0 ml).

The sample (0.5 ml) of serum or of urine (diluted 50 fold with water), standard creatinine solution, or water as a reagent and a column blank, was mixed with adsorption buffer (5.0 ml) and 5.0 ml of each diluted sample allowed to drain through a prepared column. Following a wash with distilled water (4.0 ml), the adsorbed creatinine was eluted with the appropriate eluting reagent (3.0 ml).

Measurement of protein concentration in eluates

To monitor the elution of protein from the ion-exchange columns, a control serum was processed according to the above procedure. Fractions (0.5 ml) were collected during the water wash and sodium acetate (0.5 mole/l) elution steps, and analysed as follows. Portions (0.3 ml) of each fraction or of standard protein solutions and of distilled water (to serve as blank) were added to working alkaline copper reagent (2.0 ml), mixed, and kept at room temperature for 5 min. Folin-Ciocalteu reagent (0.2 ml) was then added and the reaction mixtures were kept for a further 30 min at room temperature for colour development. The absorbances of the test and standard solutions were measured against the blank at 720 nm.

Elution of creatinine from the cation-exchange column

To monitor the elution of creatinine from the columns, an aqueous creatinine solution (0.885 mmole/l), a control serum (Wellcontrol 3) and distilled water (as a reagent and column blank) were each taken through the cation-exchange procedure described above. During the elution of creatinine with sodium acetate solution (0.5 mole/l), fractions (0.5 ml) were collected and analysed using the Jaffe reagents as follows. Portions (0.2 ml) were added to sodium hydroxide solution (1.0 ml); picric acid solution (0.2 ml) was then added, and the reaction mixtures incubated at room temperature for 30 min. The absorbances of the test solutions were measured at 490 nm against the column blank.

The elution of creatinine with ammonium acetate solutions ranging in concentration from 0.1 to 0.5 mole/l was also investigated. The Jaffe reac-

tion could not be used in this instance because of interference from ammonium ions; consequently creatinine was monitored in portions (0.5 ml) of the ammonium acetate eluates (diluted to 3.0 ml with water) by direct measurement of absorbance at 235 nm (the absorption maximum of creatinine at pH 7.0) against an appropriate blank.

Recovery experiments

The recovery of creatinine which had been added to a control serum, a pooled patients' serum, ultrafiltered serum, or urine (diluted 50 fold) was determined by diluting portions of each of these specimens (a) with an equal volume of a standard solution of creatinine (0.885 mmole/l) and (b) with an equal volume of water. Creatinine was then isolated from the samples by the ion-exchange procedure, and measured by the Jaffe reaction which was carried out as follows. A portion of each eluate (1.0 ml) was added to sodium hydroxide solution (1.5 moles/l, 1.0 ml) followed by picric acid solution (0.5 ml), the procedure was calibrated by treated creatinine standards similarly, with absorbance measurements made at 490 nm against a blank as before. The recoveries were estimated from the results so obtained.

High-performance liquid chromatography

The separation was carried out on a reversed-phase column (30 cm × 4 mm I.D.) consisting of a monomolecular layer of octadecyltrichlorosilane chemically bonded to 10 μm silica (μBondapak C_{18}, Waters Assoc., Milford, Mass., U.S.A.). Ammonium acetate solution (0.01 mol/l) was used as the mobile phase and elution was at 1 ml/min with a pressure drop of 1000 p.s.i.

For the isolation of serum and urinary creatinine, the specimens were pre-purified by the ion-exchange procedure described above. The eluate (3 ml) was then evaporated to dryness with a rotary evaporator. The residue was redissolved in 100 μl of ammonium acetate solution (0.01 mole/l) and 50 μl of the solution were injected into the chromatograph.

The peak corresponding to creatinine was collected and the solvent evaporated off as before.

Preparation and gas chromatography of O-trifluoroacetylcreatinine

The creatinine obtained by HPLC was treated with 100 μl of trifluoroacetic anhydride (TFAA) and heated for 5 min at 60°. After cooling, 2 μl of the clear solution were injected into the gas chromatograph. The column used (3.25 × 2.5 mm I.D.) was packed with 3% OV-210 on Chromosorb W, 80—100 mesh. The injector and oven temperatures were set at 200° and 130°, respectively.

This compound could be isolated as an oil by heating creatinine with TFAA and subsequent removal of excess TFAA and the trifluoroacetic acid formed with a rotary evaporator.

Preparation of creatinine trifluoroacetate

Creatinine (1 mg) was dissolved in trifluoroacetic acid (2 ml), and diethyl ether (5 ml) was added slowly with stirring until no more crystals were formed.

The crystals were filtered, washed with diethyl ether and recrystallised from choloroform—methanol (m.p. 155—156°).

Infrared spectroscopy

The spectrum of O-trifluoroacetylcreatinine was recorded as a thin film, and those of creatinine and creatinine trifluoroacetate as Nujol mulls. Potassium bromide prisms were used throughout.

Mass spectrometry

The mass spectrum of O-trifluoroacetylcreatinine was measured by injecting a sample in trifluoroacetic anhydride on to a gas chromatographic column (3.25 × 2.5 I.D.) packed with Chromosorb W, 80—100 mesh coated with 3% OV-210 coupled to the mass spectrometer. The source temperature was 200°.

RESULTS AND DISCUSSION

Isolation of creatinine by ion-exchange chromatography

When serum samples were applied to the cation-exchange columns in the manner described, only 0.1% of the protein present in the sample adsorbed to the column. Furthermore, as indicated by the elution profiles in Fig. 1, 90% of this adsorbed protein was eluted in the water wash. Consequently the final eluate, containing the desorbed creatinine, contained only 0.01% of the protein originally applied to the column.

As shown in Fig. 2, creatinine which had been adsorbed to the column from serum or aqueous solutions of creatinine was eluted (99 ± 1%) with

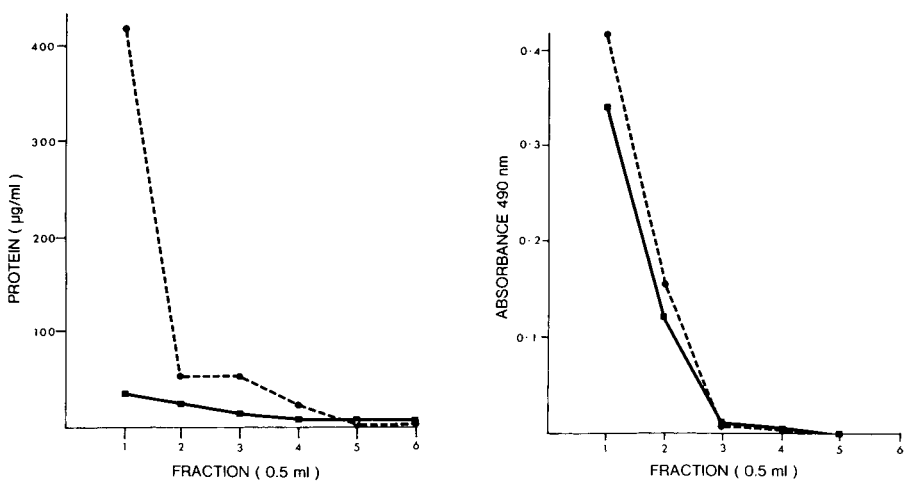


Fig.1. Elution of protein from cation-exchange column. —, protein in sodium acetate eluate; - - - -, protein elution during water wash.

Fig.2. Elution of creatinine from cation-exchange column. —, control serum; - - - -, creatinine standard.

2.5 ml of sodium acetate solution (0.1 mol/l). Similar results were obtained when ammonium acetate (0.1 mol/l) was used as the eluent.

Recoveries of creatinine which had been added to a control serum, a pooled patients' serum, ultrafiltered serum and urine (diluted 50 fold) when isolated by the cation-exchange procedure and assayed by the Jaffe reaction, were found to be 99, 98, 100 and 99%, respectively.

These results indicate that the isolation procedure was quantitative. It was considered that no significant advantage would be gained by including an ultrafiltration step [14] prior to the ion-exchange procedure since the small amount of protein remaining in the final eluate did not interfere with the subsequent HPLC isolation.

Ammonium acetate or sodium acetate eluents were selected for this study since it was considered that the strongly alkaline nature of the eluting buffers used by previous workers [7,8] could conceivably hydrolyse creatinine to creatine. Ammonium acetate is favoured as the eluent in the primary isolation procedure as this solvent proved to be satisfactory for further purification of the isolated creatinine by HPLC.

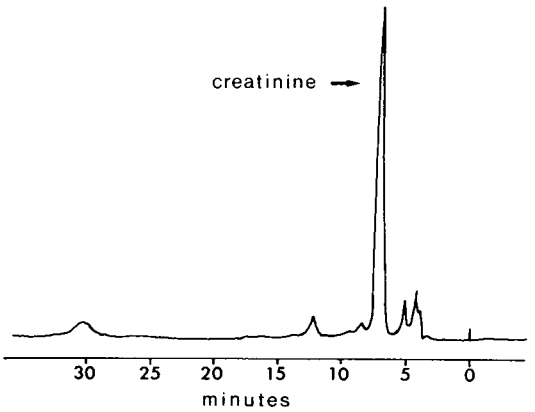
High-performance liquid chromatography

A number of solvent systems were tested before 0.01 *M* ammonium acetate solution was finally selected as the one most suitable for the separation of creatinine from biological fluids. For example, reversed-phase chromatography on a μBondapak C_{18} column with 0.005 *M* formic acid—methanol (25:1, v/v) and ion-pair chromatography with 10% methanol in PLC-B7 (containing 1-heptanesulphonic acid buffered at pH 3.5, Waters Assoc.) both gave excellent separation of synthetic creatinine standard. Unfortunately these systems proved to be unsuitable for the analysis of serum creatinine due to interfering substances from serum. Reversed-phase chromatography with ammonium acetate solution as the mobile phase effectively resolved creatinine from the interfering substances present in serum and in urine which had been observed with the other solvent systems. The concentration of the ammonium acetate solution seemed to have little influence on the retention of creatinine; thus 0.05 and 0.01 *M* solution gave virtually the same retention times, i.e. 7.5 min. It is clearly advantageous if the retention time does not vary much with slight changes in solvent concentration, since highly reproducible results can then be expected. This was shown by the analysis of over 50 serum specimens for creatinine without any alteration of retention time. Figs. 3 and 4 show typical separations of serum and urinary creatinine, respectively; it is apparent from the chromatograms that urine samples generally contained fewer interfering compounds.

The sensitivity of the UV detector allowed easy detection of 10 ng of creatinine at 0.005 a.u.f.s. without baseline drift. It was therefore possible to detect the creatinine present in 100 μl samples of normal serum.

Preparation and characterization of O-trifluoroacetylcreatinine

Since creatinine can exist in the enol form (Fig. 5), acylation of the molecule is possible. The required derivative was simply prepared by reacting creatinine with hot TFAA. The product, O-trifluoroacetylcreatinine, was



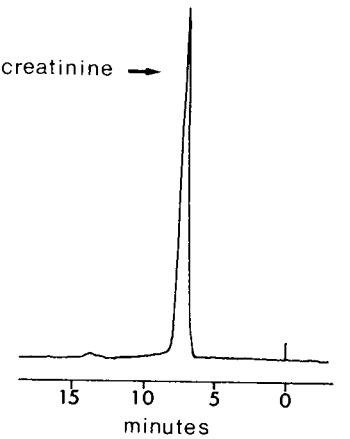


Fig.3. HPLC separation of serum creatinine.

Fig.4. HPLC separation of urinary creatinine.

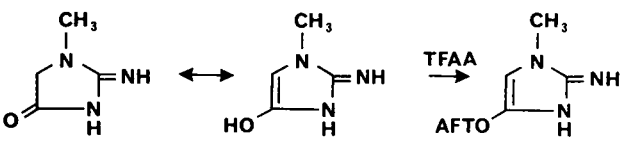


Fig.5. O-Trifluoroacylation of creatinine.

characterized by infrared (IR) spectroscopy and gas chromatography—mass spectrometry with the following results: in the IR spectra, the carbonyl absorption of creatinine at 1670 cm^{-1} was shifted to higher frequency at 1775 cm^{-1} indicating O-acylation (Fig. 6); gas chromatography on 3% OV-210 gave a single peak which, when monitored with the mass spectrometer, gave a molecular ion at M^+ = 209 confirming trifluoroacylation. Peaks at *m/e* = 140 ($M^+ - CF_3$) and *m/e* — 112 ($M^+ - COCF_3$) were also consistent with trifluoroacylation (Fig. 7). As expected from the occurrence of fluorine in only one isotopic form, it was satisfactory to find that the mass spectrum contained no peaks which might cause difficulties in isotope dilution work using [$^{15}N_3$]creatinine.

Further trifluoroacylation of the derivative was not observed. This is to be expected since after O-trifluoroacylation, hydrogen bonding of a fluorine atom of the secondary amine group of the molecule is possible (Fig. 8) and this effectively prevents further substitution. Creatinine isolated from serum and urine samples by HPLC gave the same derivative when treated with TFAA, so confirming the identity and homogeneity of the isolated compound.

O-Trifluoroacetylcreatinine was found to be stable if kept free from moisture, and was much more easily handled then tris(trimethylsilyl)creatinine. In the presence of moisture, as in "wet" organic solvents, colourless needles slowly crystallised out solutions of O-trifluoroacetylcreatinine. In the IR

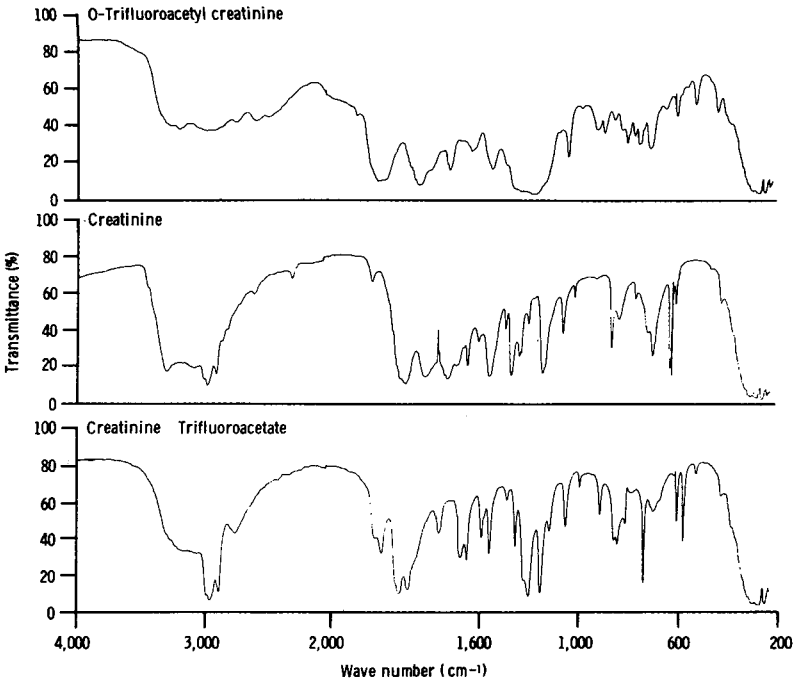


Fig.6. IR spectra of O-trifluoroacetylcreatinine, creatinine and creatinine trifluoroacetate.

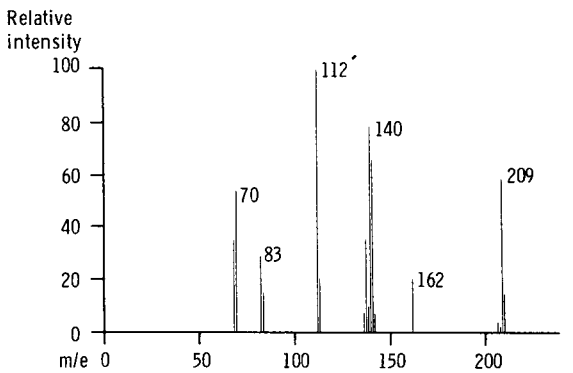


Fig.7. Mass spectrum of O-trifluoroacetylcreatinine.

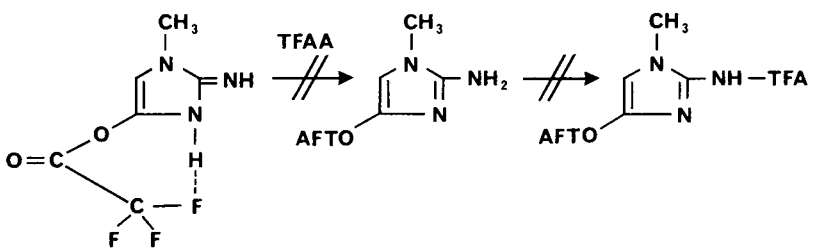


Fig.8. Scheme showing how further substitution of creatinine is prevented after O-trifluoroacylation.

spectrum of the crystalline product (Fig. 6), the carbonyl absorption of creatinine was shifted to 1710 cm^{-1} ; another carbonyl peak at 1775 cm^{-1} could be assigned to trifluoroacetic acid, since a broad hydroxyl absorption in the 3000 cm^{-1} region was typical of an acid. The structure of this product was therefore considered to be that of creatinine trifluoroacetate. This was confirmed by its conversion black to O-trifluoroacetylcreatinine by heating with TFAA and by the preparation of an identical substance on reacting pure creatinine with trifluoroacetic acid.

CONCLUSIONS

The objectives of this work were to develop an efficient method for the separation and purification of creatinine from biological fluids and a practicable means of derivatizing creatinine so that it could be characterized by gas chromatography—mass spectrometry. Both of these objectives have been realized, so that a definitive method of assay of creatinine in biological fluids is now feasible.

Successive cation-exchange chromatography and reversed-phase HPLC with UV detection offers a rapid (30 min) clean-up procedure suitable for both serum and urine. The sensitivity (detection limit 5 ng) is such that a 100-μl sample of normal serum, can be processed satisfactory. O-trifluoroacetylcreatinine is readily prepared and easily characterized by gas chromatography—mass spectrometry.

The speed and simplicity of the purification procedure suggest that by the addition of internal standard to the original specimen it would be possible to develop a reference method of assay of creatinine.

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POLYMORPHISM OF A, B AND H SUBSTANCES IN HUMAN URINE

A. FIORI, G. PANARI, G. ROSSI and D. DE MERCURIO

Istituto di Medicina Legale e delle Assicurazioni, Universita Cattolica del Sacro Cuore, Rome (Italy)

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SUMMARY

The gel-chromatographic behaviour of A, B and H substances from urine was examined and compared with that of previously described ABH salivary fractions. Urinary fractions 1 and 2 exhibited a molecular size smaller than those of salivary fractions 1 and 2. In the urine of 52 subjects a polymorphism of A, B and H substances, with four main types, was observed which is independent of the salivary one and of the so-called secretor and non-secretor status.

INTRODUCTION

Polymorphism of A, B and H substances has recently been described in human saliva [1—4] and seminal fluid [5]. Twenty gel-chromatographic types have been identified in the secretions, which are the result of various associations of five serologically active fractions of each substance, all normally contained in red-cell stroma [6]. The ABH secretory types are probably under genetic control [7].

The ABH group-specific glycoproteins in urine have been studied by Yoshida [8], Freudenberg and Eichel [9, 10], Jorpes and Norlin [11, 12], Jorpes and Thaining [13], Kobayasi et al. [14] and Yasuoka [15], and more recently by Masamune et al. [16], King et al. [17, 18], Price Evans et al. [19] and Lundblad and Berggård [20]. Low-molecular-weight ultrafiltrable group-specific compounds were then detected by King et al. [17] and subsequently identified as oligosaccharides by Lundblad [21—23] and Björndal and Lundblad [24].

In the present study the gel-chromatographic behaviour of the ABH urinary fractions was examined and compared with that of salivary fractions. Moreover, a polymorphism of A, B and H substances was observed and found to be independent of the salivary one.

MATERIALS AND METHODS

Preparation of the samples

Urine. Urine specimens were collected from 52 healthy subjects selected from those previously tested for ABH salivary types [3]. The urine of two children of a couple of true non-secretors included in the group was also examined. 250—350 ml of urine were usually obtained from each subject. In some cases, when a larger amount of group-specific substances was needed for particular analytical work, 2000—4000 ml were processed. Each sample was usually concentrated some hours after collection or, sometimes, preserved at −20° until examined. Desalting by dialysis was as a rule omitted to prevent loss of low-molecular-weight dialysable fractions. Samples were concentrated to a syrup in a rotating evaporator at 30—35°, and then kept at 4° for 5—6 h. The precipitated material was discarded by centrifugation and the liquid further concentrated to 8—10 ml. The final fluid was again maintained at 4° overnight to precipitate other salts, and an aliquot was subjected to gel chromatography. The remaining solution was kept at −20° for subsequent controls. Additional steps in the concentration process were required when 2000—4000 ml of urine were examined. Because such samples were dialysed after the first evaporation, a 100×—200× concentration was easily reached.

Saliva. Saliva specimens were obtained from the same subjects and gel chromatographed for serological assays to check the ABH secretory types as previously described [3]. Only in some cases was the presence of sub-fractions 4 and 5 [4] tested for.

Gel and ion-exchange chromatography

Sephadex G-25 (Pharmacia, Uppsala, Sweden) columns (20 × 1.2 cm) were routinely used for the determination of the individual urinary ABH patterns. Different bed sizes of the same gel (28 × 1.2 cm, or 50 × 1.2 cm) were used when grouping was performed by the automated haemagglutination-inhibition method. Distilled water was used as eluent. The flow-rates were maintained at 10—12 ml/h, and fractions of about 1—1.5 ml were collected.

For other analytical or preparative purpose, gel filtration was carried out on larger Sephadex G-25 columns (42 × 2.5 cm) and on Sephadex G-15 (28 × 1.2 cm), Sephadex G-100 (20 cm × 1.2 cm), Sephadex G-200 (40 × 1.5 cm) and on agarose Bio-Gel A-15m (100—200 mesh) and A-50m (50—100 mesh) (Bio-Rad Labs., Richmond, Calif., U.S.A.), 62 cm × 1.5 cm columns, with distilled water, or 0.05 *M* Tris—HCl buffer (pH 7.3) containing 0.9% sodium chloride, as eluents. Blue Dextran 2000, Dextran 200.000 (Pharmacia), and IgG anti-A antibodies were used as reference substances for the latter gels. The reference substances were detected by UV absorption spectrophotometry at 260 nm, by the orcinol—sulphuric acid test and by haemagglutination of A red cells, respectively.

Dialysis and ultrafiltration

Dialyses and ultrafiltrations were carried out at 4° in Visking 32/32 tubing

(Scient. Instr. Centre, London, Great Britain). XM 100 and UM 10 Diaflo membranes with an Amicon Model 52 ultrafiltration cell (Amicon, Oosterhout, The Netherlands) were also used with nitrogen at differential pressures of 0.68 kg/cm^2 and 3 kg/cm^2, respectively.

Haemagglutination-inhibition tests

The manual test-tube method previously described was used for all specimens [3].

In many cases the results obtained by the manual method were checked by the automated technique suggested by Sturgeon and McQuiston [25] and by Sturgeon et al. [26] slightly modified [27].

With the automated method some drawbacks were observed. First, there was interference of urinary pigments in the photometric determination of haemoglobin. With short Sephadex G-25 beds (20 × 1.2 cm) these pigments were eluted as a yellow band at an elution volume of 25 ml, the same as urinary group-specific fraction 2. There was separation with 28 × 1.2 cm beds and this was improved in larger columns where the pigments separated into two yellow bands, at elution volumes of 205 and 260 ml, respectively, and a red band at 235 ml.

Another drawback of the automated technique applied to eluates of urine was the irregular "agglutination base". This was caused by the slight haemolytic activity of the eluates, which could not be avoided even when they were carefully made isotonic. The haemolysis effect increased in the fractions eluted immediately before the urinary pigments.

Carbohydrate analyses

Qualitative analyses of neutral sugars and amino sugars were carried out on purified urinary ABH substances.

Purification of urinary fraction 1 was difficult owing to the presence in urine samples of a number of high-molecular-weight carbohydrate-containing compounds [28, 29]. Different methods were tried on 4000-ml samples of urine, i.e. modifications of the original methods proposed by King et al. [18] and by Kobayasi et al. [14].

Most satisfactory results were obtained by the following technique. Three 4000-ml samples of urine containing group A fraction 1 and two samples containing B fraction 1 were concentrated to 300 ml, dialysed, concentrated to 4 ml and gel chromatographed on a 42 × 2.5 cm Sephadex G-25 column. The excluded effluent was recycled on Sephadex G-200. The serologically active, excluded peak was gel filtered through Bio-Gel A-50m and the active fraction finally hydrolysed with 2 *M* hydrochloric acid for 3 h at 110°. The hydrolysate was concentrated to dryness over sodium hydroxide and phosphorus pentoxide in a vacuum dessicator. As a control, two 4000-ml samples were taken from a group A and from a group B subject lacking ABH specific substances in urine.

Neutral sugars. Neutral sugars were detected by paper chromatography and by gas—liquid chromatography.

Paper chromatography was carried out on Whatman No. 1 paper (57 × 29.5 cm) with 1-butanol—pyridine—water (6:4:2) as solvent. The reducing sugars were detected by a silver-dip reagent [30].

A modification of the method of Sweeley et al. [31] was adopted for GLC. The gas chromatograph was a Model Fractovap 2300 from Carlo Erba (Milan, Italy), equipped with a dual-flame detector system and a Speedomax Leeds & Northrup recorder.

The separation of trimethylsilyl ethers of sugars was carried out on Chromosorb W (60—80 mesh) coated with 1.5% OV-17, obtained from Carlo Erba. Glass columns were 6 ft. × 1/8 in. I.D. The columns were conditioned at 250° without gas flow for several hours and then at the same temperature with normal gas flow for an additional 2 h. The flow of the carrier gas (nitrogen) was 30 ml/min. The temperature of operation was 200°. Samples of 1 μl were used from a total of 50 μl of the silanized extract.

Hexosamines were detected by paper chromatography and GLC after the hydrolysis procedure suggested by Lundblad [21].

RESULTS

The group-specific fraction excluded from Sephadex G-200

The non-dialysable, non-ultrafiltrable fraction of the A, B and H substances [18] was found in the concentrated urine of some subjects only (see Tables I and II). This serologically active fraction, which has been called urinary fraction 1 (uF1), was excluded from Sephadex G-25 columns of various sizes and also from Sephadex G-100 and G-200 columns. The data were obtained by gel chromatographing concentrated urine in amounts ranging from 1.5 (in small columns) to 3—4 ml (in larger columns). uF1 was precipitable with four volumes of ethanol or methanol and the precipitate was readily soluble in saline or in diluted aqueous sodium acetate solution.

As this behaviour is the same as that of the high-molecular-weight salivary fraction 1 (sF1), a further chromatographic comparison between the two substances was made on Bio-Gel A-15 m, and a clear-cut difference resulted from these experiments. Specimens of saliva (3 ml) containing group A or B sF1 were gel filtered on the agarose bed, and sF1 was eluted at 52 ml. On the other hand, uF1, isolated by exclusion chromatography on Sephadex G-200, had an elution volume of 170 ml on Bio-Gel A-15m (Fig. 1). This result was confirmed by direct gel filtration on the same gel of some samples of concentrated urine containing uF1.

TABLE I

DISTRIBUTION OF THE FOUR ABH URINARY PATTERNS

Urinary fraction	Blood group			Total	Percentage
	A	B	O		
Type I uF1	5	2	2	9	18.7
Type II uF2	2	1	2	5	10.5
Type III uF1/uF2	5	6	1	12	25
Type IV uF—	3	2	17	22	45.8

COMPARISON OF THE ABH URINARY AND SALIVARY PATTERNS

Subject	Group	Salivary patterns		Urine
		so-called secretors	so-called non-secretors	
S.P.	B	B-1,2		uF1—uF2
M.G.	B	B-1,2		uF1—uF2
P.G.	A		A-2,3	uF—
G.V.G.	A	A-1		uF1
F.A.	O	H-1		uF—
D.M.D.	O	H-1,2		uF—
G.V.	O	H-1		uF—
T.F.	B		B-	uF—
B.L.	A	A-1,2,3		uF1
F.A.	B	B-1		uF1
S.A.	AB	A-1/B-1		uF2
S.V.	O		H-3	uF—
C.G.	O		H-2,3	uF—
R.G.	O	H-1,3		uF1—uF2
N.D.	O	H-1,3		uF—
A.P.	A		A-	uF1—uF2
A.M.	O		H-	uF1
M.G.	O	H-1,2,3		uF1
M.A.	O	H-1,3		uF2
R.R.	A	A-1,3		uF1—uF2
B.D.	O	H-1,3		uF—
B.M.	O	H-1		uF—
C.A.	O	H-1		uF—
B.S.	A		A-2,3	uF2
D.C.F.	O	H-1,2,3		uF—
C.L.	O	H-1		uF—
F.R.	A	A-1,2,3		uF1
G.G.	O	H-1		uF2
L.L.	A	A-1		uF1—uF2
D.A.P.	B	B-1,2,3		uF1—uF2
G.M.	B		B-	uF2
T.M.	B	B-1,2,3		uF1—uF2
A.V.	B	B-1,3		uF1—uF2
S.P.	B	B-1,3		uF1
G.M.	AB	A-1/B-1		uF1—uF2
P.R.	O	H-1,2,3		uF—
N.A.	A		A-	uF—
B.F.	A	A-1		uF1
F.S.	O	H-1		uF—
C.G.	A	A-1,3		uF2
V.R.N.	AB	A-1/B-1		uF1—uF2
D.M.L.	O	H-1		uF—
M.C.	O	H-1		uF—
C.S.	O	H-1		uF—
S.A.	A	A-1,2,3		uF—
F.A.	B	B-1,2,3		uF—
C.G.	B	B-1,2,3/4,5		uF1—uF2/3,4,5
F.M.T.	AB	A-1,2/B-1		uF1—uF2/3,4,5
B.A.	A	A-1,2,3/4,5		uF1—uF2/3,4,5
P.V.	O	H-1,3		uF—/3,4,5
C.A.	A		A-2	uF1—uF2/3
N.F.	A	A-1,3		uF1/3

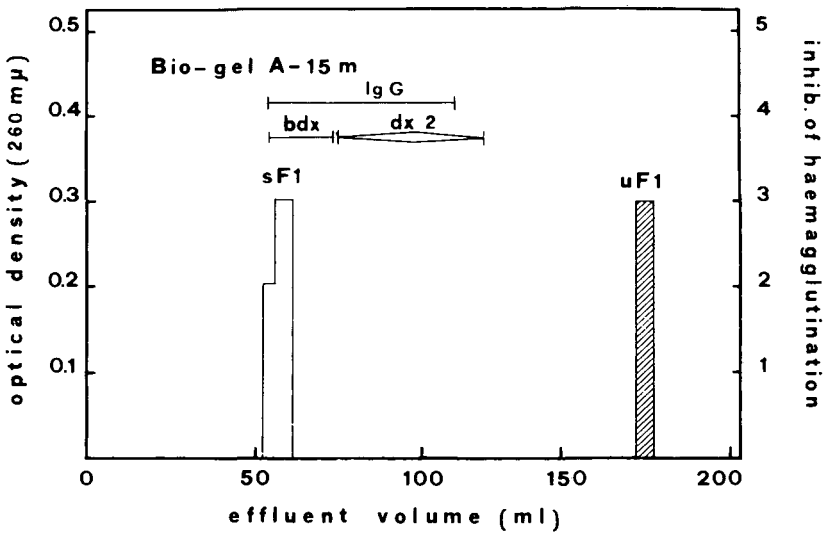


Fig.1. Gel filtration on Bio-Gel A-15m (60 × 1.5 cm) of Blue Dextran (bdx), group A salivary fraction 1 (sF1), group A urinary fraction 1 (uF1) anti-A IgG antibodies and Dextrans 200,000 (dx 2). The results of the inhibition of haemagglutination, in this figure and in Fig. 2, are scored as follows: 0 = one clump; 1 = +++; 2 = ++; 3 = +; 4 = ±; 5 = — (no agglutination).

To obtain further information about the molecular size of uF1, 500 ml of urine containing group A uF1 were dialysed and ultrafiltered through an XM-100 Diaflo membrane. The ultrafiltrate was concentrated to a few milliliters and, as well as the retentate, filtered on Sephadex G-200. Serological activity was found in the excluded effluent of the ultrafiltrate only. The experiment was repeated with a PM-10 Diaflo membrane and the group-specific activity was detected in the retentate.

The specific serological activity of uF1 eluted from short columns of Sephadex G-25 was variable when studied by the manual method. In most cases an inhibition score of 3 (i.e. agglutination +) was observed in 4—5 elution tubes. In other cases, the inhibition score was 4 (±) or 2 (++), the antiserum being selected to give inhibition 0 (clump) in negative tubes. Comparable results were obtained with the automated method.

Qualitative analysis of sugars. Qualitative analysis of sugars of uF1 were carried out with the chief aim of ascertaining the presence of glucose. The most reliable results were obtained by the purification procedure which had, as final step, gel chromatography on Bio-Gel A 50m.

Both paper and gas—liquid chromatography disclosed in all samples the presence of glucose, galactose, fucose and hexosamines. No sugar was detected on the material from the subjects lacking group-specific substances in urine.

The low-molecular-weight ABH urinary fractions

In 6 cases only (see Table II, Nos. 47, 48, 49, 50, 51 and 52) low-molec-

ular-weight, dialysable, water- and alcohol-soluble fractions were found in urine which had the same gel-chromatographic behaviour with Sephadex G-25 as fractions 3, 4 and 5 of saliva and red cells [4].

Another fraction, which was alcohol- and water-soluble, and ultrafiltrable but not dialysable, was detected in a number of subjects, alone or associated with uF1. As the elution volume (20 ml) of this fraction on Sephadex G-100 columns was only slightly different from that of salivary fraction 2 (10 ml), it was called urinary fraction 2 (uF2). However, a further study disclosed some differences between uF2 and salivary fraction 2 (sF2). sF2 was not ultrafiltrable, as demonstrated by experiments on specimens of whole saliva containing sF2. On Sephadex G-25, sF2 was excluded while uF2 was retained. The elution volumes of uF2 with this latter gel were the following: 25 ml on columns 20 cm × 1.2 cm (void volume 17 ml); 28 ml on columns 28 cm × 1.2 cm (void volume 25 ml), 178 ml on columns 42 cm × 2.5 cm (void volume 90 ml). uF2 was excluded from Sephadex G-15.

Serological activity. The specific serological activity of uF2 according to the manual test was sometimes stronger than that of uF1 contained in the same sample. An almost complete inhibition (agglutination ±) in two or three tubes, and more frequently a 3 (+) score, was observed.

Qualitative analysis of sugars. The qualitative analysis of sugars of uF2 was carried out on a group A uF2 isolated from a 5000-ml sample of urine which had been dialysed, concentrated to a few millilitres and filtered on a large Sephadex G-25 column. The active fraction was purified on Sephadex G-15 and hydrolysed with 2 *M* hydrochloric acid. Paper chromatography and gas—liquid chromatography disclosed the presence of glucose, galactose, fucose and hexosamines.

Individual gel-chromatographic patterns

Four main gel-chromatographic patterns were found in the urine of the subjects examined, as shown in Fig. 2. In some individuals only uF1 was found in the urine, in others only uF2, and in some others both uF1 and uF2. In a fourth group of subjects no active ABH fraction was detected. Only 6 persons had some additional small fractions (see Table II).

The data were all obtained by the manual serological method. In many cases, samples of the same subject taken at different times were examined, with identical results. A number of specimens was also checked by the automated technique. Fig. 3 (a,b,c) shows some of the findings.

The different distributions of the four main types are reported in Table I, where the symbol uF— indicates the absence of both uF1 and uF2.

In Table II the ABH urinary and salivary patterns of each subject are compared. Sub-fractions 4 and 5 were determined only in the saliva of those subjects who had fractions 3, 4 and 5 in the urine.

Some individuals, whose saliva contained sF1 and were therefore called secretors, had only uF2 (e.g. Nos. 19, 28, 40) or no fraction (e.g. Nos. 5, 6, 7, 15, 45, etc.) in the urine. Additional evidence of these findings was given by the study of a family (Fig. 4; the parents are Nos. 16 and 17 in Table II). The couple and the children had no group-specific fraction in their saliva, all being true non-secretors, and had uF1 or uF1/uF2 in the urine.

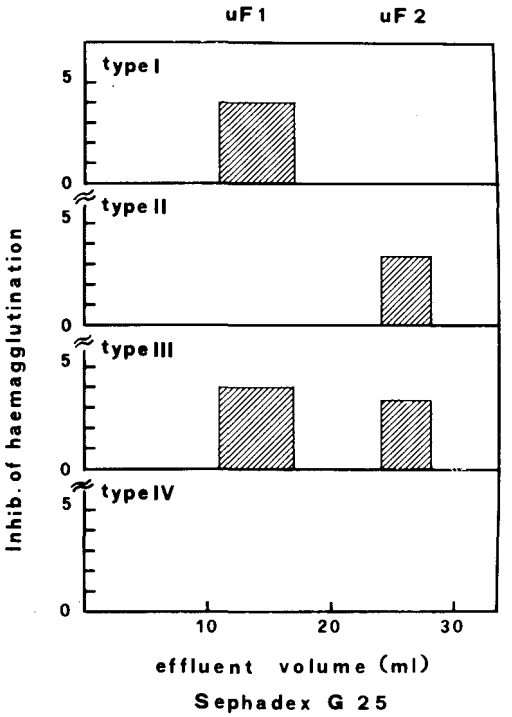
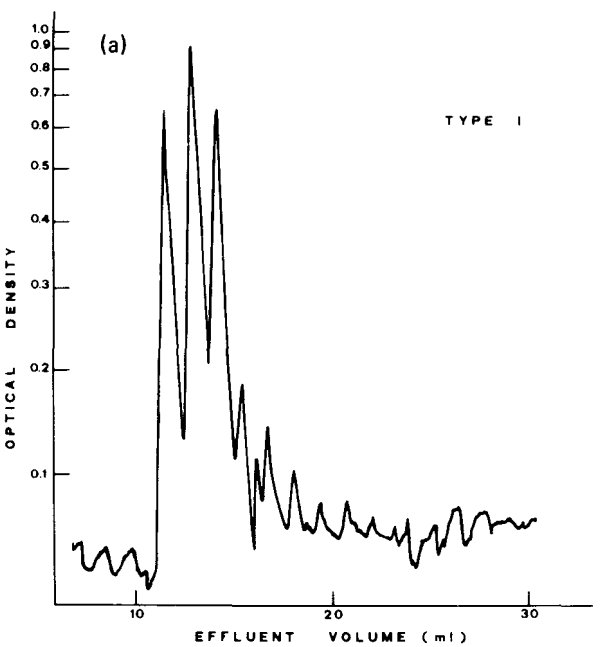


Fig.2. Schematic representation of the four urinary ABH gel-filtration types determined on 20 × 1.2 cm Sephadex G-25 columns.



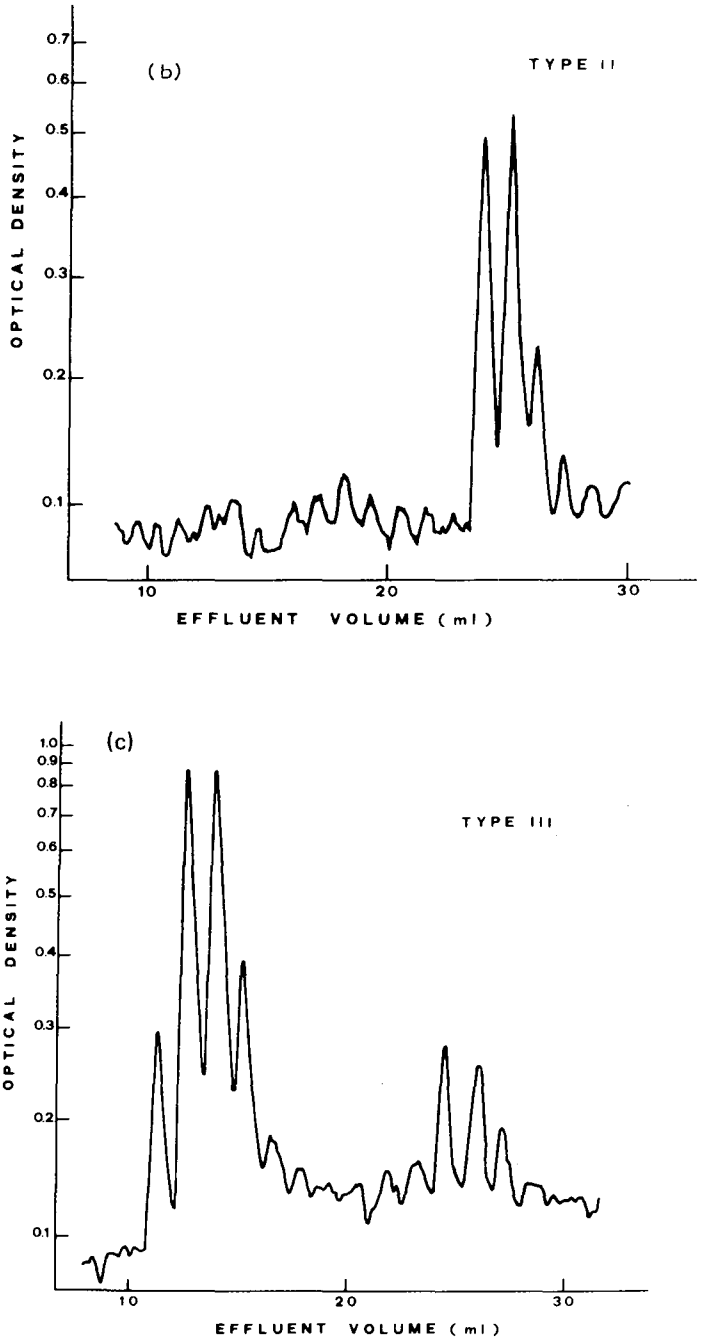


Fig.3. Some examples of urinary ABH gel-chromatographic patterns (Sephadex G-25, 28 × 1.2 cm), determined by the method of automated inhibition of haemagglutination. The peaks that rise from the irregular base line indicate the haemagglutination inhibition. In (a) a group A type I (uF1 only) urine is recorded; in (b) a group B type II (uF2 only) and in (c) a group B type III (uF1/uF2).

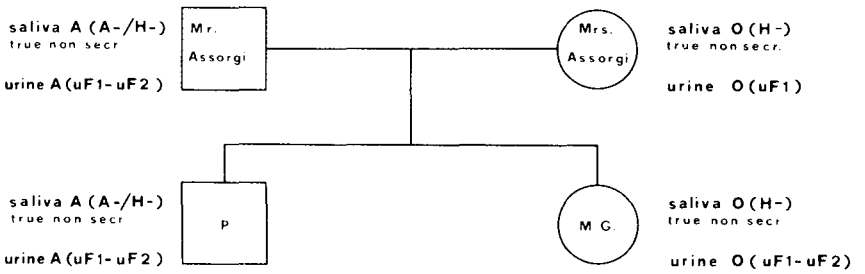


Fig.4. The ABH salivary and urinary gel chromatographic patterns of a family of true non-secretors. All the members had group-specific substances in urine.

DISCUSSION

Comparison of present findings with previous data on urinary A, B and H substances

Urinary fraction 1. uF1 of ABH substances is the same alcohol-precipitable [11], non-dialysable and non-ultrafiltrable [18—20] group-specific glycoprotein as identified by Yoshida in 1928 [8] and studied by a number of authors [9—11, 13, 14, 16, 18—20].

Urinary group-specific fraction 2. uF2 is the same non-dialysable, ultrafiltrable fraction first described by King et al. [17] and exhaustively purified and studied by Lundblad [21—23] and by Björndal and Lundblad [24], whose gel chromatographic data are comparable with ours.

Molecular size. The molecular sizes of uF1 and uF2 are different from those of salivary fractions 1 (sF1) and 2 (sF2). As far as the larger uF1 group-specific urinary glycoprotein [20] is concerned, this is excluded from Sephadex G-200 with sF1 but is eluted much later than sF1 on Bio-gel A-15m. In addition, uF1 is ultrafiltered through XM-100 Diaflo membrane as is dextran, with a molecular weight of 250,000 daltons, but sF1 is not. Finally, uF1 is retained by PM-10 Diaflo membranes that do not hold dextran with a molecular weight of 100,000 daltons.

Taking the above data together one can surmise that the molecular weight of uF1 could fall within the range 150,000—200,000.

The behaviour of uF1 on Bio-Gel A-15m, compared with that of reference substances of known molecular weight, suggests an adsorption effect.

Urinary fraction 2 (uF2) is an oligosaccharide [21—24], smaller than sF2 and larger than salivary fraction 3 (sF3). In fact, uF2 is ultrafiltrable but not dialysable and is retained on Sephadex G-25, whereas sF2 is neither dialysable nor ultrafiltrable and is excluded from the same gel; sF3 is dialysable and ultrafiltrable, and is eluted from Sephadex G-25 much later than uF2 [1, 3]. From the above data the molecular weight of uF2 could be evaluated as being in the range of 4000—5000 daltons.

The comparison of gel-chromatographic and ultrafiltration data obtained from uF1 and uF2 with those of ABH fractions from red cells [6] leads to the same conclusions as regards molecular size.

Glucose. Glucose has been detected in both urinary fractions 1 and 2, in agreement with Lundblad's results [20—24] and in contrast with previous

analytical data on urinary group-specific fractions [9, 14—16, 18]. These contrasting results should be chiefly ascribable to the different analytical procedure adopted.

The presence of glucose in both group-specific fractions is relevant for their inclusion in the class of glucose-containing ABH substances such as salivary fractions 2, 3, 4 and 5 [32] and of glycolipids of red cells. Therefore urinary fractions have no evident relationship to the glucose-free high-molecular weight salivary fraction 1 (see ref. 33).

Urinary and salivary polymorphism of A, B and H substances

It has been shown in previous papers [1—3] that the so-called secretor and non-secretor status is not dimorphic but polymorphic. Eight main types have been identified in saliva of which one type is represented by a few true non-secretors and the remainder by various associations of the group-specific fractions 1, 2 and 3. These findings have been recently confirmed by Ueda [34]. As salivary fraction 3 is sometimes associated with sub-fractions 4 and 5, or with both these small fractions, sub-types were also identified. Thus the possible patterns are twenty [4].

There is now evidence for a molecular polymorphism of A, B and H substances in urine too. In fact the same ABH pattern was found in several specimens of urine taken at different times from the same individuals, and four types of these constantly reproducible individual patterns were identified, i.e. no ABH group-specific activity, uF1 or uF2 only, both uF1 and uF2 (Fig. 2).

The ABH urinary polymorphism shows no demonstrable relationship to the salivary one. Some individuals whose saliva contains sF1 and can therefore be called secretors according to the old classification, have only uF2 (e.g. Nos. 19, 28 and 40 of Table II) or no fraction (e.g. Nos. 3, 5, 7, 15, 45 of Table II) in their urine. Good additional evidence of these findings is given by the family reported in Fig. 4 where all members are true non-secretors in saliva, but have ABH fractions in their urines.

We have no data at present to explain the presence in urine of a molecule as large as urinary group-specific glycoprotein uF1. The hypothesis could be advanced that biosynthesis and secretion of ABH urinary substances occur in some cells of the urinary system.

The possibility of a genetic determinism will now be considered to explain the distribution of the types within the ABO groups. However, the genetic analysis requires still other information not yet available.

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Biomedical Applications

CHROMBIO. 094

SEPARATION OF URINARY ULTRAVIOLET-ABSORBING METABOLITES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY USING A COMMERCIALLY AVAILABLE ANALYTICAL UNIT

F. GEERAERTS, L. SCHIMPFESSEL and R. CROKAERT

Laboratorium voor Fysiologische Scheikunde, Faculteit Geneeskunde en Farmacie, Vrije Universiteit Brussel (Belgium)

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SUMMARY

A high-pressure liquid chromatographic system for the separation of UV-absorbing and/or fluorescent urinary metabolites is presented. Thirty to forty UV-absorbing chromatographic peaks are obtained from a 200-μl urine sample using a 100 cm × 2 mm column filled with a total porous ion-exchanger. The reproducibility of the separation has been studied and eighteen important substances have been located.

INTRODUCTION

Human urine is reported to contain more than 700 molecular constituents [1]. About 150 of them absorb ultraviolet (UV) light [2]. These substances represent intermediates or end-products of metabolic pathways.

A physiological fluid as complex as urine can only be studied in detail by using a separation method with a high resolving power. A high-pressure liquid chromatographic (HPLC) system for the analysis of urine is presented, based on the fundamental work of Scott and co-workers [3—6].

Earlier HPLC systems are nowadays difficult to use because of the rapid evolution of HPLC technology and the advent of new commercial liquid chromatographs.

The analytical unit described has a very high resolving power, due to the use of a very fine, porous ion-exchanger, and gives reproducible results. It is versatile, easy to use and its components are commercially available. The detection system can be used for UV-absorbing metabolites, for reaction products absorbing in the visible region and for compounds that are naturally or artificially fluorescent.

EXPERIMENTAL

Materials

The high-pressure liquid chromatograph used is Model 8500 from Varian (Palo Alto, U.S.A.) equipped with a multilinear solvent programmer. The samples are injected via a liquid-chromatograph air-actuated sampling valve (sample loop of 200 μl, Varian) on a 100 cm × 2 mm I.D. 316 stainless-steel column. The columns are filled with Zerolit, a strongly basic, totally porous ion-exchanger. The column temperature is controlled by a Haake 22 water-bath with TP 32 programmator (Haake, Osterröde, G.F.R.).

The Variscan, a UV—vis continuous-wavelength detector with flow cells of 8 μl, and the dual-pen Techtron A-25 recorder, are associated Varian equipment. One pen of the recorder draws the absorption changes (line A in Fig. 2), the other the composition of the mobile phase expressed as the fraction of component B present in the eluent (line B in Fig. 2). The dual-wavelength detector is an ISCO UA5 absorbance monitor with a multiplexer-expander (Instrument Specialities Co., Lincoln, U.S.A.). The native fluorescence is continuously measured with a Fluorichrom (Varian).

The eluate is collected by a Redirac collector (LKB, Bromma, Sweden), which is cooled by placing it in a refrigerator (4°).

The fluorimeter is an Aminco-Bowman spectrophotofluorimeter (American Co., Silver Springs, U.S.A.).

Products

Zerolit (particle size, 5—7 μm) is a product of the Permutit (London, Great Britain) Aminex A-27 (particle size, 8—12 μm) can be obtained from Bio-Rad Laboratories (Richmond, U.S.A.). Acetic acid and ammonium acetate are pro analysi products from Merck (Darmstadt, G.F.R.). The standards are of the purest quality available from different sources.

Mobile phase

An acetic acid—ammonium acetate buffer (pH 4.4) varying from 0.015—6 *M* was used as the mobile phase.

METHODS

Fig. 1 is a schematic representation of the chromatographic system. Pumps A and B are filled, respectively, with 0.015 *M* and 6 *M* acetate buffer. Both solutions are previously de-gassed under vacuum.

The urine is collected, immediately centrifuged and 200 μl are promptly injected. The elution is programmed (Table I), the flow-rate is set at 8 ml/h and the chromatography is started. The column is maintained at 30° for the first 4 h; then the temperature is raised to 60° (1°/min) for the rest of the run. The UV absorption of the separated compounds is continuously measured and recorded at 254, 260 and 280 nm. In order to obtain a suitable absorption for the strongly absorbing components (creatinine, uric acid) as well as for the small peaks, an absorption range of 0—1 a.u.f.s. (on each detector) was found convenient for urine with a normal creatinine concentration (1000—2000 mg/24 h).

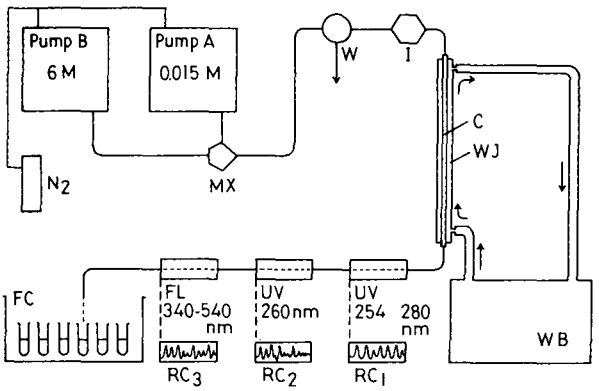

Fig.1. Schematic representation of the chromatographic system. C, column; FC, fraction collector; FL, fluorescence detector; I, injection system; MX, mixing chamber; RC, recorder; UV, UV absorbance detector; W, waste; WB, water-bath; WJ, water-jacket.

TABLE I

GRADIENT ELUTION PROGRAMME

Complete gradient elution takes 1140 min. The column can then be automatically regenerated by programming the 'reset'. In this position, the initial buffer composition (e.g. 0.015 *M*) will be pumped through the column. Regeneration can be followed by measuring the UV absorption (minimum regeneration time, 90 min).

Step*	Time (min)	Elution type	Buffer concentration (*M*)	
			beginning	end
1	120	Isocratic: (100% A + 0% B)	0.015	0.015
2	120	Linear gradient: (100% A + 0% B)— (96% A + 4% B)	0.015	0.3
3	600	Linear gradient: (96% A + 4% B)— (35% A + 65% B)	0.3	4
4,5,6	180	Isocratic: (35% A + 65% B)	4	4
7	35	Linear gradient: (35% A + 65% B)— (0% A + 100% B)	4	6
8	85	Isocratic: (0% A + 100% B)	6	6

*Step on solvent programmer

The fluorescence of the eluted compounds is continuously measured with the Fluorichrom (excitation wavelength 340 nm, emission wavelength 540 nm).

Finally, the eluate is collected into a refrigerated fraction collector in fractions of 1 ml to permit further study of the urinary metabolites by other techniques (fluorimetry, TLC, specific reactions).

RESULTS

The whole analysis takes about 20 h. The chromatograms from a fresh urine sample of a healthy adult are shown in Figs. 2 and 3. Thirty to forty UV-absorbing peaks are well separated.

The retention times (RT) of the compounds commonly present in urine have been determined and are given in Table II. Several other peaks were observed, which probably resulted from variations in the composition of the diet.

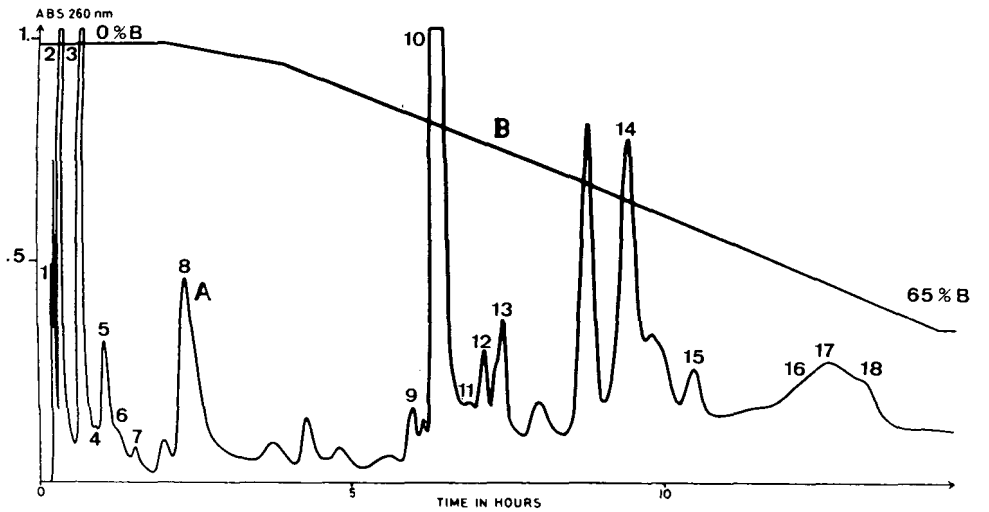

Fig.2. Typical chromatogram obtained using the Variscan detector. Conditions: flow-rate, 8 ml/h; eluant, ammonium acetate—acetic acid buffer (pH 4.4) varying from 0.015 to 6 *M* (buffer gradient as described in Table I); sensitivity, 1 a.u.f.s.; sample, 200 µl fresh urine of a healthy male subject (28 years old). Line A represents the absorbance at 260 nm, line B the buffer gradient expressed as the fraction of buffer B (6*M*) present in the mixture. Identified peaks (peak code number in parentheses): 1, N-methylnicotinamide (10); 2, creatinine (20); 3, pseudouridine (30); 4, tryptamine (31); 5, uracil (40); 6, serotonine (50); 7, urocanic acid (60); 8, nicotinamide (90); 9, hypoxanthine (130); 10, uric acid (160); 11, tryptophan (170); 12, 5-hydroxyindole acetic acid (180); 13, nicotinic acid (1801); 14, hippuric acid (240); 15, indican (250); 16, quinaldic acid (260); 17, vanillic acid (270); 18, kynurenic acid (2901).

Peak numbers are empirical, logical numbers. The RT of the urinary metabolites varies between 17 and 870 min. The peaks are well distributed over the whole chromatogram: 32% of the UV-absorbing metabolites are eluted during the first fifth of the run, 18% in the second fifth, 21% in the third, 16% in the fourth and 13% in the last fifth.

The chromatographic analysis is shown to be reproducible. The coefficients of variance lie between 8.7% (mean for the first six peaks, 7.6%) and 0.5% (mean for the last six peaks, 0.9%). As can be expected, the largest variations are noted in the first part of the chromatogram since separation in this region depends on non-ionic adsorption [8] which can be strongly affected by several factors.

The statistical study of the RTs of the separated compounds is compli-

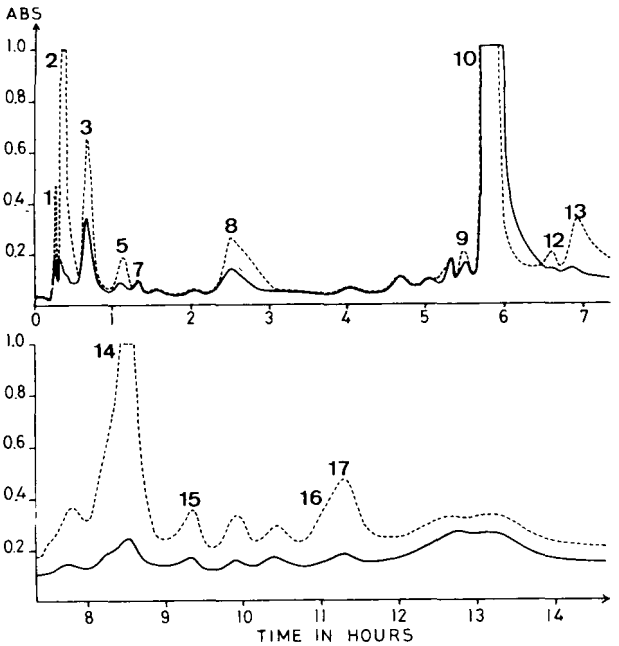

Fig.3. Chromatogram obtained using the ISCO UA5 absorbance monitor. - - - -, 254 nm; —, 280 nm. For conditions and identified peaks, see legend of Fig. 2.

TABLE II

MEAN RETENTION TIMES OF THE SEPARATED PEAKS OF THE COMMON UV-ABSORBING COMPOUNDS IN URINE

RT, retention time. S.D., standard deviation. C.V., coefficient of variation (n = 10).

Peak number	Mean RT (min)	S.D.	C.V. (%)	Peak number	Mean RT (min)	S.D.	C.V. (%)
10	16.57	1.13	6.8	160	387.87	16.06	4.2
101	19.60	1.52	7.7	170	395.50	7.37	1.9
20	21.88	1.61	7.3	180	417.50	14.06	3.4
30	44.50	3.89	8.7	1801	431.33	12.13	2.8
40	63.60	4.81	7.5	190	453.28	12.71	2.8
50	73.50	5.43	7.4	200	476.37	13.07	2.7
60	87.83	3.49	4.0	210	483.40	4.98	1.0
70	96.00	4.90	5.1	220	506.00	10.20	2.0
701	114.33	2.94	2.6	230	545.17	2.86	0.5
702	127.00	1.00	0.8	231	557.80	3.90	0.7
80	142.40	8.16	5.7	240	578.37	9.01	1.6
90	153.75	9.60	6.2	250	605.00	9.43	1.6
901	188.00	5.65	3.0	260	633.57	2.94	0.5
100	206.17	8.68	4.2	2601	666.57	7.55	1.1
110	266.80	7.43	2.8	270	709.33	7.45	1.0
120	286.57	10.87	3.8	2701	733.00	8.66	1.2
130	310.57	10.11	3.3	280	770.00	6.00	0.8
140	329.66	17.08	5.2	2901	844.25	4.19	0.5
150	353.60	11.70	3.3	2902	870.50	6.36	0.7

cated by the variability of urine samples, which change in composition from person to person and for one and the same individual from day to day (Figs. 2, 4 and 5 and Table III).

Identification of the urinary metabolites is in progress. The position of 18 of them has been determined: N-methylnicotinamide (RT, 17 min), creatinine (22 min), pseudouridine (45 min), tryptamine (58 min), uracil (64 min), serotonin (72 min), urocanic acid (89 min), nicotinamide (158 min), hypoxanthine (308 min), uric acid (380 min), tryptophan (395 min), 5-hydroxyindole acetic acid (418 min), hippuric acid (575 min), indican (610 min), quinaldic acid (634 min), indolacetamide (664 min), vanillic acid (710 min) and kynurenic acid (844 min).

This identification is based on the absorption ratio (280/254 nm), native fluorescence, co-chromatography, retention time of pure products and, if possible, fluorimetric spectra, TLC and specific reactions [10]. The identified peaks are indicated in Fig. 2.

Identification is made difficult by the fact that only small quantities of metabolites are involved. Concentration techniques, (such as lyophilisation and evaporation under vacuum), have been shown to degrade some labile compounds [10].

Fig. 6 shows the fluorescent compounds present in urine.

The buffer gradient designed for the Zerolit column can also be used with an Aminex A-27 column (100 cm × 2 mm) as illustrated in Fig. 7, although a slight modification of the flow-rate programme is necessary (see legend to Fig. 7).

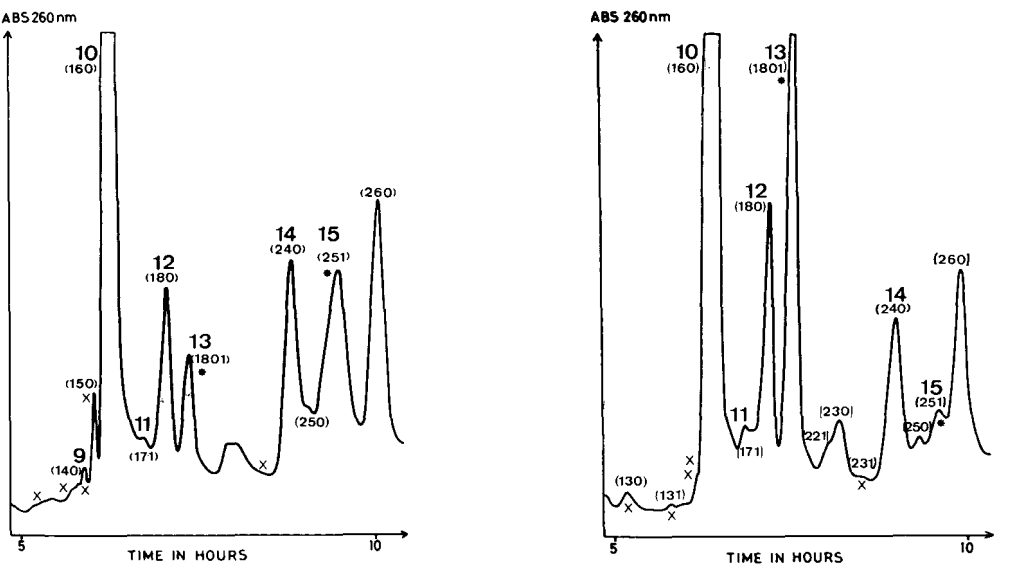

Fig.4. Detail of a chromatogram of a 200-μl urine sample from the same person as in Fig. 2. Conditions and identified peaks as described in Fig. 2. Note the quantitative (*) and qualitative (×) differences with Fig. 5. In parentheses, peak code number.

Fig.5. Detail of a chromatogram from a second male subject (45 years old). Conditions and identified peaks as described in Fig. 2. Note quantitative (*) and qualitative (×) differences with Fig. 4. Peak code numbers in parentheses.

TABLE III

COMPARISON OF PEAK AREA OF URINE SAMPLES

U_{10}, U_{20}, U_{30}, U_{40}: urine samples from 4 male subjects (age 28—50 years); U_{40}, U_{41}, U_{42}, and U_{43}: urine samples from the same person (28 years), taken on different days. Peak area was calculated using the formula: peak area = height × width at half height (No molar response factors were taken into account since some peaks are still unidentified). n.d. = not detectable; n.m. = not measurable because out of scale.

Peak code number	Peak area (mm^2)							
	Samples from different persons				Samples from one person			
	U_{10}	U_{20}	U_{30}	U_{40}	U_{40}	U_{41}	U_{42}	U_{43}
10	216	227	151	165	165	186	132	165
101	484	79	182	177	177	n.d.	176	130
40	55	n.d.	n.d.	103	103	trace	21	22
90	656	420	617	44	44	900	806	28
1701	184	150	74	n.d.	n.d.	trace	47	trace
180	234	180	632	465	465	598	198	276
1801	461	680	n.m.	21	21	601	368	trace
240	1160	539	61	1680	1680	1508	1282	1371
250	57	72	63	125	125	trace	60	99
260	469	294	691	1000	1000	531	n.d.	642

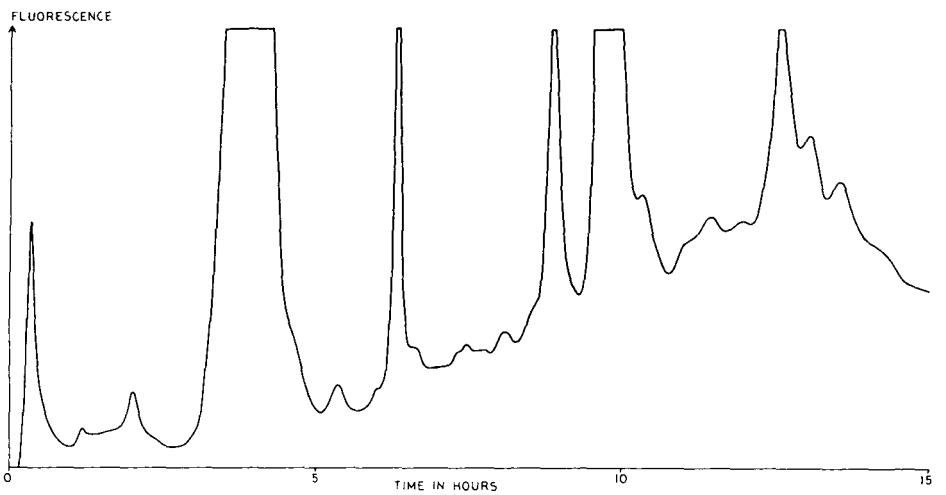

Fig.6. Chromatogram showing the fluorescent compounds in urine. Excitation wavelength 340 nm, emission wavelength 540 nm. Attenuation, 20; gain, 1. Identification of these peaks is in progress.

DISCUSSION

The whole of the chromatographic system used is commercially available (in contrast to the apparatus used by the Oak Ridge group, which was a self-constructed high-pressure liquid chromatograph).

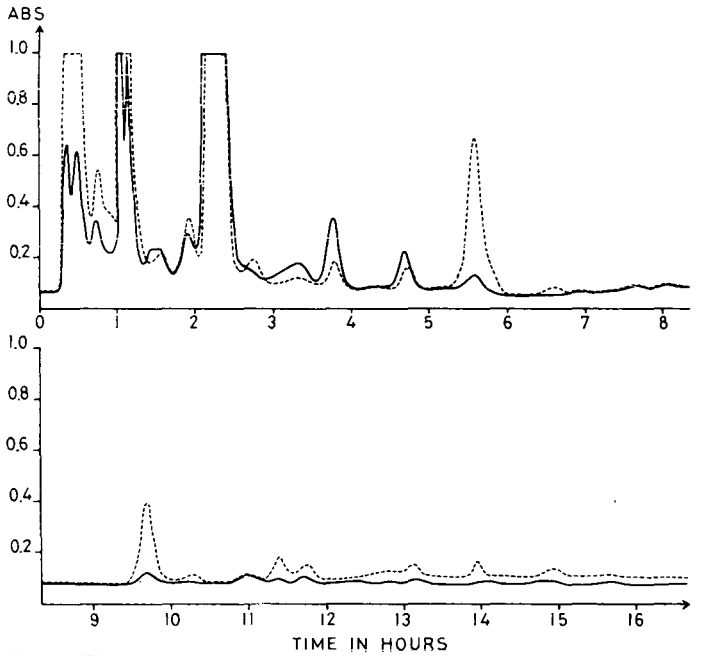

Fig.7. Chromatogram of a urine sample separated on Aminex A-27. Flow rate programme: 0—2 h, 4 ml/h; 2—19 h, 8 ml/h. Gradient programme: see Table I. - - - -, 254 nm. ——, 280 nm.

The buffer system is that chosen by Scott et al. [2], and it has also been used by Burtis [9]. As the components of the buffer (acetic acid and ammonium acetate) have the advantage of being volatile, they can be evaporated during concentration procedures (lyophilisation) in order to avoid eventual interference during TLC and other procedures where the presence of salts is not desirable.

The buffer gradient and the flow-rate have been determined experimentally [10] and result from a compromise between separation performance and speed for the resin used.

Recently, Zerolit and Aminex A-27 have been compared [7]. The plate height for the purified Zerolit (which has been used as the packing material of the column) has been shown to be about seven times smaller than that with Aminex A-27.

Raising the temperature from 30° to 60° after 4 h chromatography has two advantages: a 50% decrease in the RT of strongly adsorbed components [7], and a lowering of the viscosity of the concentrated buffer, which results in a fall in the pressure drop.

Liquid chromatography is shown to be a useful technique for the separation of urinary constituents. The method is reproducible and the detectors presently available allow detection at the nanogram level, which formerly could only be attained by gas chromatography. However, this separation technique requires the transformation of non-volatile compounds into volatile derivatives. With HPLC, a urine sample can be analysed without derivatization, which eliminates the risk of degradation prior to chromatography.

With regard to the stability of the column packing material, daily use for 6—7 months is possible. Tailing and rise in pressure (normal starting pressure is about 2000 p.s.i., end pressure about 4000 p.s.i.) are signs that the column needs to be changed. Intercolumn differences are within the normal chromatogram-to-chromatogram variation.

The HPLC system described here has been used in our laboratory for more than a year without presenting the great practical difficulties often mentioned which until now have retarded the introduction of this new technique into pharmacological and clinical laboratories.

ACKNOWLEDGEMENT

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Biomedical Application

CHROMBIO. 080

SIMPLIFIED MICRO-METHOD FOR THE QUANTITATIVE ANALYSIS OF PUTRESCINE, SPERMIDINE AND SPERMINE IN URINE

OLLE HEBY and GUNNAR ANDERSSON

Department of Zoophysiology, University of Lund, Helgonavägen 3B, S-223 62 Lund (Sweden)

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SUMMARY

A simplified micro-method for the quantitative analysis of urinary polyamines is described. After acid hydrolysis of urine, the polyamines are converted to fluorescent 1-dimethylaminonaphthalene-5-sulfonyl (Dns; dansyl) derivatives and separated by means of thin-layer chromatography. Dns-NH_2, which has been reported to interfere with the determination of putrescine, is well separated from di-Dns-putrescine. Putrescine, spermidine and spermine are quantitated by in situ scanning of their fluorescent spots on the chromatogram.

The present method is both sensitive and reproducible. It eliminates a number of time-consuming steps and thus reduces preparative losses. Yet an adequate chromatographic resolution is obtained. Representative polyamine analyses of urine from normal volunteers and from cancer patients are reported. Elevated levels occur in the urines of pregnant women and of patients with various types of cancer.

INTRODUCTION

A relationship between polyamine levels in physiological fluids and cancer has been established. Thus, elevated levels of the polyamines putrescine, spermidine and spermine have been observed in cerebrospinal fluid of patients with central nervous system tumors [1—3], and in serum [4, 5] and urine [4, 6—10] of patients with many other forms of cancer. Present data suggest that determinations of polyamine levels in physiological fluids may provide a valuable test both as an adjunct to the diagnosis of cancer and for monitoring therapy.

Methods using thin-layer chromatography (TLC) [11, 12], gas chromatography [6] and automated high-pressure liquid chromatography [13—15] have been devised for the quantitative analysis or urinary polyamines. Several methods require tedious and time-consuming sample preparation as well as

lengthy analysis, whereas other methods require expensive instrumentation. The purpose of the present study was to develop a simple method for polyamine analysis involving a minimal number of preparative steps, thus facilitating the analysis of large numbers of clinical samples. The method described allows for minimal sample handling and rapid analysis with high sensitivity and reproducibility, and adequate separation of the polyamines from interfering compounds.

MATERIAL AND METHODS

Sample collection

Urine specimens from normal individuals (volunteers not suffering from acute or chronic disease) and from hospitalized patients with cancer (in an advanced clinical stage) were collected before the morning meal. Immediately after collection, two 10-ml aliquots were centrifuged at 1,000 *g* for 10 min at 2° to remove any cells that may have been present. Then the supernatants were analyzed for their creatinine (according to Løken [16]) and polyamine concentrations. All specimens were then stored at −25°.

Chemicals

TLC plates (20 × 20 cm) precoated with 250 μm silica gel 60 layers (Merck), and analytical grade reagents were used.

Polyamine analysis

Urine (1 ml) was hydrolyzed with an equal volume of concentrated HCl (12 *M*) in a PTFE-sealed test tube at 110° for 14—16 h. After acid hydrolysis, the sample was neutralized with solid Na_2CO_3 and centrifuged at 2,000 *g* for 5 min. A 200-μl aliquot of the supernatant was supplemented with 400 μl of Dns-Cl (30 mg/ml acetone). To the reaction mixture was added 100 μl of a saturated Na_2CO_3 solution and the mixture was sonicated for 2—3 h in an ultrasonic cleaner. The excess of Dns-Cl was converted to Dns-proline by reaction with 100 μl of the amino acid (250 mg of L-proline per ml). This reaction was quantitative after 3 min of sonication. The excess of Dns-Cl must be removed inasmuch as it is easily hydrolyzed by silica gel to 1-dimethylamino-naphthalene-5-sulfonic acid (Dns-OH), which causes blue-green fluorescent streaks on the chromatogram. The sulfonamides (Dns-amides) were extracted into 500 μl of toluene and the layers were separated by centrifugation. The principle for the dansylation technique has been described in detail by Seiler [17, 18] and by Seiler and Wiechmann [19].

TLC separation of Dns-amides

Aliquots (5—20 μl) of the toluene extracts were applied to pre-activated (110° for 1 h) silica gel 60 plates divided into 12 bands by scoring. A 25-μl Hamilton micro-syringe pipet with disposable PTFE tips was used for the application. Toluene was used as solvent since it does not move the Dns-amides chromatographically and thus results in small application spots [18]. Dansylated polyamine standards at 3 concentrations were included on each plate. These concentrations were selected so that they encompassed the range found

in the urine samples. The dansyl derivatives were separated in 1 h by one-dimensional ascending chromatography in chloroform—triethylamine (5:1) according to Seiler and Wiechmann [20]. After chromatographic separation, the plate was carefully sprayed with 20 ml of a solution of triethanolamine—propan-2-ol (1:4) according to Seiler and Wiechmann [21], and dried in vacuo for 16 h at room temperature in a desiccator containing silica gel as desiccant. This procedure considerably increased the fluorescence intensity and the stability of the Dns derivatives. Apparently triethanolamine reduces the adsorption of the dansyl derivatives to the polar and acidic silica gel and thus increases the quantum yield of fluorescence [17]. The removal of water and possibly some volatile quenching substances by desiccation may contribute to the increased fluorescence intensity [17]. To avoid quenching of the fluorescence by water adsorption during scanning, the plates were equilibrated for 30 min at room atmosphere.

Instrumentation

After equilibration the TLC plate was analyzed by fluorometry in situ in an Aminco-Bowman spectrophotofluorometer (model J4-8950) equipped with a TLC scanner and an XY recorder. This instrument uses two monochromators and therefore both the activation and emission spectra can be determined directly on the TLC plates. Thus valuable qualitative information about the separated substances is provided in addition to the quantitative information. The scanning accessory used, operates by measuring the reflected fluorescence light, at variance with the device used for example by Seiler [22] in which fluorescence measurements are achieved by transmittance. The excitation maximum was at 340 nm and the emission maximum at 505 nm for the dansylated polyamine derivatives. Scoring of the TLC plates allowed for perfectly linear development of the chromatograms, a pre-requisite for obtaining accurate results with the scanning device used.

RESULTS AND DISCUSSION

The present method shows that it is possible to analyze urinary polyamines quantitatively without previous concentration of the urine and without removal of ammonium contained in the urine. Furthermore, the method allows for minimal sample preparation, and rapid analysis with high sensitivity and reproducibility.

Reduced sample preparation was achieved by omitting several concentration steps as well as the extraction of polyamines (as free bases) into 1-butanol or isoamyl alcohol (3-methyl-1-butanol). These steps are not obligatory for the analysis of urinary polyamines. Furthermore, the removal of ammonia by urease treatment or evaporation in alkaline solution was found to be unnecessary and was omitted for the purpose of minimizing losses. Other workers, however, have not achieved adequate separation between the Dns-derivatives of ammonia and putrescine, thus requiring that their urine samples be either incubated with urease and aerated before hydrolysis in acid [11] or dried in the alkaline state [12]. However, with the present method, excellent separation between these two derivatives is obtained (Fig. 1). Recoveries of polyamines

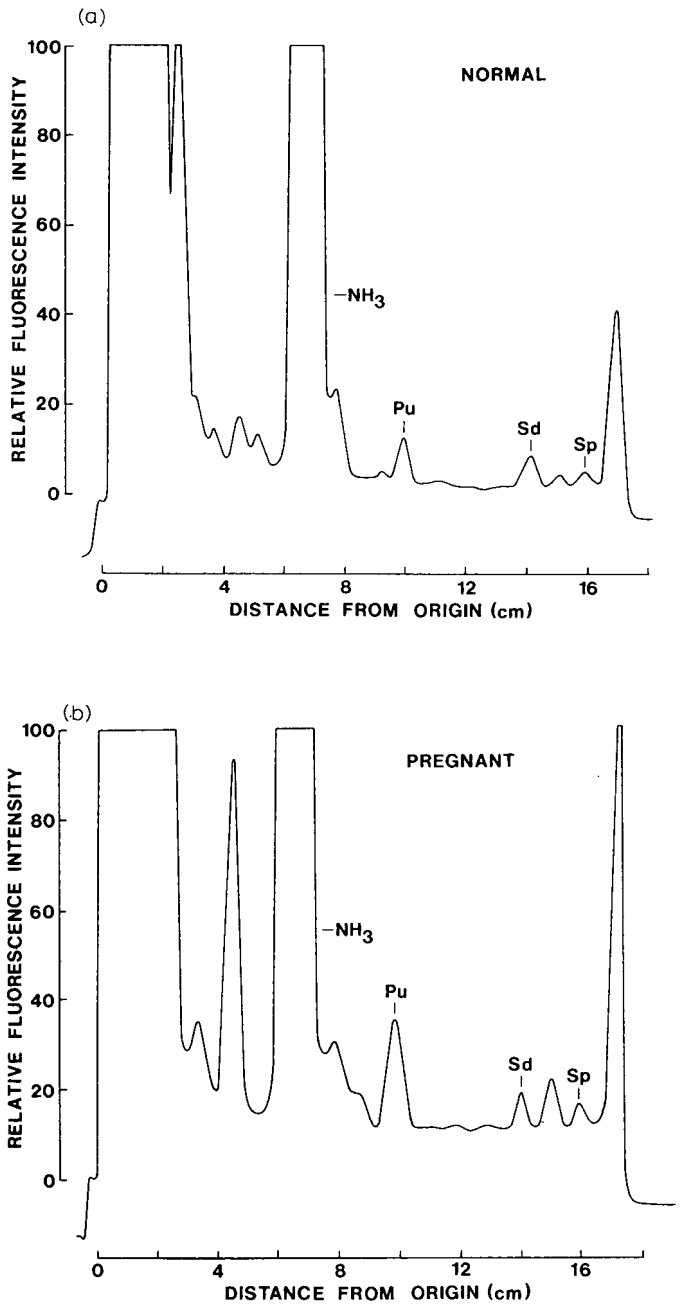
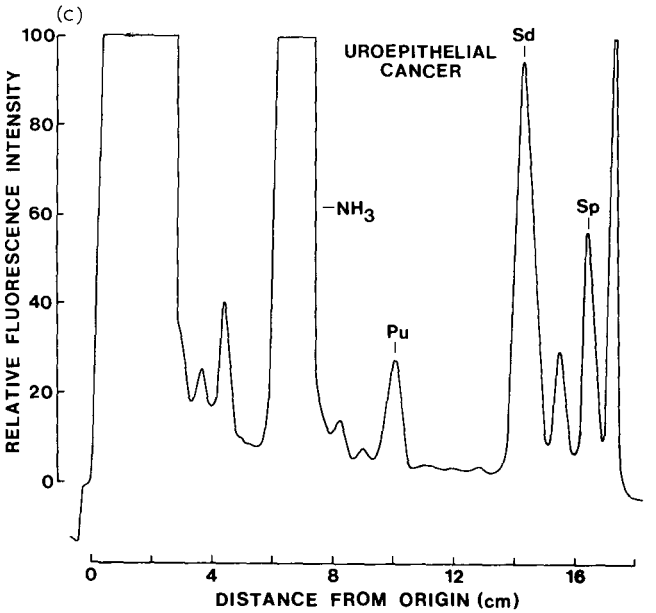


Fig. 1. Chromatographic separation of the dansylated derivatives of putrescine (Pu), spermidine (Sd) and spermine (Sp) contained in (a) urine from a normal female with a 5.8 m*M* creatinine concentration; (b) urine from a woman in the 8th month of pregnancy with a 6.1 m*M* creatinine concentration; (c) urine from a patient with uroepithelial cancer with a 5.4 m*M* creatinine concentration; and (d) urine from a patient with disseminated mammary carcinoma with a 6.8 m*M* creatinine concentration. The dotted curve (0.1×) represents a 10-fold damping of the normal deflection (1.0×).


(c)
UROEPITHELIAL CANCER
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Sp
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4
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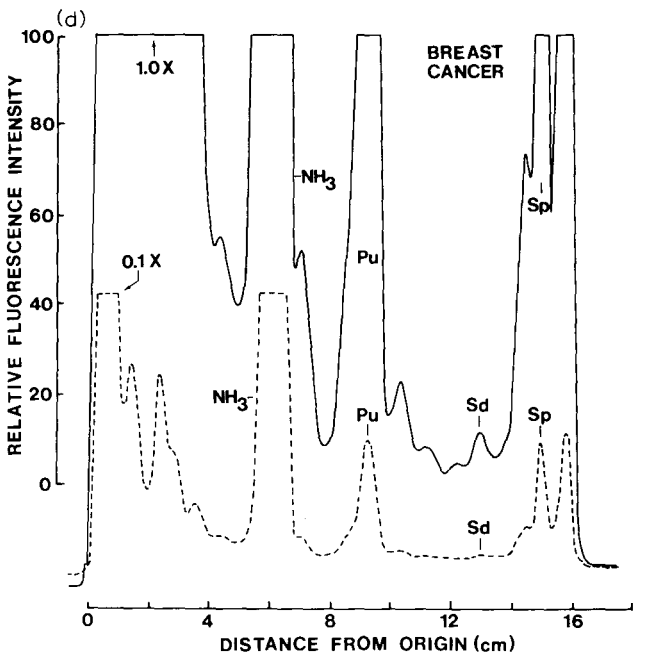
12
16
DISTANCE FROM ORIGIN (cm)


(d)
BREAST CANCER
1.0 X
0.1 X
NH_3
NH_3
Pu
Pu
Sd
Sd
Sp
Sp
RELATIVE FLUORESCENCE INTENSITY
100
80
60
40
20
0
0
4
8
12
16
DISTANCE FROM ORIGIN (cm)

added to the urine samples were 98% for putrescine, 95% for spermidine and 89% for spermine.

Rapid analysis was accomplished by using one-dimensional TLC. Yet an adequate separation of the polyamines was obtained. The Dns derivatives of putrescine, spermidine and spermine appeared as well-defined and well-separated spots and there was no apparent interference from other urinary constituents.

The high sensitivity was achieved by using a fluorometric technique, in which the urinary polyamines were dansylated according to the procedure described by Seiler [17—22]. This is probably one of the most sensitive methods for the determination of polyamines. It involves the formation of Dns derivatives of polyamines and their separation on TLC plates followed by the measurement of the fluorescence intensity of the spot corresponding to each amine by scanning in situ.

For obvious reasons, this method allows for the analysis of free as well as total (free plus conjugated) polyamines in urine. When the free polyamines are to be determined, the only modification that has to be done is the elimination of the hydrolysis and neutralization steps.

Linear calibration curves were obtained between 25 and 500 pmoles per spot. The coefficients of variation for the analysis of mixtures of the polyamines in this range (25—500 pmoles) were better than 10% (CV = S.D./mean, n=5). To obtain this reproducibility special precautions have to be observed at two steps of the procedure: first, the dansylated derivatives have to be applied without the TLC plate being touched with the pipette tip; and second, the TLC plate must be carefully and evenly sprayed with the reagent which increases the fluorescence intensity and stability of the Dns derivatives. When the relative merits of methods developed for the analysis of urinary polyamines are compared, it is apparent that the present method provides definite advantages over gas chromatographic techniques, which require tedious and time-consuming clean-up steps. Compared with automated ion-exchange chromatography, TLC spectrophotofluorometry appears to have the advantage of being more reliable in running. Furthermore, automated amino acid analyzers adapted for polyamine analysis are very expensive both to obtain and to maintain.

Figs. 1a—d show comparative chromatograms (similar creatinine values) of the separation of putrescine, spermidine and spermine in urine specimens from two normal individuals (a non-pregnant woman and a pregnant woman) and from two cancer patients (uroepithelial cancer or breast cancer). The identity of the putrescine, spermidine and spermine peaks was corroborated by inclusion of commercial products. The chromatograms usually show peaks other than those of putrescine, spermidine and spermine. Frequently, a peak is observed just before the peak of putrescine, which appears to be 1,3-diaminopropane. When added to urine it coincided with this peak. In no case has the peak which appears to be due to 1,3-diaminopropane been large enough to interfere with the quantitation of putrescine. In most chromatograms we observed an extra peak between spermidine and spermine. Thus far, however, the identity of this peak has not been revealed. Occasionally, the chromatograms displayed an additional peak near the peak for cadaverine. We have made no effort to identify positively the compound responsible for this peak. Only on rare occasions have these additional peaks interfered with the determination of putrescine, spermidine and spermine.

TABLE I

POLYAMINE CONCENTRATIONS IN THE URINE OF NORMAL INDIVIDUALS

Group		Putrescine	Spermidine (μg/mg creatinine)	Spermine*
Normal		2.9	2.0	0.5
		1.9	1.5	±
		2.4	1.9	±
		2.3	1.7	–
		3.9	2.7	1.1
		2.7	2.4	0.5
		3.3	1.9	0.6
		2.0	2.2	–
		4.1	2.6	0.9
		4.3	2.9	1.7
		2.5	1.8	±
		3.7	2.5	0.8
	Mean ± S.D.	3.0 ± 0.84	2.2 ± 0.44	
Pregnancy**		5.1	1.8	0.6
		4.3	2.1	–
		3.5	2.5	0.7
		7.0	3.0	0.9
		3.7	1.9	+
	Mean ± S.D.	4.7 ± 1.4	2.3 ± 0.49	
Postpartum,	3 days	3.6	2.4	–
	6 days	2.9	2.6	0.8
	9 days	2.3	2.1	–

* +, definitely detectable, but not quantifiable; ± just detectable; – not detectable

** Specimens were collected during the last month of pregnancy.

The concentrations of the polyamines were estimated with the present method in urine of normal individuals (Table I) and of patients with a variety of disease entities (Table II). Putrescine and spermidine were found in all urine samples analyzed whereas spermine was not detectable in some of the samples. Furthermore, we observed that pregnant women, in addition to patients with cancer in an advanced clinical stage, showed elevated urinary polyamine levels. However, it appears that only putrescine increases significantly during pregnancy. In cancer patients the spermidine and/or spermine concentrations were markedly increased, in addition to the putrescine concentration, when compared with the levels of these compounds in normal urine.

Elevated levels of the urinary polyamines have now been observed in a number of independent studies involving large numbers of patients [4, 6—10]. The results obtained have led to an increasing interest in extracellular polyamines and suggest a possible use of urinary polyamine levels as a clinical test in the diagnosis, management and follow-up of patients with cancer. To meet the need for a simpler, yet reliable method we have developed the present technique, which obviates some of the drawbacks noticed for similar analytical procedures.

TABLE II

POLYAMINE CONCENTRATIONS IN THE URINE OF PATIENTS WITH VARIOUS TYPES OF CANCER IN ADVANCED CLINICAL STAGES

All specimens were collected from patients with active disease in an advanced clinical stage, and always before treatment.

Diagnosis	Putrescine	Spermidine (μg/mg creatinine)	Spermine
Hematological tumors			
Acute lymphoblastic leukemia	31.5	24.0	4.2
Acute myelogenous leukemia	8.3	7.8	3.6
Hodgkin's disease	12.1	15.3	2.1
Lymphosarcoma	6.4	8.7	1.5
Reticulum cell sarcoma	11.9	10.2	6.6
Multiple myeloma	7.7	3.8	0.9
Solid tumors			
Undifferentiated small cell carcinoma (oat cell) of the lung	5.5	5.1	0.5
Mammary carcinoma	48.9	2.9	22.0
Transitional cell carcinoma of the bladder	4.6	20.7	8.9
Osteogenic sarcoma	7.1	4.8	1.1
Malignant melanoma of the skin	9.3	5.1	1.4

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Biomedical Applications

CHROMBIO. 086

DETERMINATION OF WATER SOLUBLE IMIDAZO-1,4-BENZODIAZEPINES IN BLOOD BY ELECTRON- CAPTURE GAS—LIQUID CHROMATOGRAPHY AND IN URINE BY DIFFERENTIAL PULSE POLAROGRAPHY

CARL V. PUGLISI, JOHN C. MEYER, LUCIUS D'ARCONTE, MARVIN A. BROOKS and J. ARTHUR F. de SILVA

Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, N.J. 07110 (U.S.A.)

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SUMMARY

A sensitive and specific electron-capture gas—liquid chromatographic (GLC—ECD) assay was developed for the determination of 8-chloro-6-(2′-fluorophenyl)-1-methyl-4H-imidazo(1,5*a*)(1,4)benzodiazepine (I) or 8-chloro-1,4-dimethyl-6-(2′-fluorophenyl)-4H-imidazo(1,5*a*)(1,4)benzodiazepine (II) in blood. The assay for both compounds involves extraction into benzene—methylene chloride (9:1) from blood buffered to pH 12.6. The overall recovery of I and II from blood is 86%± 5.0 (S.D.) and the sensitivity limit of detection is of the order of 2 to 3 ng of I or II per millilitre of blood.

The major urinary metabolite of I is 8-chloro-6-(2′-fluorophenyl)-1-hydroxymethyl-4H-imidazo(1,5*a*)(1,4)benzodiazepine, (IA) present as a glucuronide conjugate while 8-chloro-6-(2′-fluorophenyl)-4-hydroxyl-1-methyl-4H-imidazo(1,5*a*)(1,4)benzodiazepine, (IB) and 8-chloro-6-(2′-fluorophenyl)-4-hydroxy-1-hydroxymethyl-4H-imidazo(1,5*a*)(1,4) benzodiazepine, (IC) are minor metabolites. The major metabolite IA is extracted into benzene—methylene chloride (9:1) from urine buffered to pH 11.0 (after incubation with glucuronidase—sulfatase at pH 5.0), and analyzed by differential pulse polarography (DPP) in 0.1 *M* phosphate buffer (pH 3). The overall recovery of IA is 84 ± 3.0% (S.D.) with a sensitivity limit of 50 ng per millilitre of urine. The metabolites of compound II have not as yet been elucidated. The GLC—ECD and DPP assays were applied to the determination of blood levels and urinary excretion in dogs following single 10 mg/kg intravenous and oral doses of I and following single 6 mg/kg intravenous and 10 mg/kg oral doses of II. Blood levels of compound I were also evaluated in man following intravenous infusion of single 10 mg doses.

INTRODUCTION

The imidazo-1,4-benzodiazepines, 8-chloro-6-(2′-fluorophenyl)-1-methyl-4H-imidazo(1,5*a*)(1,4)benzodiazepine, (I)·maleate, and 8-chloro-1,4-dimethyl-6-(2′-fluorophenyl)-4H-imidazo(1,5*a*)(1,4)benzodiazepine, (II)·hydrochloride are members of a series of the water soluble benzodiazepine analogs synthesized by Walser et al. [1]. They are of clinical interest as anti-anxiety agents of short duration of activity.

TABLE I

CHEMICAL NAMES AND PHYSICAL PROPERTIES OF THE COMPOUNDS

Compound	Chemical Name	MW	M.P.
I	8-chloro-6-(2′-fluorophenyl)-1-methyl-4H-imidazo [1,5*a*] [1,4] benzodiazepine	325.77	152—154
IA	8-chloro-6-(2′-fluorophenyl)-1-hydroxymethyl-4H-imidazo[1,5*a*] [1,4] benzodiazepine	341.78	258—260
IB	8-chloro-6-(2′-fluorophenyl)-4-hydroxy-1-methyl-4H-imidazo[1,5*a*] [1,4] benzodiazepine	341.77	185—186
IC	8-chloro-6-(2′-fluorophenyl)-4-hydroxy-1-hydroxymethyl-4H-imidazo[1,5*a*] [1,4] benzodiazepine	357.78	238—240
II	8-chloro-1,4-dimethyl-6-(2′-fluorophenyl)-4H-imadazo-[1,5*a*] [1,4] benzodiazepine	339.80	247—250 (decomp.)
III*	7-chloro-5-(2′-chlorophenyl)-1,3-dihydro-1-(2-dimethylaminoethyl)-2H-1,4-benzodiazepin-2-one	376.29	178—180
IV	5-Aminomethyl-1-[4-chloro-2-(2′-fluorobenzoyl)-phenyl] -2-methylimidazole dihydrochloride	416.74	300—302
V**	5-(1-Aminoethyl)-1-[4-chloro-2-(2′-fluorobenzoyl)-phenyl] -2-methylimidazole	430.77	

*Compound III is the reference standard in the GLC—ECD assay.
**Compound V has not as yet been synthesized.

The chemical names and physical properties of I and its major metabolites and II are given in Table I, and their chemical structures are given in Fig. 1. In vitro studies [2] on the biotransformation of I showed that the compound was mainly metabolized by hydroxylation, producing significant amounts of the 1-hydroxymethyl, (IA) and smaller amounts of the 4-hydroxy (IB) and the 1-hydroxymethyl-4-hydroxy (IC) analogs respectively. The metabolites of compound II have not as yet been elucidated. The parent compounds I and II are amenable to rapid and sensitive electron-capture gas—liquid chromatographic (GLC) analysis from blood as previously demonstrated for other benzodiazepines [3, 4]. The assay employs OV-1 as the liquid phase and a ^{63}Ni electron-capture detector (ECD) used in conjunction with an electron-capture linearizer for the determination of I or II with nanogram sensitivity. The reference standard in the assay is 7-chloro-5-(2′-chlorophenyl)-1,3-dihydro-1-(2-dimethylaminoethyl)-2H-1,4-benzodiazepin-2-one, III.

The urinary excretion of I and its metabolites, IA, IB and IC was determined by GLC—ECD following enzymatic deconjugation and derivatization with bis-trimethylsilyl acetamide (BSA). The major urinary metabolite, IA, which is present in amounts far in excess of the minor metabolites, was also detemined by differential pulse polarography (DPP).

EXPERIMENTAL

GLC—ECD of I or II in blood

Column conditions. The column packing was a pre-tested phase containing 3% OV-1 on 60—80 mesh Gas-chrom Q (Applied Science Labs., State College,

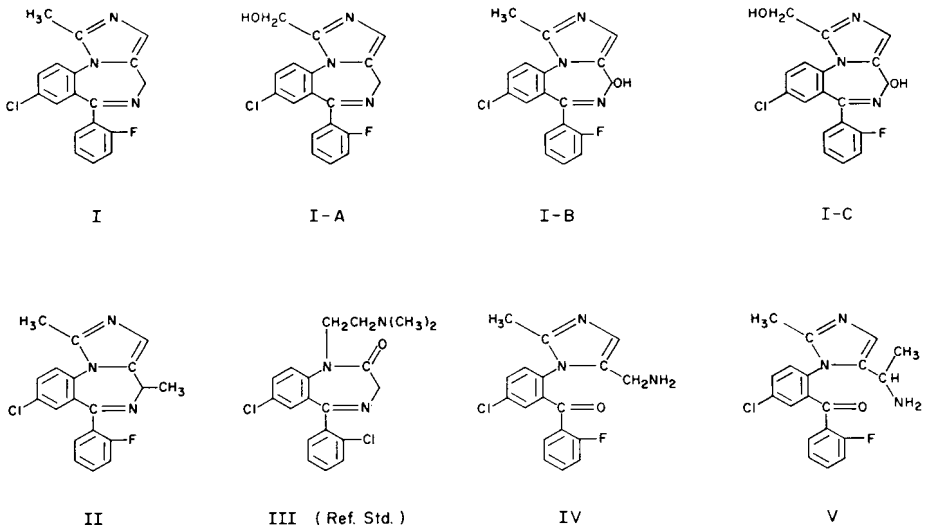

Fig. 1. Chemical structures of compounds referred to in Table I and in text.

Pa., (U.S.A.) packed in a U-shaped, 4 ft × 4 mm I.D. borosilicate glass column. The glass column was treated before packing with a 1% solution of Siliclad (Clay-Adams, New York, N.Y. U.S.A.) for 10 min, thoroughly rinsed with distilled water and dried for at least 1 h at 100°. The packed column was conditioned at 325° under "no flow" conditions for 4 h and then at 265° for at least 18 h with a nitrogen flow-rate of 40 ml/min.

Instrumental parameters. A Tracor Model 222 gas chromatograph, equipped with a ^{63}Ni ECD containing a 15 mCi ^{63}Ni β-ionization source was used. Argon—methane (9:1), (Matheson Gas Products, East Rutherford, N.J. U.S.A.) was used as the carrier gas and the column head pressure was pre-set at 40 p.s.i.g., with a column flow of 65 ml/min and a detector purge of 20 ml/min. The temperature settings were as follows: oven, 225°; injection port, 260°; detector, 325°. The conditions of flow-rate and column temperature must be adjusted to obtain a retention time of 5.5—6 min for I or II. Under these conditions, the reference standard III has a retention time of 9.5—10.5 min. Typical chromatograms for I and III recovered from dog blood and human blood are shown in Figs. 2 and 3, respectively. Chromatograms for II recovered from dog blood are similar to those shown in Fig. 2.

The ECD linearizer Model 114460 standing current was adjusted to $0.5 \cdot 10^{-9}$ A, the relative pulse width was adjusted to 0.15 which corresponds to 0.75 μsec and the attenuation was set at 16. The chart speed was 30 in. per hour and the time constant on the 1.0-mV Honeywell recorder (Model 194) was 1 sec (f.s.d.). Under these conditions 2.0 ng of I, 2.4 ng of II, and 1.0 ng of III give nearly full scale pen response on the 1.0-mV recorder. The minimum detectable amount of I or II is 2—3 ng/ml of blood.

Preparation of standard solutions. Weight out 10.00 mg each of the free base

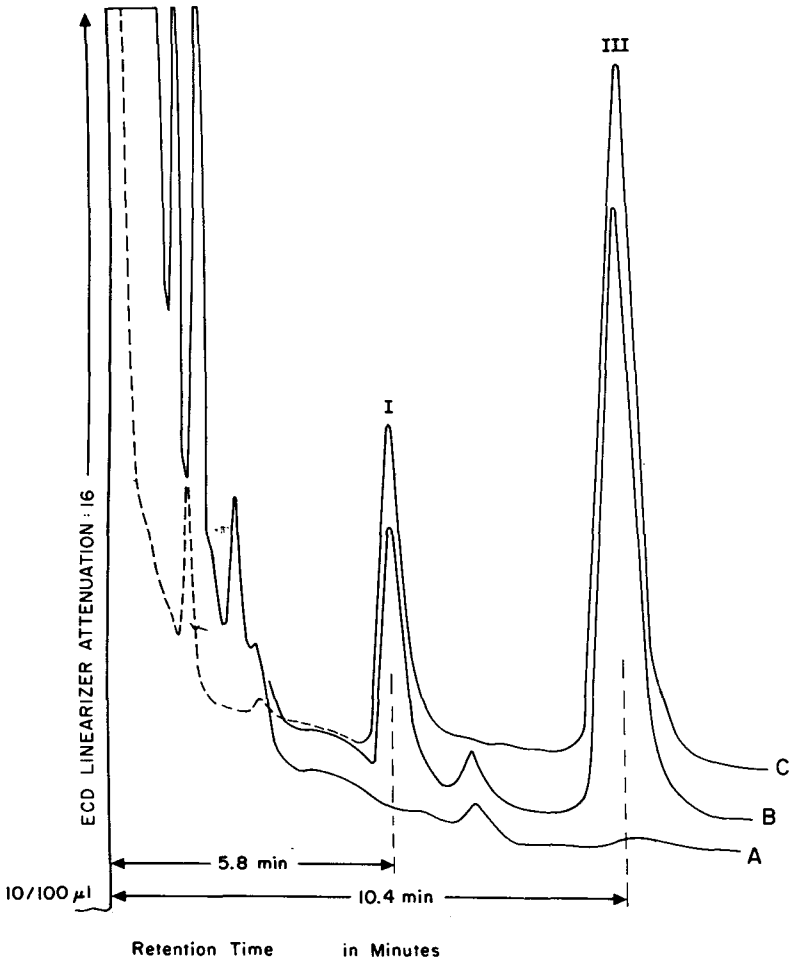

Fig. 2. Chromatograms of (A) control dog blood extract, (B) control blood extract containing added authentic standards, and (C) authentic standards of compounds I and III.

of I or II and the reference standard III into separate 10-ml volumetric flasks. Dissolve in 2.0 ml of acetone and make up to volume with *n*-hexane. These stock solutions contain 1.0 mg/ml and are used to prepare serial 1:10 dilutions in acetone—*n*-hexane (1:4) to yield solutions containing 1 µg/ml. Combine suitable aliquots of these solutions to prepare working standards containing the following concentrations per 100 µl of acetone—*n*-hexane.

(a) Four standard solutions for compound I, containing: 5.0, 10.0, 15.0 or 20.0 ng I, and 10.0 ng of III 100 µl of each solution (Std. 1, 2, 3 and 4, respectively).

(b) Four standard solutions for compound II, containing: 6.0, 12.0, 18.0 and 24.0 ng II and 10.0 ng of III 100 µl of each (Std. A, B, C and D, respectively).

Aliquots (100 µl) of each standard solution of either I or II are added

directly to blood (do not evaporate solvent) as internal standards for recovery determinations and for the construction of an internal standard curve from which the concentration in the unknowns is determined by interpolation.

Aliquots (10 μl) of each of the standard solutions of either I or II are directly analyzed by GLC—ECD to establish the external calibration curve.

Calibration of I, II and III by GLC—ECD. A calibration (external standard) curve of the peak area ratios of I or II to III versus concentration of I or II per 100 μl of acetone—*n*-hexane (1:4) is constructed. The external standard calibration curve is used to establish the parameters for GLC—ECD analysis while the recovered internal standards are used to prepare a calibration curve for the quantitation of the concentration of I or II in biological specimens. The internal standards must be run with each set of unknowns.

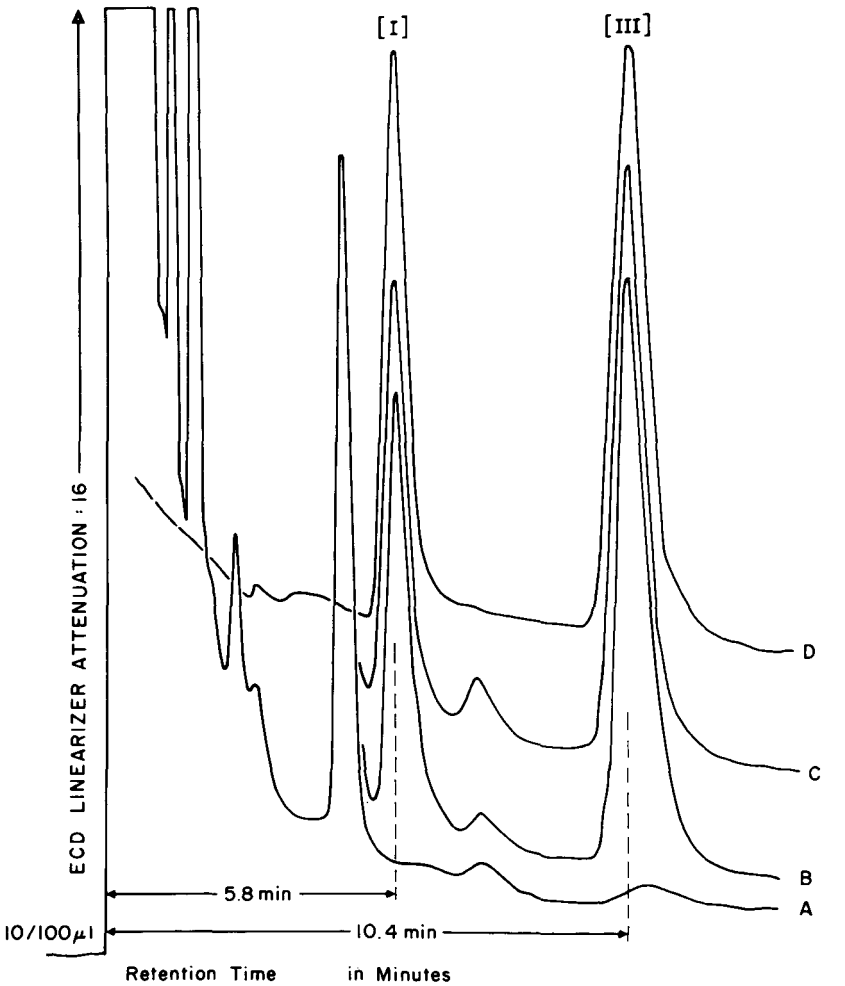

Fig. 3. Chromatograms of (A) control human blood extract, (B) control blood extract containing added authentic standards, (C) subject post-dose blood extract, and (D) authentic standards of compounds I and III.

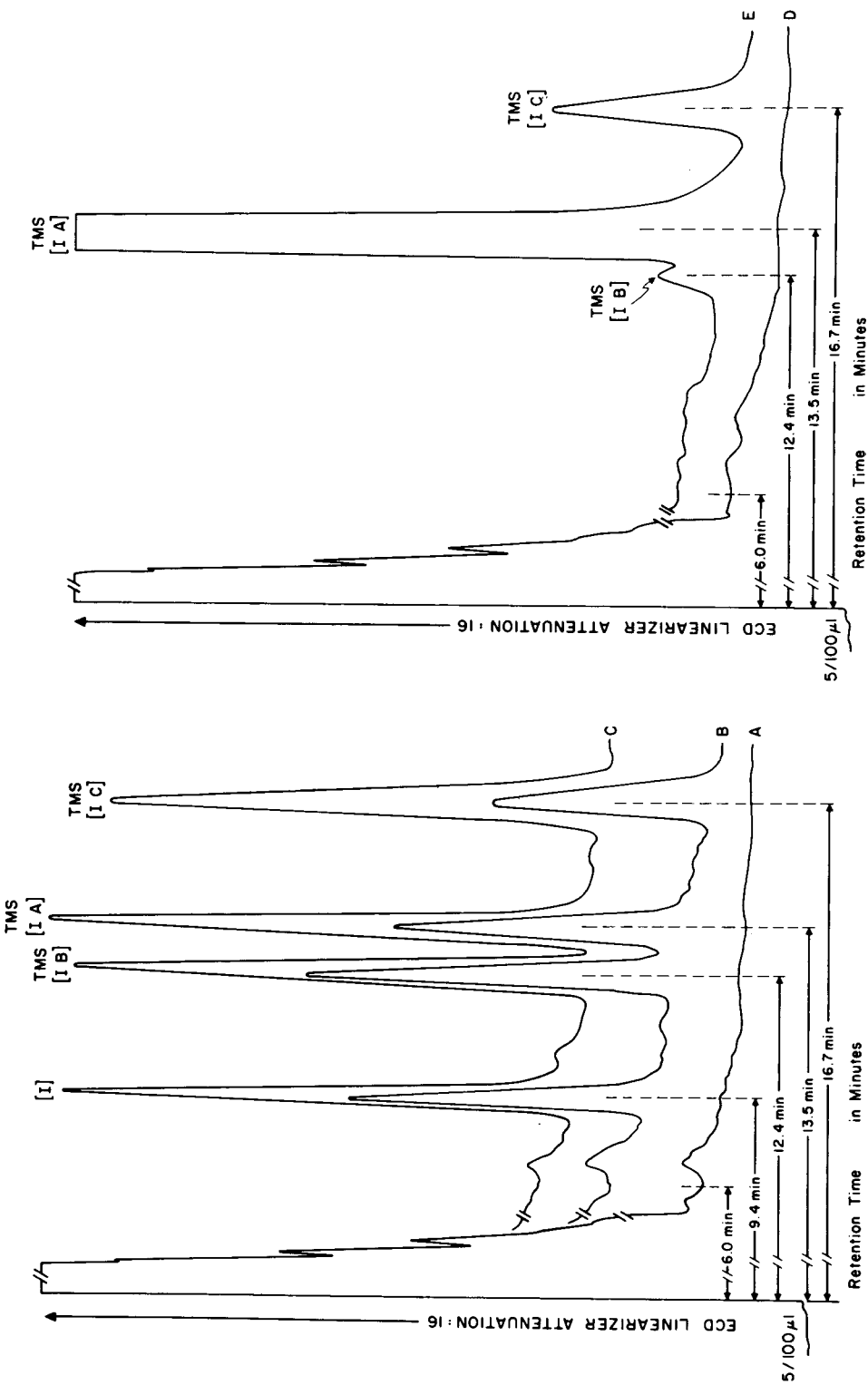

Fig. 4. Chromatograms of (A) patient 0-h urine, (B) patient 0-h urine plus added authentic standards, (C) authentic Standards, (D) directly extractable fraction in 0—12 h urine, and (E) post-glusulase extractable fraction in 0—12 h urine.

Reagents. All reagents were of analytical reagent grade (> 99% purity), and all inorganic reagents were prepared in distilled, deionized water.

Saturated solution of Na_3PO_4, (pH 12.6). Add 200 g of $Na_3PO_4 \cdot 12H_2O$ to 500 ml of distilled water and stir vigorously for 30 min. Allow the crystals to settle to the bottom of the container and remove the supernatant reagent as needed.

The organic reagents used are mixtures of the following: benzene—methylene chloride (9:1), the extraction solvent, benzene—acetone—methanol (17:2:1) and acetone—*n*-hexane (1:4), the solvents for GLC—ECD analysis which are stored over anhydrous sodium sulphate. Benzene and methylene chloride are nanograde (Mallinkrodt, St. Louis, Ma., U.S.A.) and *n*-hexane is 99 mol.% pure, "H-301" from Fisher (Pittsburgh, Pa., U.S.A.).

Extraction of blood for the determination of I or II by GLC—ECD. Into a 15-ml centrifuge tube (PTFE stoppered), add 1.0 ml of whole blood, 2.0 ml of pH 12.6 saturated Na_3PO_4 buffer (mix well), and extract with 8 ml of benzene—methylene chloride by shaking at 80—100 strokes per min for 15 min on a reciprocating shaker (Eberbach Corp., Ann Arbor, Mich., U.S.A.). Along with the samples, run a specimen of control blood (taken preferably from the subject prior to medication) and four specimens of control blood containing either 100 μl of standard solutions 1, 2, 3, or 4 for compound I, or standard solutions A, B, C, or D for compound II added as the respective internal standards. Centrifuge the samples for 10 min at 1500 *g* in a refrigerated centrifuge (Damon/IEC Model PR-J, Needham, Mass., U.S.A. rotor No. 253, 2400 rpm) at 5°, and transfer a 7.5-ml aliquot of the upper organic layer into a clean 15-ml conical centrifuge tube. Evaporate to dryness at 55° in a N-Evap Model N-07 evaporator (Organomation Assoc., Worcester, Mass., U.S.A.) under a stream of clean, dry nitrogen. Vacuum dry the residues over Drierite pellets in a vacuum desiccator for 15 min to remove all traces of moisture and dissolve the residues in 100 μl of benzene—acetone—methanol (17:2:1). Inject a 10-μl aliquot for GLC—ECD analysis. The peaks due to I or II and the reference standard III are identified by their respective retention times (Figs. 2 and 3).

Calculations. The concentration of I or II in the unknows represented by their respective peak area ratios is interpolated directly from the blood recovered internal standard curves in which the peak area ratios of either I or II to III are plotted graphically versus total concentration added per millilitre of blood. The absolute recovery of I or II is determined from the ratio of the slope value [peak area/ng] of the internal to that of the external standard curves.

Analysis of urinary metabolites of I

Intact I is not excreted in the urine. The major urinary metabolite of I is the 1-hydroxymethyl analog (IA) (Fig. 1) which is excreted as a glucuronide conjugate. Two other minor metabolites, the 4-hydroxy analog, (IB) and the 1-hydroxymethyl-4-hydroxy analog, (IC) are also excreted as glucuronide conjugates [2]. None of the above compounds are excreted in the free or unconjugated form. All three metabolites are extracted into benzene—meth-

ylene chloride (9:1) from urine buffered to pH 11.0 (after incubation with glucuronidase-sulfatase at pH 5.3), the residue of which is silylated with BSA in acetonitrile and analyzed by GLC—ECD. Since the 1-hydroxymethyl metabolite (IA) is present in concentrations far in excess of the other two minor metabolites, it can be more conveniently analyzed by DPP in 0.1 *M* pH 3.0 phosphate buffer as the supporting electrolyte, using the reduction peak of the azomethine group for quantitation.

GLC—ECD analysis. The parameters used in the assay in blood were used with the following modifications: column, 6 ft. × 4 mm I.D.; borosilicate glass column containing 5% OV-1 on 100—120 mesh Gas-Chrom Q; oven temperature, 250°: column flow, 75 ml/min. The conditions of flow-rate and column temperature must be adjusted to obtain a retention time of 9.4 min for compound I and 12.4, 13.5 and 16.7 min for the TMS derivatives of compounds IA, IB, and IC, respectively. No reference standard is used in this assay.

Standard solutions. The analytical standards required are given in Table I.

Dissolve a weight equivalent to 10.0 mg (free base) of compounds I, IA, IB, and IC separately in 10 ml of methanol to yield stock solutions containing 1 mg/ml. Make serial 1:10 dilutions of these stock solutions to yield working solutions in the following concentrations: Std. 5, containing: 7 ng I, 7 ng IA, 4 ng IB, 5 ng IC; Std. 6, containing: 14 ng I, 14 ng IA, 8 ng IB, 10 ng IC; Std. 7, containing: 21 ng I, 21 ng IA, 12 ng ng IB, 15 ng IC: and Std. 8, containing: 28 ng I, 28 ng IA, 16 ng IB, 20 ng IC per 100 μl of methanol.

Aliquots (100 μl) of each standard solution are added directly to urine as internal standards for recovery determinations and for the construction of an internal standard curve as their TMS derivatives, from which the concentration in the unknowns are determined by interpolation.

Aliquots (100 μl) of each standard solution are also directly silylated and 5 μl aliquots are analyzed by GLC—ECD as the respective TMS derivatives to establish the external calibration curve.

Extraction of urine. Into a 15-ml conical centrifuge tube add 0.2 ml of urine, 0.8 ml of 0.2 *M* (pH 5.3), sodium acetate buffer, and 1% (v/v) (10 μl) of Glusulase enzyme preparation containing 100,000 units of β-glucuronidase and 50,000 units of sulfatase per ml (Endo Labs., Garden City, N.Y., U.S.A.). The tubes are gently agitated to mix the reagents homogeneously, then stoppered loosely and placed in a Dubnoff incubation shaker (Precision Scientific, Chicago, Ill. U.S.A.) at 37° for 2 h to effect enzymatic deconjugation. Along with the unknowns, process a 0.2 ml specimen of control urine and four 0.2 ml specimens of control urine containing 100 μl of standard solutions 5, 6, 7, or 8. Cool the samples to room temperature, add 2 ml of 1.0 *M* (pH 11.0) phosphate buffer, and extract with 8 ml of benzene—methylene chloride (9:1). Centrifuge the samples as in the blood assay and transfer a 7.5-ml aliquot of the supernatant into another 15-ml tube, evaporate to dryness and vacuum dry the residue for 15 min in a vacuum dessicator. Dissolve the dry residue in 100 μl of BSA (Pierce, Rockford, Ill., U.S.A.) in acetonitrile (1:4) to form the TMS derivatives of compounds IA, IB, and IC. Compound I does not derivatize and is determined as its intact moiety. Inject a 5-μl aliquot for GLC—ECD

analysis. The peaks due to I and the TMS derivatives of IA, IB, and IC are identified by their respective retention times (Fig. 4).

Calculations. The concentration of compound I and the TMS derivatives of compounds IA, IB and IC in the unknown is determined by interpolation from the calibration curve of the internal standards processed along with the unknowns, using the direct calibration (peak area versus concentration) technique. The percent recovery of the internal standards is determined by comparing the slope value [peak area (cm^2) per ng of compound] of the internal standards to that of the external standard curve.

DPP analysis

Conditions for polarographic analysis. A Model 174 polarographic analyzer with a Model 172A drop timer (Princeton Applied Research Corp., Princeton, N.J., U.S.A.) were used in the differential pulse mode in conjunction with a three-electrode semi-micro polarographic cell consisting of a dropping mercury electrode (DME), a saturated calomel electrode (SCE) and a platinum wire as the auxiliary electrode as previously described [5]. The pulse amplitude was —50 mV, the drop time was 2.0 sec, and the drop-rate was 2.42 mg/sec. ($m^{2/3} \cdot t^{1/6} = 1.803$). The current range was between 0.5 and 5.0 μA for a peak response of full scale deflection, the scan range was 1.5 V, and the scan-rate was 1 mV/sec. The samples were scanned between —0.450 V and —0.750 V versus SCE, and the polarograms were recorded on a Houston Omnigraph Model 2200-3-3 (X—Y) recorder (Houston Instruments, Bellaire, Texas, U.S.A.). The analytical peak due to the reduction of the azomethine group ($>C_5 = \bar{N}_4$) of IA occurs at —0.640 V versus SCE in a supporting electrolyte consisting of 0.1 *M* (pH 3) phosphate buffer containing 0.005% methoxypolyethyleneglycol-550 as the maximum suppressor. Typical polarograms for the analysis of IA in urine are shown in Fig. 5.

Sample preparation. Into a 50 ml centrifuge tube add 2.0 ml of urine and 3 ml of 0.2 *M* (pH 5.3) sodium acetate buffer. Adjust to pH 5.0 with glacial acetic acid and mix well on a Vortex super mixer. Add 1% by volume of Glusulase enzyme preparation and shake gently to produce a homogeneous mixture. Along with the samples process a 2.0 ml specimen of control urine and separate 2.0 ml specimens of control urine containing 0.4, 0.8, 1.2 or 1.6 μg of IA as the internal standards. Stopper the tubes and place in a Dubnoff metabolic shaking incubator at 37° for 2 h. Cool the samples to room temperature and adjust the pH to 11.0 (using a pH meter) by titrating the samples dropwise with 2.5 *M* NaOH and adding 1 ml of 1 *M* (pH 11) phosphate buffer. Extract the samples with 16 ml of benzene—methylene chloride (9:1) by shaking for 10 min at a moderate speed on a reciprocating shaker. Centrifuge the samples in a refrigerated centrifuge at 5° for 5 min at 1500 *g*, and transfer a 14-ml aliquot of the upper organic layer into a 15-ml conical centrifuge tube. Evaporate to dryness at 55° under a stream of clean, dry nitrogen. Dissolve the residues in 100 μl of methanol and add 2 ml of 0.1 *M* (pH 3) phosphate buffer containing 0.005% methoxypolyethyleneglycol-550 as the maximum suppressor. Mix well in a Vortex action mixer and deoxygenate the samples for 1 to 2 min with nitrogen bubbled through a microporosity filter stick (No. JD-5385-01) coarse porosity (SGA Scientific Inc., Bloomfield, N.J.,

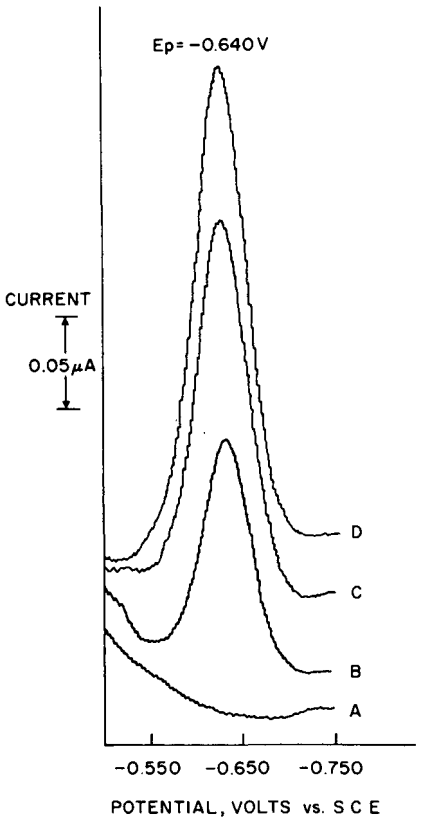

Fig. 5. DPP of de-conjugated compound IA. (A) Control Urine, (B) authentic standard recovered from urine, (C) authentic standard, (D) 0—12 h post-dose urine.

U.S.A.). Transfer the deoxygenated sample into the polarographic cell containing the three operational electrodes and analyze the samples for IA by scanning between —0.450 and —0.750 V versus SCE (Fig. 5).

Calculations. The current (μA) resulting from IA and its overall recovery is determined as described previously [5]. The concentration of IA in the unknowns is determined by interpolation from its respective internal standard curves, making the necessary corrections for the aliquots taken.

RESULTS AND DISCUSSION

A sensitive and specific GLC—ECD assay was developed for the determination of compounds I or II from 1 ml or less of blood. Compound I undergoes biotransformation in the dog and in man yielding three urinary metabolites.

The parent drug I is equally well extracted from blood at pH 9, 11, or 12.6, into benzene—methylene chloride (9:1). Extraction at pH 12.6 yielded chromatograms devoid of interfering peaks in the retention areas of interest and was therefore chosen as the pH of choice for extraction from blood. The urinary

metabolites IA, IB, and IC are quantitatively extracted at pH 11. The metabolites of compound II have not as yet been characterized.

The GLC—ECD behaviour of compounds I or II manifested adsorption losses upon chromatographic analysis. These losses were corrected by deactivating the glass column with a 1% solution of Siliclad in water. Compound III was chosen as the reference standard for the blood-assay due to its similar GLC—ECD and extraction behaviour to compounds I and II. Under the above operational conditions, the life span of the column is usually 5—6 months of continuous use.

Recovery and sensitivity limits of the GLC—ECD assay

The overall recovery of I and II from blood is 86 ± 5.0% (S.D.), and the sensitivity limit is of the order of 2 to 3 ng of I or II per millilitre of blood. The direct extraction procedure is limited to 1 ml or less of blood.

The overall recovery of compound I and the TMS derivatives of IA, IB and IC from urine is 92 ± 5.3% (S.D.) with sensitivity limits of 10—15 ng of each compound per millilitre of urine analyzed.

DPP analysis

The analysis of 1,4-benzodiazepines by DPP is well documented [5, 6, 7]. Compounds I and II exhibited two polarographic peaks in 0.1 *M* sulphuric acid but only one peak in buffers ranging from pH 3 to pH 4. This phenomenon is due to the hydrolysis of the [$>C_5 = N\overline{4}$] azomethine bond to yield "open ketones", compounds IV and V (Fig. 1). DPP was used to determine the rate of hydrolysis of compounds I and II in acidic solutions, its reversibility and stability in solutions of varying pH.

It was found that an equilibrium exists between the parent compounds I and II and their respective open ketones, IV and V, that this equilibrium is strongly pH dependent and that from pH 3.0 through pH 11.0 (pH 11 is used for extraction) only the intact compounds are present. Similar phenomena of pH dependence on chemical stability were reported for flurazepam [6].

The overall recovery of metabolite IA from urine determined by DPP is 84.3 ± 3% (S.D.). The sensitivity limit is 50 ng of IA per millilitre of urine using a 2 ml specimen per analysis.

Application of the GLC—ECD and DPP assays in biological specimens

Studies in the dog. Blood level profiles of compound I were determined in a dog following the intravenous and oral administration of a 10 mg/kg dose of compound I, whereas those of compound II were determined in a dog following a 6 mg/kg intravenous dose, and a 10 mg/kg oral dose, respectively. The blood levels of compounds I and II following the intravenous administration were measurable through 12 and 30 h, respectively. After a 10 mg/kg oral dose, peak levels of 0.99 μg/ml were observed at 30 min for compound I whereas peak levels of 2.87 μg/ml were observed at 1.5 h for compound II, see Figs. 6 and 7, respectively.

The urinary excretion of compound I and IA, IB and IC was determined by DPP following the 10 mg/kg intravenous and oral doses of I. Direct extraction

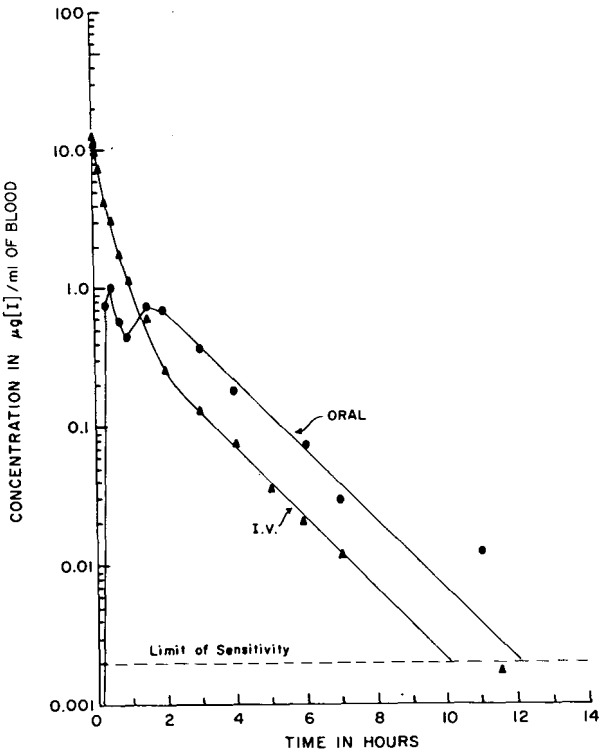

Fig. 6. Blood level fall-off curves of I in the dog following a 10 mg/kg dose of the maleate salt of I by intravenous and oral routes.

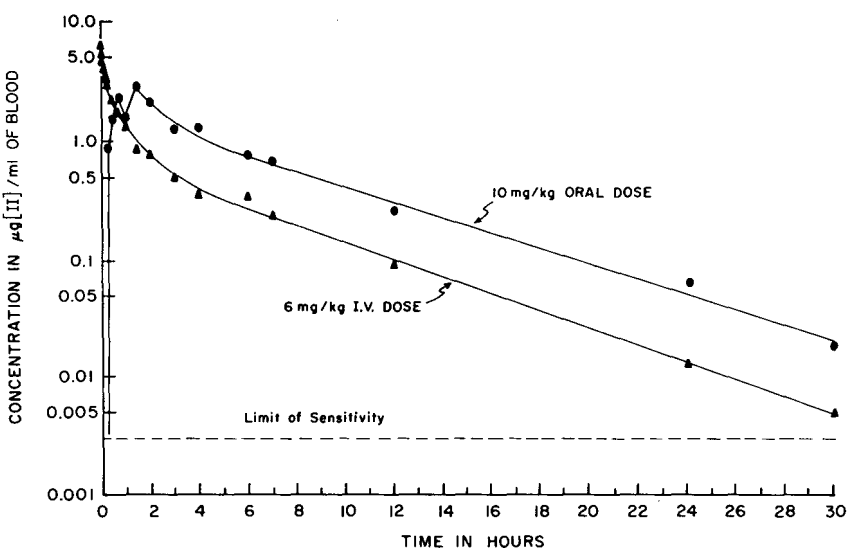

Fig. 7. Blood level fall-off curves of II following intravenous and oral administration of II·HCl in a dog.

at pH 11.0 showed that less than 0.3% of the administered dose was excreted as I, IA, IB, and IC in the unconjugated form.

The major urinary metabolite of I, IA, is present as a glucuronide or sulfate conjugate and accounts for 4.82% and 5.49% of the 10 mg/kg intravenous and oral doses respectively (Table II). The metabolites of compound II have not as yet been characterized.

Studies in man. A pilot study was conducted in which five healthy volunteers were administered a single 10 mg intravenous infusion of I as the maleate salt at a rate of 10 mg/min for one minute (total dose 10 mg free base). Blood samples were collected from the five subjects and pooled urine specimens were collected from subjects 3, 4, and 5.

TABLE II

URINARY EXCRETION OF IA IN THE DOG FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION OF A 10 MG/KG DOSE OF I·MALEATE

Determined by DPP after enzymatic deconjugation. n.m. = not measureable (<50 ng/ml of urine).

Excretion period (h)	% of dose excreted as IA	
	Intravenous	Oral (Capsule)
0—24	3.2	4.76
24—48	1.36	0.73
48—72	0.26	n.m.
Total	4.82	5.49

(A) Blood level profile. The blood concentration data for subjects 1—5 are plotted semilogarithmically versus time in Fig. 8.

The blood level profile indicates an initial rapid disposition of I, with blood levels declining 5 to 10 fold within the first hour following administration of the dose. The drug is also rapidly eliminated from the body, with "apparent" half-lives ranging from 1.3 to 2.2 h in the five subjects studied.

(B) Urinary excretion profile. Urine specimens from subjects 3, 4, and 5 were analyzed for intact I, IA, IB and IC in the directly extractable unconjugated form using the GLC—ECD assay. No measurable levels of either the intact drug or any of the above metabolites were seen in these subjects (Fig. 4D). The levels (if any) were below the sensitivity limit of the GLC—ECD assay (less than 10 to 15 ng/ml of urine). The samples were re-analyzed by GLC—ECD following glucuronidase-sulfatase incubation.

Trace amounts of IB and approximately 1% of the dose as IC were seen in the conjugated fraction. The major metabolite was IA (Fig. 4E) which accounted for approximately 50% of the administered dose, of which up to 96% was excreted during the first twelve hours. Since this compound was excreted in amounts far in excess of the other two minor metabolites, the urinary excretion profile of IA was determined by DPP and the data summarized in Table

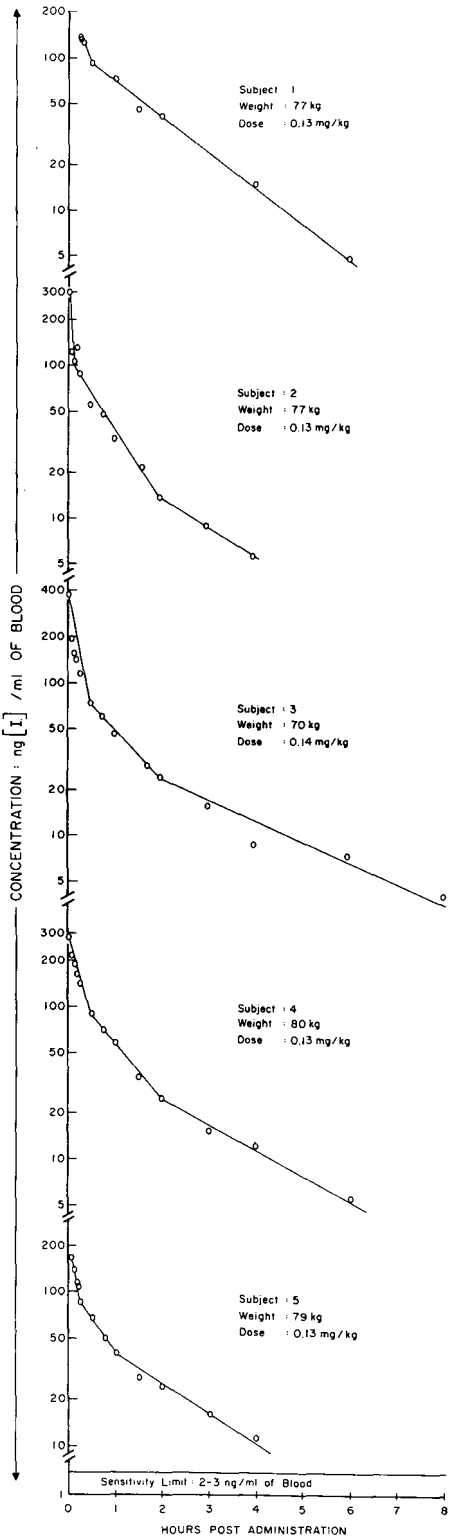

Fig. 8. Blood level fall-off curves in five subjects following the intravenous infusion of a single 10 mg dose of I-maleate.

III. Extensive elimination occurs in the 0—12 h period, paralleling the blood level fall off profile, with substantially smaller amounts being eliminated in the 12—24 h period and thereafter. The overall recovery of the administered dose in the 0—72 h period as this metabolite ranged from 45 to 57% of the dose.

The urine levels of IA obtained by GLC—ECD were statistically evaluated against those obtained by DPP using linear regression analysis. The resulting least square line (r = 0.99) indicated that a slope of 1 and a intercept of 0 fell within the 95% confidence limits (Table IV) showing that the two assays were equivalent.

TABLE III

URINARY EXCRETION OF IA IN MAN FOLLOWING A 10-MG INTRAVENOUS INFUSION OF I·MALEATE

Determined by DPP after enzymatic deconjugation. n.m. = not measureable (<50 ng/ml of urine).

Excretion period (h)	% Dose excreted		
	Subject 3	Subject 4	Subject 5
0—12	41.0	43.2	27.1
12—24	11.4	1.7	14.0
24—48	3.8	n.m.	3.8
48—72	1.0	n.m.	n.m.
Total	57.2	44.9	44.9

TABLE IV

CORRELATION OF GLC—ECD AND DPP IN THE ANALYSIS OF IA IN URINE

Correlation coefficient $r = 0.99$

Subject	Excretion period (h)	Concentrations μg/ml or urine	
		GLC—ECD	DPP
3	0—12	1.20	1.20
3	12—24	0.98	0.95
4	0—12	1.37	1.44
4	12—24	0.15	0.14
5	0—12	1.35	1.23
5	12—24	1.17	1.17

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GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF CARBAMAZEPINE AND PHENYLETHYLMALONAMIDE IN PLASMA AFTER REACTION WITH DIMETHYLFORMAMIDE DIMETHYLACETAL

ROBERT J. PERCHALSKI*

Research Service, Veteran Administration Hospital, Gainesville, Fla. 32602 (U.S.A.)

and

B.J. WILDER

Neurology Service, Veterans Administration Hospital, and Department of Neurology, College of Medicine, University of Florida, Gainesville, Fla. 32610 (U.S.A.)

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SUMMARY

A previously published procedure for the gas chromatographic analysis of carbamazepine has been modified and expanded to allow simultaneous determination of phenylethylmalonamide, a metabolite of primidone. Internal standards that closely resemble each compound are used, and derivatives are made by reaction with dimethylformamide dimethylacetal. This change of internal standard for carbamazepine and the use of a commercial, pretested column-packing material eliminate the major pitfalls of the original method.

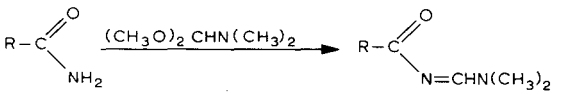
INTRODUCTION

In the early 1970s, the dimethylformamide dialkylacetals were investigated for analytical use in derivatization of fatty acids and amino acids [1, 2]. Although these original reports indicated that reactions were easy, fast, and quantitative, the reagents were never used for actual assays in biological systems. Apparently problems with reagent purity and multiple products became evident after further study.

The reagent was later found to undergo rapid quantitative reaction with primary amides, and this property was used in the quantitation of carbamazepine (now one of the primary antiepileptic drugs) in plasma [3, 4]. Structural studies of the derivatives of carbamazepine, 10,11-dihydrocarbamazepine, and

*To whom correspondence should be addressed.

Cyheptamide (dibenzo[*a*,*d*] [1,4] cycloheptadiene-5-carboxamide) [5] showed that the reaction product was formed by coupling of the amide nitrogen through the acetal linkage of the reagent, to give an N-dimethylaminomethylene structure.



This method has been improved and expanded by changing the internal standard for carbamazepine to a compound that is easily synthesized in one step and more closely resembles carbamazepine than does Cyheptamide, the standard previously used. The column packing has been changed to one that is commercially available and has a guaranteed performance. Finally, a new internal standard, *p*-methylphenylethylmalonamide, has been incorporated to allow simple, accurate quantitation of phenylethylmalonamide (PEMA), one of the metabolites of primidone.

Since the initial studies of Gallagher et al. [6] and Baumel et al. [7, 8], almost no clinical information about PEMA has been published. This can be directly related to the scarcity of analytical information. The early techniques [8—10], which were developed for brief clinical studies, were long, involved, and unsuited to routine use. The most promising method published to date [11] involves a long extraction and uses an internal standard that is chemically unrelated to most of the drugs determined. Use of the usual internal standards is impossible because the drugs are chromatographed as trimethylsilyl derivatives, and under most conditions these phenytoin and carbamazepine derivatives are unresolved from the carbamazepine and phenytoin internal standards, respectively.

The present method, along with reliable on-column methylation techniques will allow rapid, accurate quantitation of all the major antiepileptic drugs and their metabolites that are present in plasma. With these two basic techniques, compounds are grouped rationally according to functional groups and concentration, and are analyzed as valid derivatives rather than as degradation products.

MATERIALS AND METHODS

Apparatus

A Varian Model 2100 gas chromatograph equipped with flame ionization detectors was used for this study. The column (91.5 cm × 2 mm I.D.) was a glass U-tube, packed with 3% OV-225 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The injection port, column oven, and detector were heated to 250°, 235°, and 270°, respectively. Nitrogen carrier gas was adjusted to about 70 ml/min to give a retention time for the carbamazepine derivative of 3.0 ± 0.2 min.

The mass spectra for the derivative of phenylethylmalonamide were run on a DuPont Model 490-F single-focusing mass spectrometer, operated in the

electron impact mode. Samples were probe-distilled directly into the ionization chamber. The ion source was operated at 235° with an ionizing voltage of 70 eV.

Reagents

Dimethylformamide dimethylacetal (DMF-DMA) (Aldrich, Milwaukee, Wisc., U.S.A.) was redistilled through a 15-cm distillation column before use. The product was collected between 105° and 107° in an amber bottle, which was then closed with a PTFE-lined screw cap and stored in the refrigerator. Carbon disulfilde was also redistilled and stored in the same type of bottle. All solvents and chemicals were analytical reagent grade. Carbamazepine was obtained as 200-mg tablets of Tegretol (Geigy Pharmaceuticals, Ardsley, N.Y., U.S.A.) and recrystallized from isopropanol to give a product that melted at 191—192°. The internal standard for carbamazepine, 10,11-dihydrocarbamazepine, was made by the procedure of Palmer et al. [12] and was recrystallized from isopropanol to give a product that melted at 200--202°. Phenylethylmalonamide* and *p*-methylphenylethylmalonamide** were used as received. The internal standard solution is made by dissolving about 1.5 mg of 10,11-dihydrocarbamazepine and 2.5 mg of *p*-methylphenylethylmalonamide in 10 ml of methanol and quantitatively adding this solution to 190 ml of 0.1 *M* ascorbic acid. The solution is stored under refrigeration in an amber bottle with a PTFE-lined screw cap and is stable for at least two months.

Procedure

In a culture tube (13 × 100 mm) having a PTFE-lined screw cap, 1 ml of plasma is combined with 0.50 ml of the internal standard, 0.5 ml of a 1 *M* K_3PO_4 solution and 3 ml of benzene—ethyl acetate (3:2, v/v). The mixture is shaken for 10 min and centrifuged to separate the layers. The organic phase is then transferred to a 5-ml Mini-vial (Alltech Assoc. Arlington Heights, Ill., U.S.A.) and evaporated at 50° under a stream of dry, filtered air or nitrogen. The residue is allowed to react for 10 min at 100° in a closed vial with 50 μl of DMF-DMA. The vial is then set aside to cool. Immediately before sample injection, the reagent is evaporated at room temperature under an air or nitrogen stream and the residue is dissolved in 25 μl of carbon disulfide. With a clean, dry syringe, about 1 μl is injected into the gas chromatograph.

RESULTS AND DISCUSSION

After publication of the method on carbamazepine determination by derivatization with DMF-DMA [3], several problems arose as people tried to duplicate the work. Initially, the main problem was the layered liquid phase column of OV-1 over OV-210. This was not a mixed liquid phase, but each phase was coated separately on the solid support to retain its individual advantage. The OV-210, which was solution-coated on the bare solid support, gave good resolution of the peaks of interest and minimum tailing. The OV-1,

*Supplied by Dr. C.E. Pippinger.
**Supplied by Dr. Kenneth H. Dudley.

which acted to greatly decrease the retention times of the peaks, was layered over the OV-210 by the pan-coating technique. For this step, a solvent (toluene) was used that would not dissolve the layer of OV-210 already on the solid support. A second problem arose later when Cyheptamide, the internal standard, became unavailable. The purposes of this work were (i) to expand the method to include phenylethylmalonamide, the primary amide metabolite of primidone, and (ii) to make the method routinely available by using internal standards that could be easily made or bought, and by using a commercial column packing of guaranteed performance.

Without any change in the basic method, these goals were all reached by changing the column packing to 3% OV-225 on 100—120 mesh Gas-Chrom Q, which is available as a pretested packing material, by substituting 10,11-dihydrocarbamazepine for Cyheptamide as the internal standard for carbamazepine, and by incorporating *p*-methylphenylethylmalonamide as a new internal standard for PEMA. Unlike the Cyheptamide derivative, the dihydrocarbamazepine derivative is stable in carbon disulphide, and it is easily made by a simple catalytic hydrogenation procedure [12]. The internal standard for PEMA is commercially available (Aldrich; Cat. No. 19,496-4).

The chromatograms in Fig. 1 show extraction of plasma from a patient on a regimen of phenobarbital (60 mg/day), primidone (500 mg/day), ethosuximide (750 mg/day), and carbamazepine (600 mg/day) and of drug-free plasma. The barbiturate, hydantoin, and succinimide anticonvulsants, when reacted with DMF-DMA, generally give multiple products, which elute within 1.5 min under the present conditions. Measured drug concentrations in the patient's plasma were 58.4, 8.2, 17.7, 5.91, and 8.97 μg/ml for phenobarbital, primidone, ethosuximide, carbamazepine, and PEMA, respectively. Pheno-

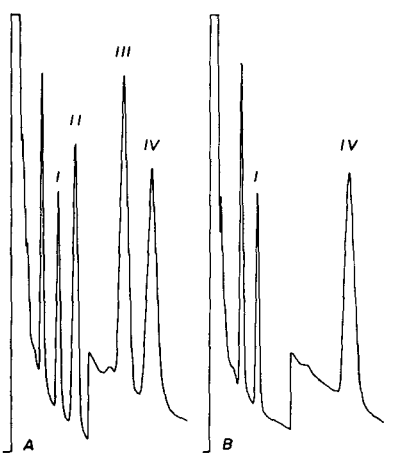
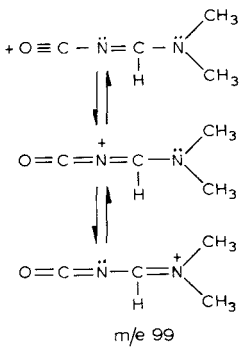


Fig. 1. Chromatograms of extracts of plasma from a patient receiving carbamazepine and primidone (A) and a person receiving no drugs (B). Designated peaks are N-dimethylaminomethylene derivatives of 10,11-dihydrocarbamazepine (I), carbamazepine (II), phenylethylmalonamide (III), and p-methylphenylethylmalonamide (IV). Measured concentrations of carbamazepine and phenylethylmalonamide were 5.91 μg/ml and 8.97 μg/ml, respectively.

barbital, primidone, and ethosuximide were determined by a modification of the method of Perchalski and Wilder [14]. The discontinuity in the baseline between the carbamazepine and PEMA peaks indicates a fourfold decrease in attenuation.

The derivative of PEMA, formed by reaction with DMF-DMA, was subjected to electron impact mass spectrometry. As in the previous study of these derivatives [5], the base peak at *m/e* 99 was by far the major peak in the spectrum. The molecular ion peak was clearly visible (1.8%) at *m/e* 316, which indicated that both nitrogens of PEMA are derivatized to the N-dimethylaminomethylene structure. No peak was visible at *m/e* 261, which would have been characteristic of the singly derivatized molecule.

The high relative intensity of the peak at *m/e* 99 is probably due to resonance stabilization of the ion through the conjugated oxygen-nitrogen system.



This characteristic spectrum makes these derivatives ideally suited to single ion monitoring. A method based on this mass spectrometric technique could probably be developed and would require only about 10 μl of plasma.

Reproducibility of the technique was evaluated by extracting ten replicates of a plasma sample that contained 4.53 μg/ml and 7.17 μg/ml of carbamazepine and phenylethylmalonamide, respectively. The coefficient of variation (CV) of the carbamazepine results was 1.9% and that of the PEMA results was 2.2%. Two of these samples were run over the 90-min period after evaporation of the derivatizing reagent, and again at 21 h. The CVs of the carbamazepine results were 1.1% for 90 min and 1.2% over the 21 h. That of the PEMA results was 4% over 90 min. After 21 h the peaks for PEMA and its internal standard were barely detectable.

Quality control samples, containing low (2.33 and 3.53 μg/ml) and high (9.32 and 14.1 μg/ml) concentrations of carbamazepine and phenylethylmalonamide, respectively, were run daily. Over a 20-day period, results had CVs of 6% and 1.8% (means, 2.44 and 9.47 μg/ml) for the low and high carbamazepine samples, respectively; and 4% and 5% (means, 3.55 and 14.2 μg/ml) for the low and high PEMA samples, respectively.

Recovery of carbamazepine from plasma was 93.5 ± 2.4% over the range of 2—10 μg/ml, whereas that of phenylethylmalonamide was much less, at 41.0 ± 1.0% over the range of 3—15 μg/ml. Reduction of the aqueous volume by

cutting plasma, standard, and buffer volumes in half did not significantly increase the recovery of carbamazepine. Recovery of PEMA was increased to about 55%; however, the smaller sample size was not adequate for detection of low drug levels.

The extracting solvent used in the original method, benzene—ethyl acetate (4:1, v/v), gave a slightly lower recovery of carbamazepine; however, the recovery of PEMA was only about 25%. Various other lighter-than-water solvents and solvent mixtures — made from cyclopentane, benzene, ethyl acetate, ether, methylene chloride, and ethylene dichloride — were tried, but no combination enhanced the recovery of PEMA without introducing interferences.

Such recovery could not normally be tolerated because low recoveries generally result in poor precision and accuracy. In this case, however, use of an internal standard, which has exactly the same functional groups in the same relative positions as PEMA, compensates for the recovery problem and maintains a high level of precision for the method. Even with the low recovery, the limit of detection of PEMA is 0.5 μg/ml, and, as before, that of carbamazepine is 0.2 μg/ml.

A random series of samples from 21 patients who were receiving various doses of primidone were analyzed for primidone and PEMA. A plot of primidone concentration versus that of PEMA had a correlation coefficient of 0.888 and a slope of 0.779, in excellent agreement with the values of 0.814 and 0.727, respectively, reported by Gallagher and Baumel [13]. The ratio of concentrations of primidone to PEMA for these samples was 1.0 ± 0.4. This finding along with the relatively longer half-life of PEMA (about 32 h as opposed to 6 h for primidone [13] may indicate that the PEMA concentration is a more consistent gauge of the primidone activity than is primidone itself. Further studies are under way with a more controlled group of patients to determine if this ratio has some correlation with dosage, seizure control, or metabolic abnormalities.

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CHROMBIO. 090

ASSAY OF UNDERIVATIZED NITRAZEPAM AND CLONAZEPAM IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY APPLIED TO PHARMACOKINETIC AND BIOAVAILABILITY STUDIES IN HUMANS

A.G. de BOER, J. RÖST-KAISER, H. BRACHT and D.D. BREIMER

Department of Pharmacology, Subfaculty of Pharmacy, Sylvius Laboratories, Wassenaarseweg 72, Leiden (The Netherlands)

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SUMMARY

The assay procedure of underivatized, intact nitrazepam and clonazepam in human plasma is described, using gas chromatography with a support-coated open tubular column (OV-17), a solid injection system and electron-capture detection. Clonazepam is used as internal standard in the assay of nitrazepam and vice versa. Linear calibration curves after a single extraction step were obtained in the concentration range 10—100 ng/ml plasma, with standard deviations less than 4.9%. The sensitivity limit of the method is about 1 ng/ml plasma for both drugs.

The method was applied to pharmacokinetic and bioavailability studies of nitrazepam in humans. Seven healthy volunteers received two nitrazepam-containing tablet preparations (5 mg) and plasma concentrations were determined regularly from 15 min to 80 h following drug administration. The mean elimination half-life of nitrazepam was 27 h (range 13—34 h). Considerable intra-individual differences in peak level times between the two preparations were observed, whereas the extent of bioavailability was rather similar.

INTRODUCTION

Various methods for the determination of nitrazepam in plasma have been described in the literature, comprising thin-layer chromatography [1—5], photometric [6, 7], fluorimetric [8, 9] and radioactivity [10] measurements, high-pressure liquid chromatographic [11, 12] and gas chromatographic (GC) methods [13—20]. Howver, due to their lack of sensitivity or specificity, several of these methods cannot be applied to pharmacokinetic investigations when therapeutic doses are used. Of the GC methods some determine the benzodiazepines after acid hydrolysis as benzophenones [13, 17—19], which

leads to loss of specificity. Others require derivatization, e.g. methylation [15, 16] and trimethylsilylation [20]. In addition, laborious extraction procedures are often required for the isolation of the compounds from blood or plasma, which, in addition to chemical manipulation and long retention times, can make such methods very time-consuming.

It was our aim to develop a method for the assay of nitrazepam in plasma that could be applied to pharmacokinetic and bioavailability studies in man. The method should therefore be specific and sensitive and also rapid because of the great number of samples to be analyzed. The use of a so-called SCOT column (support-coated open tubular column) together with electron-capture detection proved to be suitable for our purpose. Next to nitrazepam, it was found that clonazepam could be analyzed equally well with this method.

MATERIALS AND METHODS

Cab-O-Sil (fumed silica, non-silanized, Grade M5; Carbot Corp., Boston, Mass., U.S.A. Duran 50 glass (Schott-Ruhrglas, Bayreuth, G.F.R.); benzyltriphenylphosphonium chloride (Aldrich, Milwaukee, Wisc., U.S.A.); Carbowax 20M (Chrompack, Middelburg, The Netherlands); OV-17 (Chrompack); distilled dichloromethane (Baker, Phillipsburgh, N.S., U.S.A.); distilled light petroleum (b.p. 40—60°, AnalaR grade; BDH, Poole, Great Britain); distilled ethyl acetate (Baker); nitrazepam (Hoffmann-La Roche, Basle, Switzerland); Mogadon (Hoffmann-La Roche) tablets containing 5 mg nitrazepam; Sameko tablets (Sameko, Katwijk, The Netherlands) containing 5 mg nitrazepam; clonazepam was a gift from Dr. T.B. Vree, Laboratory of Clinical Pharmacy, Radboud hospital, Nijmegen.

Extraction procedure

To 1.0 ml plasma in a centrifuge tube were added 25 μl ethanol containing 25.0 ng clonazepam (internal standard) and 1.0 ml borate buffer (0.2 *M*) pH 9.0. After homogenization the mixture was extracted twice with 5 ml light petroleum (b.p. 40—60°)—dichloromethane (1:1) on a Cenco whirlmixer for 20 sec. After centrifuging for 5 min at 2500 *g*, the upper organic layer was removed with a pasteur pipette and transferred to a conical evaporation tube. The solvent was evaporated to dryness at 50—60° in a flow of dry nitrogen on a water bath. The residue was dissolved in 50 μl ethyl acetate and 1—2 μl of this solution were brought onto the needle of the solid GC injection system. After evaporation of the ethyl acetate the residue was injected into the gas chromatograph. The whole extraction procedure is represented in Fig. 1.

Apparatus

A Hewlett-Packard Model 5713A gas chromatograph, equipped with a ^{63}Ni-pulse-modified electron-capture detector, was used. The solid injection system was a modified pyrolysis system (Becker, model 767) which has been used by Driessen and Emonds [21] for the determination of antiepileptic drugs. Temperatures: injection port, 250°; column, 230°, detector, 300°. Gas flow-rates: through the column 10 ml/min, argon—methane (95:5); auxiliary gas,

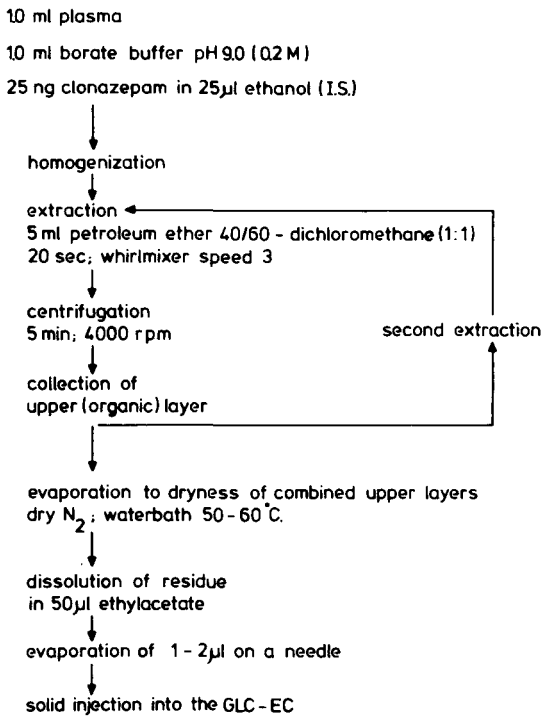

Fig. 1. Extraction scheme for the isolation of nitrazepam from plasma and the subsequent gas chromatographic determination.

argon—methane (95:5) was added at the end of the column to obtain a total flow-rate through the detector of 35 ml/min. Capillary columns were prepared using a Hupe and Busch glass-drawing machine. The glass column (Duran 50) had a length of 10 m, and I.D. of 0.40 mm and an O.D. of 0.80 mm. The column was cleaned with acetone and carbon tetrachloride. The support material (0.5 g Cab-O-Sil Grade M5) was deactivated with 2 ml of a 1% solution of benzyl triphenylphosphonium chloride (BTPPC) in dichloromethane. The excess BTPPC was removed by washing the support material two times with 10 ml of dichloromethane. After centrifugation the dichloromethane was decanted and the support material was suspended in 15 ml carbon tetrachloride by placing it in an ultrasonic bath for 15 min. The column was coated with this suspension at a rate of about 10 cm/sec. Before coating a small plug (± 40 cm) of carbon tetrachloride was brought into the column and immediately followed by the suspension in order to prevent blocking of the column. At the end of the column a dummy column (25% of the column length) was attached in order to prevent a sudden rise in the coating rate when the suspension starts to leave the column. After the suspension had left the column the flow through the column was increased for 3 h for drying and also to prevent droplets forming.

The column was then deactivated by coating dynamically with a 1% solution of Carbowax 20M in dichloromethane at a rate of 5 cm/sec. After drying,

the column was coated dynamically with a 3% solution of OV-17 in dichloromethane at a rate of 5 cm/sec. The column was dried for 3 h with a nitrogen flow of 20 ml/min. The column was conditioned overnight by starting with temperature programming from 50° up to 270° at a rate of 1°/min and then being kept at 270° overnight. A similar column preparation has been described previously [22].

For the identification of the compounds eluting from the gas chromatograph an LKB-2091 combined gas chromatograph—mass spectrometer equipped with a PDP-11 computer system was used.

Preparation of calibration curves

The concentration of nitrazepam in plasma was calculated with the aid of calibration curves prepared by adding known amounts of nitrazepam to 1.0 ml blank plasma. These standard samples were analyzed by the same procedure as described above and the ratios of the peak areas of nitrazepam to internal standard were plotted against the known concentrations of nitrazepam. The same procedure was followed for estimating the extraction yield of nitrazepam from plasma at various concentrations, except that clonazepam was used as an external standard (25.0 ng). The ratios found were compared to the ratios of standard amounts of the drugs. Calculation was carried out manually by calculating the peak area (peak height × peak width at half peak height). Before analysis of a sample series, calibration was always carried out using two plasma samples containing known concentrations of nitrazepam. Stock solutions of nitrazepam and clonazepam were stored in the refrigerator at 4° to avoid possible decomposition [23].

Human studies

Seven healthy male volunteers (aged 20—23 years, body-weight 64—86 kg) participated in the study after they had been medically examined. At an interval of two weeks each volunteer received one tablet containing 5 mg nitrazepam. Two different brands (Mogadon and Sameko) were used in a cross-over design. Subjects were instructed not to take other drugs or alcohol from 24 h before, until 24 h after, the beginning of the experiments. In addition they were not allowed to drive a motor vehicle for 48 h following drug administration.

After an overnight fast, at 9 a.m. the volunteers swallowed the intact tablet with 150 ml tap water. Initially they were asked to remain in an upright position for 15 min and then to lie down for at least 3 h. This procedure was undertaken in order to simulate as closely as possible the situation of taking a hypnotic drug and going to bed. No food, fluid or tobacco was allowed for 3 h after drug administration. Blood samples were taken at ¼, ½, ¾, 1, 1¼, 1¾, 2, 2½, 3, 4, 6, 8, 24, 32, 48, 56, 72, and 80 h from a forearm vein, for the first three hours of the experiment by means of a flexible venous cannula with injection valve. After three hours blood samples were taken by venous puncture. Blood clotting was prevented by adding a small drop of heparin solution (5000 I.U./ml) to the samples. After centrifugation the plasma samples were stored in the refrigerator at −20° until analysis.

RESULTS AND DISCUSSION

Assay procedure

Fig. 2 shows gas chromatograms of plasma extracts of plasma samples taken 1½ and 80 h after oral ingestion of a tablet containg 5 mg nitrazepam, as well as the gas chromatogram of a blank extract. There is no interference from endogenous plasma substances or metabolites and retention times are short. Clonazepam was chosen as an internal standard in the assay of nitrazepam and the two peaks are well separated at low and high concentrations.

Identification of the compounds eluting from the gas chromatograph was carried out by means of combined gas chromatography—mass spectrometry (LKB-2091 with PDP-11 computer system). Figs. 3 and 4 show the computer plots of the mass spectra of nitrazepam and clonazepam respectively. By comparison of these mass spectra with direct inlet mass spectra of the pure reference substances it can be concluded that nitrazepam and clonazepam both leave the gas chromatographic column unchanged, so that they are being determined in intact form.

According to the standard curve (Fig. 5) there is a linearity between the detector response (peak area nitrazepam/peak area clonazepam) and the concentration of nitrazepam between 10 and 100 ng/ml plasma. Also in Fig. 5 are given recovery values for the same concentration range. In spite of the short extraction time (20 sec) the recovery of nitrazepam from plasma is high: average of 92% (S.D. at each concentration 3.5% or less; n = 4). The reliability of the whole procedure is also reflected in Fig. 5, which shows the mean graph of three calibration graphs made on different occasions; the highest value for the S.D. was 4.9%. The present procedure can also be used

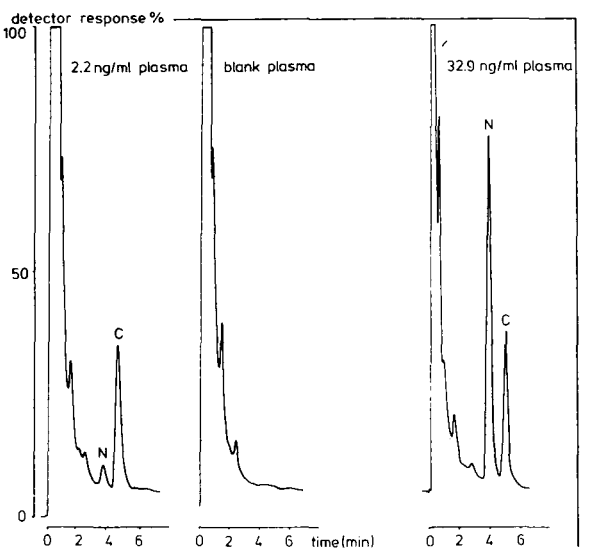

Fig. 2. Gas chromatograms of a 1-ml plasma extract obtained from a volunteer immediately before (middle), and 1.5 h(right) and 80 h (left) after, receiving 5 mg nitrazepam orally. N = Nitrazepam, C = clonazepam (internal standard, 25.0 ng/ml plasma).

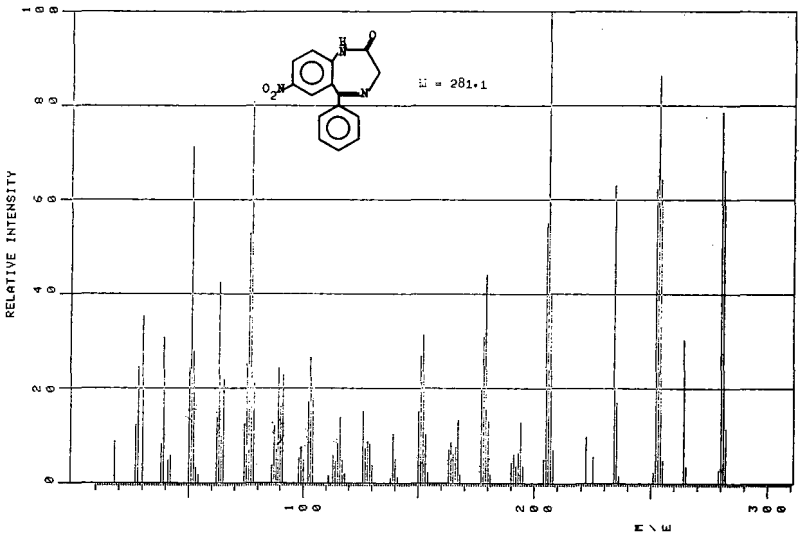

Fig. 3. Normalized electron-impact mass spectrum of nitrazepam obtained by applying a plasma extract to the LKB-2091 gas chromatograph—mass spectrometer.

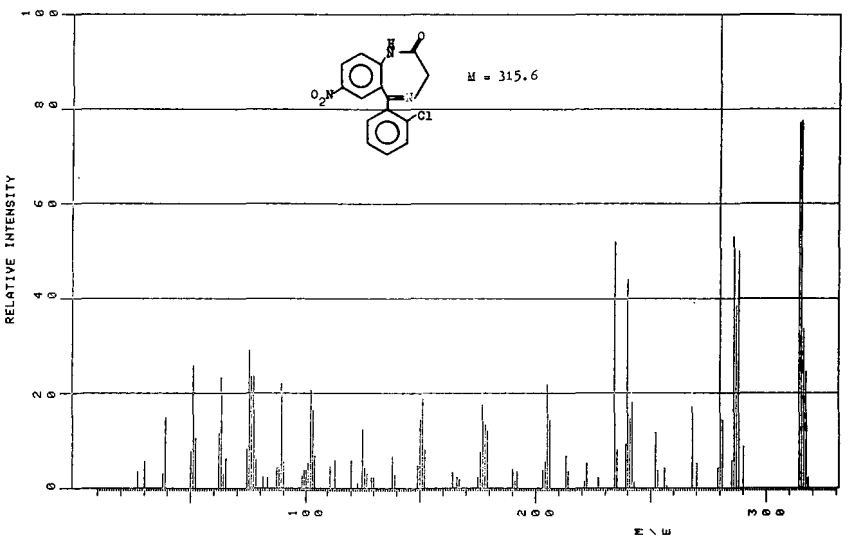

Fig. 4. Normalized electron-impact mass spectrum of clonazepam obtained by applying a plasma extract to the LKB-2091 gas chromatograph—mass spectrometer.

for the determination of underivatized clonazepam in plasma, using nitrazepam as an internal standard (25.0 ng/ml). The mean recovery of clonazepam from plasma by the same extraction procedure was 75% (highest S.D. 5.9%; n = 4). The mean of three calibration graphs for clonazepam made on different occasions showed a highest S.D. value of 4.2%.

It appears that the present method permits the accurate and specific determination of underivatized nitrazepam and clonazepam in plasma in relatively low concentrations. The detection limit is about 1 ng nitrazepam or clonaze-

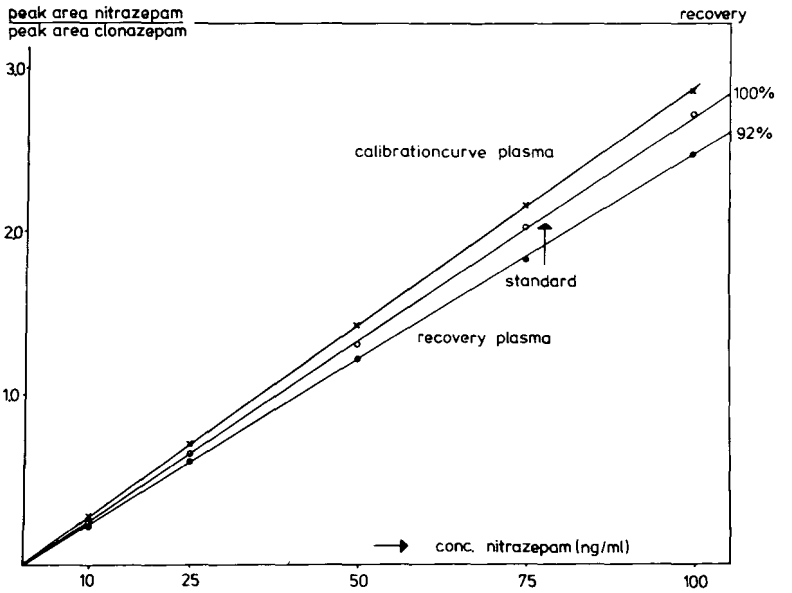

Fig. 5. Peak area ratio of nitrazepam to clonazepam (25.0 ng) as a function of known nitrazepam concentrations. The standard curve was obtained with stock solutions of the two drugs; the recovery line was obtained by extraction of nitrazepam from plasma, using clonazepam as external standard; the calibration graph was obtained by extraction of nitrazepam from plasma, using clonazepam as internal standard.

pam per ml of plasma. The use of the capillary OV-17 SCOT column appears to be a definite improvement in the analysis of these two benzodiazepines and many samples can be analyzed in short time. An addition advantage in using capillary columns is the low column bleeding which is particularly important when using electron-capture detection. A solid injection system is required in order to prevent deterioration of the column support, caused by organic solvents. Whether the present procedure is also suitable for monitoring nitrazepam or clonazepam in clinical situations requires further investigation, especially with reference to interference by co-administered medications. So far, interference by oxazepam, lorazepam, flurazepam, chlordiazepoxide, nordiazepam, diazepam, hydroxydiazepam and medazepam can be excluded. Preliminary studies on the metabolism of nitrazepam in humans have indicated that its major metabolites do not interfere with the assay of the parent compounds.

Pharmacokinetic and bioavailability studies in humans

The present assay procedure was primarily developed because of the need for more information concerning the pharmacokinetics and disposition of the extensively used hypnotic drug nitrazepam in humans following therapeutic dosage. So far, only the investigations by Rieder [9] have yielded reliable data on plasma elimination half-lives of nitrazepam in man. Apart from drug disposition data, there is a growing need for bioavailability deter-

minations of nitrazepam-containing pharmaceutical preparations. An increasing number of these preparations are becoming commercially available and it is important to show their bioequivalence, with respect both to their rate and extent of bioavailability. A rapid rate of absorption is expecially important in hypnotic drug therapy, because if early sleep is not obtained (due to slow absorption of the active ingredient) the patient may be tempted to take a second dose, which may lead to overdosage and prolonged drug effects [24, 25]. It is for this reason that in the present study many blood samples were taken during the first three hours following drug intake. From previous studies [9, 13] detailed information concerning the absorption rate of nitrazepam cannot be obtained.

Two tablet preparations, each containing 5 mg nitrazepam (Mogadon and Sameko), were compared in a cross-over way. The results for one volunteer are shown in Fig. 6. Nitrazepam is in this case more rapidly absorbed from the Sameko preparation (t_{max} = 45 min) than from the Mogadon tablet ($t_{max.}$ = 2 h). After termination of absorption there was an initial rapid decline of the plasma concentration, which is probably due primarily to distribution of the drug in the tissues. Subsequently, a definite increase in plasma concentration occurred in most cases between 4 and 7 h following drug administration. This may be caused by a redistribution process initiated by intercurrent food intake, as was recently shown to occur also for diazepam [26, 27].

Later the plasma concentration time course followed a monoexponential decay (Fig. 6), from which elimination half-lives could be calculated. The relevant pharmacokinetic parameters are summarized in Table I. The areas under the curve were normalized for the differences in elimination half-life

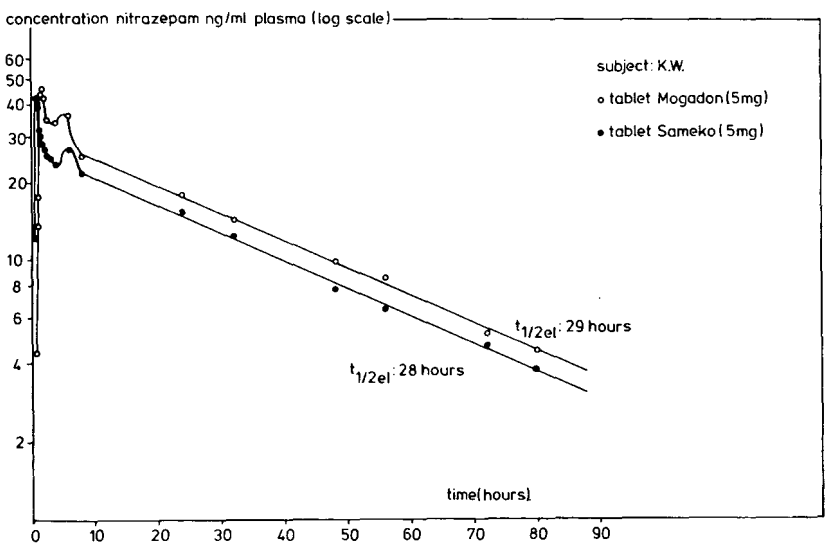

Fig. 6. Plasma concentration curves on semi-logarithmic scale of nitrazepam in a healthy volunteer following administration of a Mogadon tablet and of a Sameko tablet. The various pharmacokinetic parameters for this volunteer are given in Table I.

in the same individual and also for the undetermined area until infinity [28]. The elimination half-life of nitrazepam varied between 13 h and 34 h, with a mean value of 27 h, which result is in good agreement with that reported by Rieder [9]. There was less intra- than intersubject variability in elimination half-life (Table I). The average peak level times ($t_{max.}$) were 88 and 38 min for the Mogadon and Sameko tablets respectively. However, these values appeared not to be statistically significantly different (paired t test). With respect to the extent of bioavailability there is no important difference (despite substantial intersubject variation) between the two preparations.

TABLE I

VARIOUS PHARMACOKINETIC PARAMETERS

Elimination half-lives ($t_{½el.}$), peak level times ($t_{max.}$), areas under the plasma concentration curves (AUC), and relative bioavailability (%) of nitrazepam following the administration of Mogadon tablets (Mo) and Sameko tablets (Sa), each containing 5 mg nitrazepam.

Subject	$t_{½el.}$(h)		$t_{max.}$(min)		AUC(mg·h·l^{-1})		$\frac{AUC(Sa)}{AUC(Mo)} \times 100\%$
	Mo	Sa	Mo	Sa	Mo	Sa	
K.W.	28	28	120	45	1361	1101	81
L.B.	32	34	45	45	1301	1613	124
J.L.	32	33	30	30	909	1004	110
S.V.	28	28	30	45	1006	969	96
B.R.	26	25	60	45	815	754	92
P.B.	34	25	240	30	1318	1115	85
G.B.	18	13	90	30	972	566	58
mean values	28	27	88	38	1097	1017	92

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CHROMBIO. 087

RAPID, DIRECT DETERMINATION OF TRACE AMOUNTS OF SALICYLIC ACID IN DEPROTEINIZED SERUM BY MEANS OF HIGH-PRESSURE LIQUID—LIQUID CHROMATOGRAPHY

C.P. TERWEIJ-GROEN, T. VAHLKAMP and J.C. KRAAK

Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, Amsterdam (The Netherlands)

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SUMMARY

A simple method for the quantitative analysis of salicylic acid in blood serum is described. A liquid—liquid chromatographic system, consisting of a long-chain aliphatic amine as the stationary phase and dilute aqueous perchloric acid as the mobile phase, enables the direct injection of deproteinized serum into the system. No change in the chromatographic properties of the system was noticed after 2000 injections of deproteinized serum.

Quantitative analysis is possible using peak area or peak height measurements. The method has a high precision: relative standard deviations of 0.4% and 5% are found for samples containing 10 μg and 10 ng injected salicylic acid respectively. The detection limit is found to be about 1 ng slicylic acid, corresponding to 40 ppb* salicylic acid in serum.

Simultaneously administered drugs such as indomethacin, acetylsalicylic acid, caffeine and phenacetin, and metabolites of salicylic acid do not interfere with the analysis. The time course of the concentration of salicylic acid in serum is demonstrated after oral administration of 1 g sodium-salicylate. The phase system was also found to be suitable for the analysis of salicylic acid in urine.

INTRODUCTION

The favourable effect of aspirin in general and of sodium salicylate in particular as a medicine for the treatment of rheumatism is commonly accepted [1]. As a result of the very short half-life of aspirin (ASA) in man [2] and the rapid excretion of possibly formed metabolites [3,4], salicylic acid (SA) has to be considered as the active compound.

A rapid determination of SA in serum is essential for the adjustment of a medication schedule. This adjustment is necessary in order to find a suitable compromise between the therapeutic action and unfavourable side effects [5].

*Throughout this article, the American billion (10^9) is meant.

Usually, laborious extraction procedures are applied prior to the colorimetric or fluorimetric determination of SA [6,7]. Recently, gas—liquid chromatographic (GLC) methods for the simultaneous determination of ASA and SA in biological fluids have been described [2,8]. However, time-consuming extractions and derivatization are necessary in these methods. High-performance liquid chromatography (HPLC) has proved to be an excellent method for the determination of non-volatile acidic compounds in body fluids [9—11]. Until now, some papers have appeared dealing with the analysis of ASA, SA and other constituents in tablet preparations of these drugs by HPLC [12, 13].

In the present paper a rapid determination of SA in deproteinized serum using high-pressure liquid—liquid chromatography with UV detection is described.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of: reciprocating membrane pump (Orlita DMP 1515, Giessen, G.F.R.); flow-through manometer as damping device; stainless-steel 316 precolumn (350 mm × 10 mm I.D.); high-pressure sampling valve (Valco CV-6-UHPa) with a loop of 135 μl; thick-walled glass column (150 mm × 3 mm I.D., 12 mm O.D.) or stainless-steel 316 column (150 mm × 3 mm I.D., 6.4 mm O.D.); UV detector (variable wavelength, Zeiss PM 2 DLC, Zeiss, Oberkochen, G.F.R.); linear potentiometric recorder (Goertz, Servogor 542) combined with an integrator (Spectra-Physics Autolab System I).

In order to resist the acidic medium all connections were made of stainless-steel 316 capillary tubing and stainless-steel Swagelok couplings. The experiments were carried out at room temperature. The wavelength was adjusted to 235 nm.

Materials

In all experiments double-distilled water was used. Tri-*n*-octylamine (TOA) was from Fluka (Buchs, Switzerland). As solid support for the glass column diatomite (5—7 μm Kieselguhr, Merck, Darmstadt, G.R.F.) was used, and for the stainless-steel column low-surface-area silica (4—6 μm Spherosil XOC 005, Rhône-Poulenc, Neuilly-sur-Seine, France) was used. Materials of appropiate particle size range were prepared by means of an air classifier (Alpine MZR, Augsburg, G.F.R.). Spherosil XOB 015 (100—200 μm, Rhône-Poulenc) was used as solid support for the precolumn. All chemicals were commercially available and of analytical grade.

Procedures

Coating procedure. The solid supports were coated by a solvent evaporation technique. To 1 g solid support 0.04 g TOA dissolved in 10 ml dichloromethane was added. After stirring the slurry, the dichloromethane was removed by evaporation while stirring under a stream of dry nitrogen until the support was completely dry.

Packing procedures. The glass columns were dry-packed with coated diato-

mite. Small portions of about 10 mg coated support were put into the glass tube and compressed by hand with a teflon-tipped plunger.

The stainless-steel columns were packed by a high-pressure slurry technique using a packing apparatus as shown in Fig. 1. The column was connected to a metallic mixing vessel filled with a slurry of coated Spherosil XOC 005 in 0.05 *M* aqueous perchloric acid (2%, w/w) saturated with TOA. The slurry was pumped upward into the column at a liquid stream of 3 ml/min up to a pressure of 500 atm.

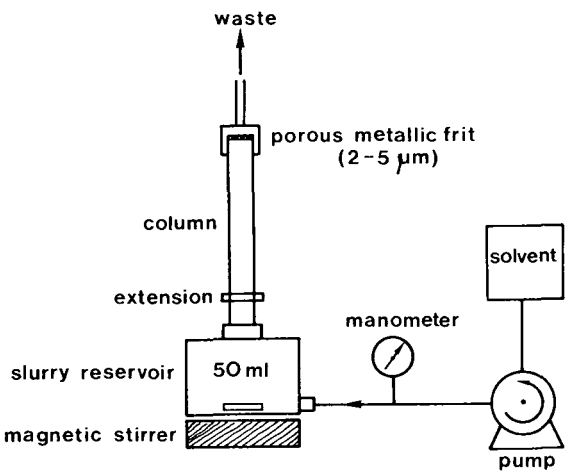

Fig. 1. Apparatus for slurry packing procedures in HPLC.

The precolumn was loosely dry-packed with Spherosil XOB 015 coated with TOA (10% w/w). Before use 250 ml of the eluent were pumped through in order to saturate the stationary phase with perchloric acid.

Mobile phase preparation. The mobile phase was prepared by diluting a weighed amount of perchloric acid (70%, w/w) with double-distilled water. The eluent was saturated with TOA and ultrasonicated to remove air.

Sample preparation. In all experiments centrifuged (10 min, 300 *g*) blood serum, free of exogenous compounds, was used. Batches of serum were stored in a deep-freeze.

To deproteinize 0.2 ml serum was mixed with 1 ml 0.3 *M* perchloric acid (pH 0.7) in a plastic centrifuge tube (10 ml). After 10 min the proteins were removed by centrifugation (10 min, 300 *g*). The supernatant was injected directly into the column using a sample loop of 135 µl volume. The loop was rinsed with water after each injection to prevent contamination and memory effects.

RESULTS AND DISCUSSION

Phase system

The determination of SA in serum was investigated using a liquid–liquid

system consisting of TOA as the stationary phase and dilute aqueous perchloric acid as the mobile phase [14,15].

The most simple method for the analysis of SA should be direct injection of diluted serum into the chromatographic system. In practice, however, this restricts the choice of the mobile phase as precipitation occurs at pH < 3, thus blocking the column.

Direct injection of 30-times diluted serum, using 0.05 *M* $HClO_4$ + 0.055 *M* Na_2HPO_4 (pH 5.5) as the mobile phase, seriously disturbed the chromatographic system as a result of the very large background. Repeated injections gave rise to baseline drifts and column blocking probably caused by precipitation of proteins at the top of the column.

The serum must therefore be deproteinized before injection. This prevents precipitation of proteins and significantly improves the background. This allows, moreover, the use of a more acidic eluent. This is favourable as the deproteinization can be performed with $HClO_4$ of such concentration that the supernatant has an identical pH and anion concentration as the eluent, while the dilution of the sample is kept minimal.

For this reason 0.25 *M* $HClO_4$ (pH 0.7) was chosen as the mobile phase. As shown in Fig. 2, with this phase system SA can be separated from residual serum compounds within six minutes.

Quantitative aspects of the method

The influence of serum constituents on the quantitative analysis of SA was investigated. A constant volume (135 µl) of solutions of SA in the eluent or in the supernatant of deproteinized serum was injected into the column and the

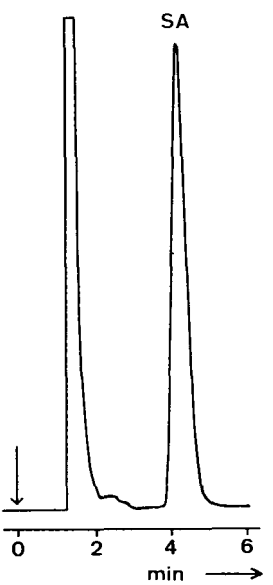

Fig. 2. Analysis of SA in deproteinized serum.
Phase system 4% (w/w) TOA on Kieselguhr—0.25 *M* $HClO_4$, pH 0.7. Glass column, 150 × 3 mm; injection volume 135 µl, Δp = 100 bar.

peak areas were measured. The relative standard deviation of the chromatographic procedure was found to be 0.4% (n=20) for injections of a standard solution of SA (4 μg/ml) in the eluent. No difference could be noticed between the linear regressions of peak area versus injected amount obtained with SA dissolved in the eluent or in deproteinized serum. Both linear regressions showed an equal slope over the range 10—20.000 ng SA with a correlation coefficient of 0.9999.

Relative standard deviations of 0.4% and 5% were found for samples containing 10 μg and 10 ng injected SA respectively (n=4).

To investigate the effect of deproteinization on the recovery of SA, known amounts of SA dissolved in water were added to serum before deproteinization. The recovery of SA varied between 90% in the μg range to 85% in the ng range. The recovery can be increased to 90—97% by washing the precipitated proteins two times with 1.2 ml eluent. In the determination of SA in serum after deproteinization there was a linear relationship between peak area and amount of SA injected over the range 10—20.000 ng, with a correlation coefficient of 0.9999. Since the same relative standard deviations were found as for SA dissolved in the eluent, the reliability of the method is not affected by the deproteinization.

Since the peak of SA in the phase system TOA—$HClO_4$ is very symmetrical, peak heights instead of peak areas can be used for quantitative analysis. The linear regression of peak height versus amount of SA injected measured within the range 20—10.000 ng showed a correlation coefficient of 0.9999, indicating a high degree of linearity.

The detection limit of SA, defined as 3 times the standard deviation of the noise, is found to be about 1 ng. This corresponds with 40 ppb SA in serum for the given injection volume of 135 μl, the largest volume that can be injected without loss of column efficiency.

Pharmacokinetic study

Simultaneously administered drugs such as caffeine, phenacetin and ASA do not disturb the analysis of SA since they are well resolved from SA.

Fig. 3 shows the analysis of serum from a human subject, analysed 45 min after oral administration of 200 mg ASA, phenacetin and caffeine.

SA and a little phenacetin were found while caffeine and possible residues of ASA, compounds eluted before SA, disappear into the serum background peak.

The phase system used was found also to be suitable for the analysis of SA in urine. The urine of the subject was collected 2—4 h after drug administration as above. Fig. 4 shows the presence of SA and phenacetin when the urine is injected directly into the column.

The background can be improved by successive extractions of the urine with hexane at pH 10 (discard the hexane layer) and with diethylether at pH 1 (discard the water layer). After evaporation of the diethylether the extract is dissolved in the eluent [15].

Improvement of the resolution of SA and phenacetin can be achieved by modifying the mobile phase (0.05 M $HClO_4$, pH 1.5 instead of 0.25 M $HClO_4$, pH 0.7) [15].

In order to investigate the time course of SA, the serum of a rheumatic patient was examined. The patient was fasted overnight and a blood sample was

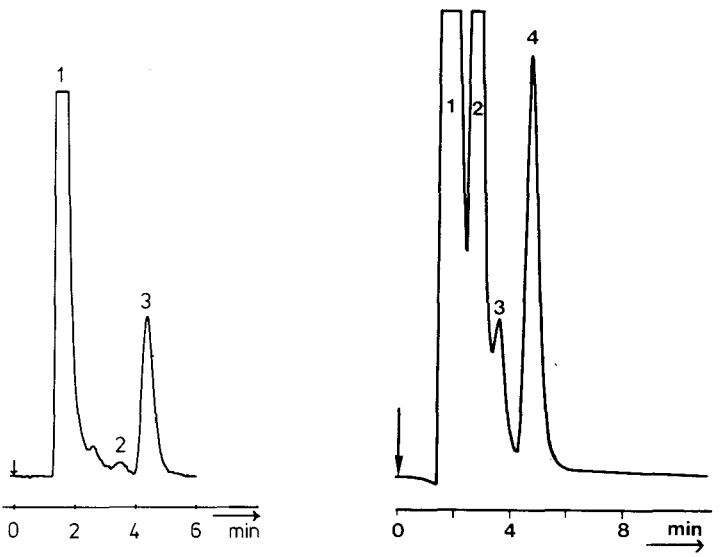

Fig. 3. Chromatogram of deproteinized serum, taken 45 min after oral administration of 200 mg ASA (aspirin), phenacetin, and caffeine. Conditions as in Fig. 2. 1 = Serum background + caffeine + ASA; 2 = phenacetin; 3 = SA.

Fig. 4. Direct injection of urine collected 2—4 h after oral administration of 200 mg caffeine, ASA and phenacetin. Conditions as in Fig. 2. 1 and 2 = Urine background; 3 = phenacetin; 4 = SA.

then taken. Blood samples were subsequently taken at time intervals of ½, 1, 2, 3½, 6 and 8 h after an oral administration of 10 ml medicine containing 1 g sodium salicylate. No heparin or citrate was added to the blood samples before centrifugation (10 min, 300 *g*). In accordance with the results of others [1], an exponential decrease in the SA concentration in serum was found, as demonstrated in Fig. 5. At the most sensitive detector attenuation (0.02 a.u.f.s.) no metabolites of SA could be detected in the chromatograms of the samples. Considering the more polar character of these possible metabolites it might be assumed that they are not retained by the phase system used. If one is interested in particular metabolites, the phase system TOA—Na_2HPO_4—$HClO_4$, pH 5.5 is more suitable as the retention at this pH can be influenced by the anion concentration [14,15].

It was noticed that a higher percentage of SA was found when heparin was added to the blood sample before centrifugation (probably as a result of adsorption of SA at the clod). In blood plasma (heparin added) 8% more SA was found than in serum.

For rheumatic patients, indomethacin is used as a medicine combined with sodium salicylate [6,16]. Recently, HPLC analysis of indomethacin in plasma has been reported [17]. However, SA was left out of consideration. The phase system used in the present paper showed a very large capacity ratio for indomethacin. In order to determine indomethacin and SA simultaneously, column switching or addition of methanol to the eluent [15] is necessary.

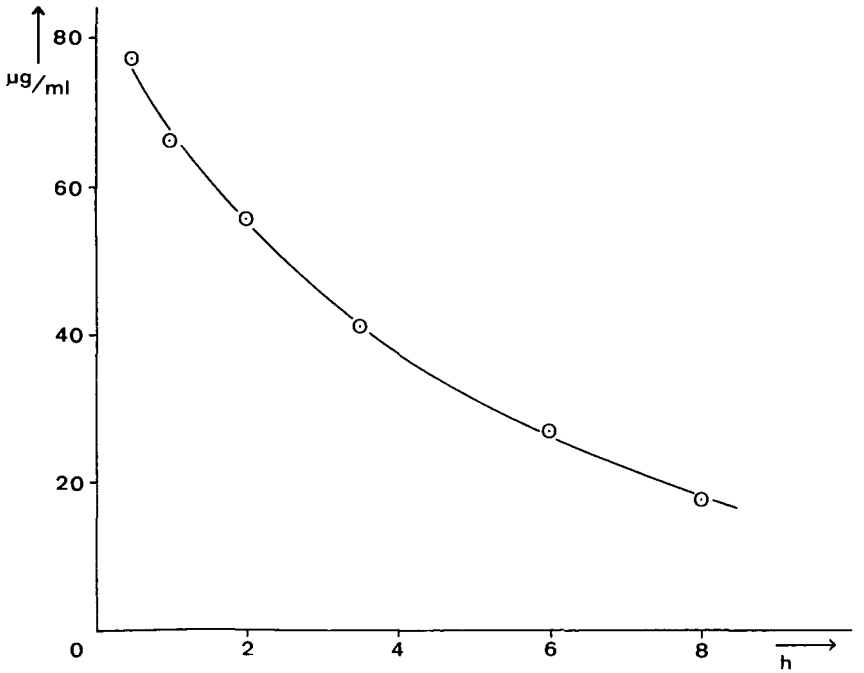

Fig. 5. Time course of SA in serum after oral administration of 1 g sodium salicylate.

CONCLUSIONS

Only a small amount of blood serum is required to determine salicylic acid down to the ppb level by means of direct injection of deproteinized serum into the chromatographic system.

The method is simple and reliable. Many samples can be handled in a short time. The phase system showed remarkable stability and no change in the chromatographic properties was noticed after 2000 injections of deproteinized serum. These qualifications favour routine analysis.

Future research will be devoted to the simultaneous determination of salicylic acid and indomethacin.

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Note

New optimized method for the determination of esterolytic activity in serum by gas—solid chromatography

OYIN SOMORIN

Chemistry Department, University of Lagos, Lagos (Nigeria)

PŘEMYSL MAREŠ

Gastroenterology Research Unit I, Faculty of Medicine, Charles University, Prague (Czechoslovakia)

and

JIŘÍ SKOŘEPA

4th Chair of Internal Medicine, Faculty of Medicine, Charles University, Prague (Czechoslovakia)

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Serum levels of esterolytic activity have been a useful tool in the diagnosis of pathological disorders, especially in that of carcinoma [1]. As all the methods for the determination of esterolytic activity currently in use are lacking either sensitivity or specificity [2—8], there is need for a new sensitive and specific method with good accuracy and precision for measuring slight differences in serum levels of esterolytic activity for purposes of detecting early stages of pathological disorders. The technique reported here, which is based on gas—solid chromatography (GSC) with a special synthetic support and detection by a flame ionization detector appears to meet such need.

EXPERIMENTAL

Reagents

Ethyl butyrate of analytical grade (Koch-Light Labs., (Colnbrook, Great

Britain) was further purified as follows. Traces of fatty acids were removed by treatment with 5% aqueous sodium hydrogen carbonate followed by washing five times with distilled water. The ester was then distilled over a 40-plate column, and the fraction with boiling-point range 120.5—121° was collected and stored in a glass-stoppered dark bottle.

Chromatographically purified *n*-octane (purity 99%) was obtained as a gift from the Technological Institute, Prague, Czechoslovakia.

Analytical reagent grade butyric acid (Lachema, Brno, Czechoslovakia) was further purified by preparative gas—liquid chromatography (GLC) to achieve purity $\geqslant$ 99%, which was checked by GLC of the methyl ester.

Diethyl ether of analytical reagent grade was purchased from Lachema.

Apparatus, instrumental and chromatographic conditions

Gas chromatographic analyses were performed on a Perkin-Elmer Model F 33 gas chromatograph equipped with an all-glass system, flame ionization detector and a glass column, 100 cm × 2 mm I.D., packed with adsorbent Spheron-BD, 75—120 mesh (synthetic material developed by the Research and Development Chemical Department of The Laboratory Instruments Works, Prague, Czechoslovakia). Further information about this support will be reported elsewhere.

The column was conditioned at 170° for 12 h with a nitrogen flow-rate of 39.2 ml/min. The operating conditions were: oven temperature, 170°; detector temperature, 225°; nitrogen flow-rate, 39.2 ml/min; air flow-rate, 400 ml/min; and hydrogen flow-rate, 52 ml/min.

A Perkin-Elmer Model 56 recorder was used with a chart speed of 5 mm/min and sensitivity set at 2 mV.

Calibration

The standard solutions of butyric acid were prepared by dissolving known amounts of butyric acid in diethyl ether containing 0.02% (v/v) of *n*-octane as internal standard. The concentration range of the butyric acid standards was 0.31 to 2.76 μmoles per μl. A 5.0-μl aliquot of each standard was injected into the gas chromatograph and the ratio of the butyric acid and *n*-octane peak areas was determined and plotted against the amount of butyric acid injected. A typical standard curve is shown in Fig. 1, curve A.

In a similar manner, standard aqueous solutions of butyric acid were prepared in 0.5 *M* phosphate buffer (pH 8.0); 1 ml of each solution was mixed with 1 ml of 1 *M* orthophosphoric acid and extracted into diethyl ether containing internal standard as described in the next section. A 5-μl aliquot of each diethyl ether extract was injected into the gas chromatograph and the ratio of the butyric acid and *n*-octane peak areas was determined and plotted against the amount of butyric acid injected. A typical standard curve is presented in Fig. 1, curve B.

Assay of biological sample for esterolytic activity

An assay procedure similar to that reported by Skořepa *et al.* [2] was used. Serum and buffered substrate were equilibrated for 5 min at 37°. Serum (0.5 ml) was added to 3.78×10^{-2} *M* buffered substrate (4.5 ml) in a glass-

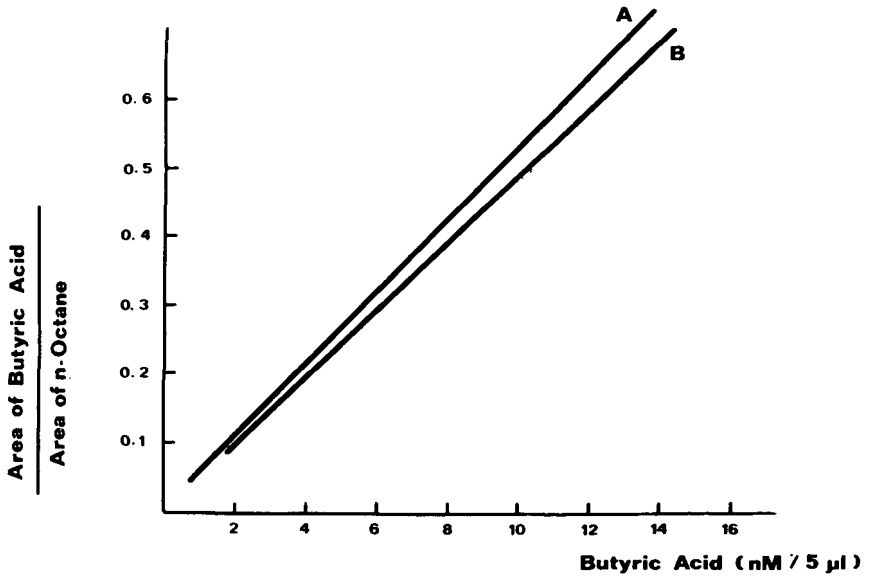

Fig.1. Calibration and extraction curves for quantitative analysis of butyric acid. Curve A: diethyl ether solution of butyric acid with internal standard was chromatographed directly. Curve B: butyric acid was extracted from buffered serum solution into diethyl ether solution of internal standard.

stoppered test-tube (180×14 mm), and the mixture was incubated in a water-bath at 37°. The enzymic reaction was stopped after a definite period by pipetting 1 ml of the incubated mixture into another glass-stoppered test-tube (150×12 mm) which contained 1 ml of 1 *M* orthophosphoric acid. The mixture was shaken, then stored in a refrigerator at 4° for 5 min before it was extracted into 2 ml of cooled diethyl ether solution which contained 0.02% (v/v) of *n*-octane as internal standard. The mixture was well shaken and stored in the refrigerator for at least 5 min before the injection of 5-µl aliquots of the upper diethyl ether layer into the gas chromatograph.

In the control test-tube, heat-inactivated serum or physiological saline was used instead of serum. Esterolytic activity assays were carried out in duplicate.

The pH optimum was determined by measuring the extent of hydrolysis of substrate dissolved in buffer solutions of different pH values (6.5—9.5).

The period of incubation was fixed at 2 h. Similarly, esterolytic activity was determined in buffer solutions of different ionic strengths (0.006—1.6 *M*).

Determination of the effect of dilution was carried out by diluting the serum with heat-inactivated sera or physiological saline.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 2. Butyric acid is conveniently separated from the solvent (diethyl ether), and the substrate (ethyl butyrate).

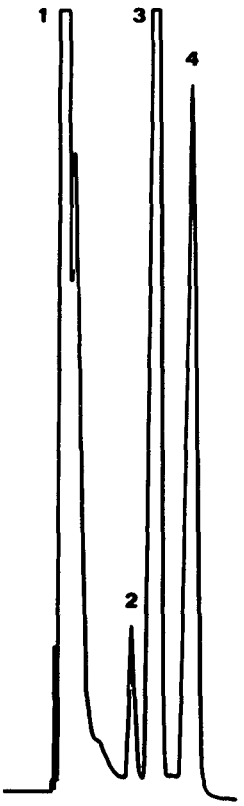

Fig.2. Typical pattern of separation of a mixture of butyric acid, ethyl butyrate, diethyl ether (solvent) and *n*-octane (internal standard). GSC conditions are as described under Experimental. 1=diethyl ether; 2=butyric acid; 3=ethyl butyrate; 4=*n*-octane.

Calibration and extraction efficiency

Standard samples of butyric acid dissolved in diethyl ether in concentrations ranging from 0.31 to 2.76 nmoles per μl were assayed. The calibration curve shown in Fig. 1, curve A, was linear. To determine the efficiency of the extraction of butyric acid from buffered aqueous solutions into diethyl ether, buffered serum solution supplemented with butyric acid concentrations ranging from 0.246 to 2.46 nmoles per μl were assayed after a one-step extraction into diethyl ether according to the procedure described. The calibration curve shown in Fig. 1, curve B, was linear over this range, which covers the range of concentrations of butyric acid produced on hydrolysis of ethyl butyrate by serum for 1—4 h. The single diethyl ether extraction proved to be effective enough, the recovery of butyric acid being 92.50± 0.81%. Since the calibration curve shown in Fig. 1, curve B, was constructed under experimental conditions similar to those used in the assay of butyric acid from hydrolysis of ethyl butyrate by biological samples, this calibration curve rather than Fig. 1, curve A, was used as the standard calibration curve in the determination of esterolytic activity in biological samples.

Reproducibility and stability

The reproducibility of chromatographic analysis was determined by injection of ten samples of 5 μl of the same solution. Three solutions with different concentrations of butyric acid were analysed and evaluated statistically. For each concentration the coefficient of variation (C.V.) did not exceed 1.8%.

The stability of the serum hydrolysate after extraction into diethyl ether was checked by injecting 5-μl samples of three mixtures that were incubated for 1, 2 and 3 h, respectively, after the following periods of storage in the refrigerator at 4°: 5 min, 30 min, 1 h, 3 h, 6, 24 h, 48 h, and 72 h. The mixtures were stable at 4°, as is evident from the results in Table I.

The high reproducibility of the method deserves comment. Heptane [9] and toluene [10] have previously been used for the extraction of free fatty acids. Under the conditions of the GSC procedure, these solvents were not suitable on elution, their peaks interfering with the elution peaks of either butyric acid or ethyl butyrate. Diethyl ether as an extraction solvent gave quantitative yields in a one-step procedure. After storage of the solution in the refrigerator at 4°, the reproducibility of results was better than ±3% Table I).

Effect of enzyme concentration on the rate of hydrolysis

The rate of esterolytic activity measured by hydrolysis of ethyl butyrate was directly proportional to the volume of serum over a 15-fold range up to 1.5 ml.

Results presented in Fig. 3 show a linear relationship between the amount of hydrolysis product, butyric acid and time up to 4 h with undiluted and diluted sera. There was no significant difference in the rate of hydrolysis when heat-inactivated serum or physiological saline was used for diluting the serum, which indicates that the blood serum probably does not contain any inhibitor of esterolytic activity.

TABLE I

EFFECT OF STORAGE ON THE SERUM HYDROLYSATE IN DIETHYL ETHER SOLUTION

Serum hydrolysate in ether	Extent of hydrolysis (nmoles per 5-μl aliquot)								Remarks
	Period of storage								
	5 min	30 min	1 h	3 h	6 h	24 h	48 h	72 h	
1-h hydrolysate	2.80	2.90	2.88	2.80	2.92	2.88	2.90	2.90	Average: 2.87 C.V.: 1.62
2-h hydrolysate	5.70	5.72	5.68	5.72	5.90	5.70	5.70	5.72	Average: 5.73 C.V.: 1.22
3-h hydrolysate	8.50	8.64	8.84	8.50	8.90	8.84	8.70	8.70	Average: 8.70 C.V.: 1.75

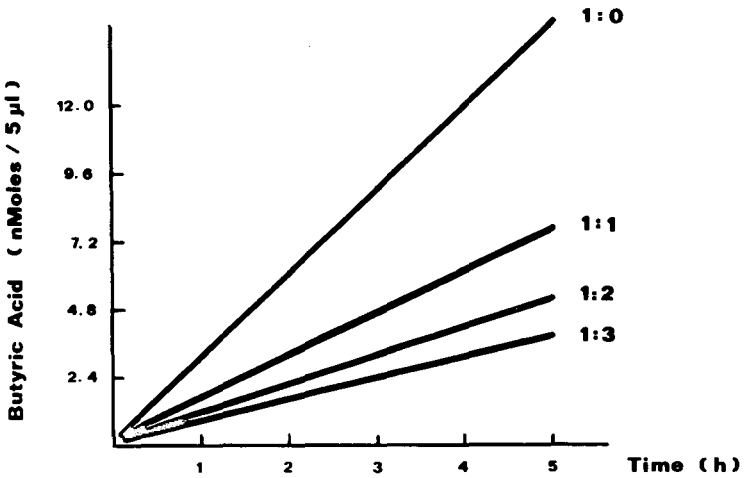

Fig.3. Time course of hydrolysis of ethyl butyrate by undiluted serum and serum diluted with heat-inactivated serum in the ratio indicated.

Effect of pH and ionic strength

The pH—activity curve for serum hydrolysis of ethyl butyrate is shown in Fig. 4. Optimal activity occurred near pH 8.0. The curve is rather broad near its optimum and in this respect resembles the pH—activity curve for tryptic hydrolysis of benzoylarginine methyl ester [11]. At pH 8.0, non-enzymic hydrolysis was almost negligible, being 0.005% of the substrate in 1 h at 37°. The stability of ethyl butyrate to non-enzymic hydrolysis makes it a suitable choice as a substrate for the determination of esterolytic activity, unlike methyl butyrate which was more volatile and far more readily split by non-enzymic hydrolysis. Other workers [12] have also reported appreciable rates of non-enzymic hydrolysis of methyl butyrate even at pH 6.8 and 37°.

Table II shows that the optimal ionic strength for the esterolytic activity of blood serum on ethyl butyrate was 0.5 *M*.

Accuracy and sensitivity of the GSC method

The accuracy of the GSC method was determined from duplicates of esterolytic assays performed on twenty different samples of blood sera. The coefficient of variation was 0.43%.

The detection limit of butyric acid was about 1 µg/ml

CONCLUSION

The GSC method proposed has several advantages in comparison with methods in current use which include: titration [2], colorimetry [3—5], fluorimetry [6], and manometry [7, 8]. The restrictions in these methods have been reported by Ikezawa *et al.* [12]. These authors proposed a GLC method which itself has a few limitations. First, the methyl butyrate used

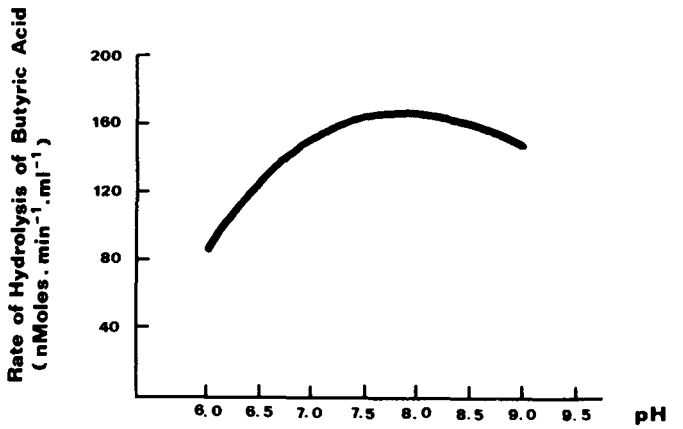

Fig.4. The pH—activity curve for the hydrolysis of ethyl butyrate by serum.

TABLE II

RATE OF SERUM HYDROLYSIS OF ETHYL BUTYRATE AS A FUNCTION OF IONIC STRENGTH

Ionic strength of phosphate buffer (pH 8.0) (*M*)	Rate of hydrolysis (nmoles of butyric acid produced per minute per ml of serum)
1.0	195.0
0.75	233.3
0.50	240.0
0.20	201.6
0.10	188.3
0.05	175.0
0.025	155.0
0.0125	141.7
0.006	135.0

in their GLC method is not a suitable substrate because of its high rate of non-enzymic hydrolysis as previously discussed [12]. Secondly, the method proposed included determination of esterolytic activity by assay of the substrate concentration. An enzymic reaction shows the desirable characteristic linear relationship between the substrate transformed and time only if it is zero order with respect to the substrate concentration. This implies only slight changes in substrate concentration and such changes cannot be determined with as high a precision as the change in the concentration of the product.

The accuracy of the method proposed is about ten times as high as that of the micro-titration method since the coefficient of variation of the method described was estimated to be 0.43% and that of the micro-titration method was 4.5%. The activities measured with the optimized GSC method were

higher than those previously reported from this laboratory for the micro-titration method [2]. This increase can be explained by the fact that the assays using the GSC method were performed under conditions of optimal pH and ionic strength.

The proposed method has good sensitivity, reproducibility and high accuracy. The speed of analysis permits this method to be proposed for routine clinical assay of esterolytic activity in blood serum.

Normal values

The average value for esterolytic activity of serum as determined by the hydrolysis of ethyl butyrate in the micro-titrimetric method was 82.98± 9.51 nmoles·min^{-1}·ml^{-1} serum and the normal values statistically evaluated were 59.18—106.78 nmoles·min^{-1}·ml^{-1}.

Preliminary results with this method gave an average value of 235.30± 61.70 nmoles·min^{-1}·ml^{-1} and a statistically evaluated normal range of 81—389 nmoles·min^{-1}·ml^{-1} serum. The differences in these values can be accounted for by reasons already discussed.

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Note

Gas chromatographic determination of 18-hydroxy-11-deoxycorticosterone

Z. TOMSOVÁ, I. GREGOROVÁ, K. HORKÝ and J. DVOŘÁKOVÁ

Laboratory for Endocrinology and Metabolism, Third Medical Clinic, Charles University Faculty of medicine, 128 21 Prague (Czechoslovakia)

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Besides aldosterone and deoxycorticosterone, a further mineralocorticoid 18-hydroxy-11-deoxycorticosterone (18,21-dihydroxy-pregn-4-en-3,20-dione; 18-OH-DOC) is also secreted by the human adrenal cortex. Its determination is of importance for the differential diagnosis of some adrenocortical disorders and of some forms of arterial hypertension [1]. However, 18-OH-DOC determination in biological material is difficult because of its easy conversion into multipolar compounds [2], ketalic forms [3] and possibly "dimers" [4,5]. Gas chromatography represents the most usual approach to the determination of 18-OH-DOC in plasma. Rapp [6] and Palem et al [7] determined it as the γ-lactone, Mason and Fraser [8] as the γ-lactone heptafluorobutyric derivative (HFB). The formation of this derivative (Fig. 1) offers a possibility to increase the sensitivity of the method, linearity of the response and precision of the determination.

This paper describes a modification of the method for 18-OH-DOC determination by gas chromatography described by Mason and Fraser [8]. The use of canrenone as an internal standard reduced the time of the analysis, since the HFB derivative of canrenone has a shorter elution time than the HFB derivative of aldosterone γ-lactone.

EXPERIMENTAL

Reagents

The 18-OH-DOC standard was kindly supplied by the Clinical Research Institute of Montreal, Canada, and later purchased from Searle de Mexico, (Naucalpon, Mexico).

[1,2-$^3H_2$]-18-OH-DOC, specific activity 51 Ci/mmole was obtained from The Radiochemical Centre (Amersham, Great Britain). The commercial product supplied in benzene—methanol (9:1) solution should be purified before

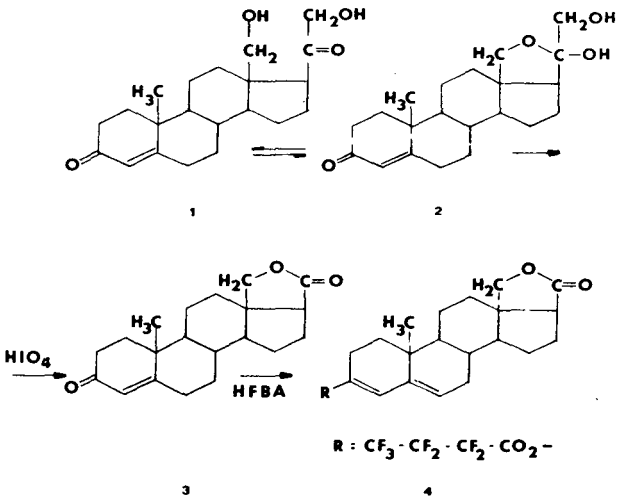

Fig.1. Formation of the γ-lactone HFB derivative of 18-OH-DOC. 1 = 18-OH-DOC; 2 = 18-20 hemiketal configuration; 3 = 18-OH-DOC γ-lactone; and 4 = 18-OH-DOC γ-lactone HFB derivative.

use by thin-layer chromatography and the eluate should be kept in a non-polar solvent [2].

Canrenone γ-lactone (3-oxo-17β-hydroxy-4,6-androstadien-17α-ylpropionic acid was from Boehringer (Mannheim, G.F.R.).

Heptafluorobutyric anhydride (HFBA) was from Serva (Heidelberg, G.F.R.).

All solvents were analytical-reagent grade and were purified by distillation before use.

Paper and thin-layer chromatography

Paper, Whatman No 1, 47 cm long, was pre-washed with methanol for two days before use and air dried. Aluminium foils coated with 0.1 to 0.14-mm silica gel layer (150 × 150 mm, Silufol, UV 254 Kavalier) were pre-washed three times with benzene—acetone (3:1) before use and air dried.

Gas chromatography

A Carlo Erba Fractovap 2200 chromatograph was used with a ^{63}Ni electron-capture detector, supplied with 25 V d.c. A 2-m glass column with an internal diameter of 4 mm was packed with Gas-Chrom Q 100—120 mesh (Serva) coated with 1% SE-30 (Applied Science Labs., State College, Pa., U.S.A.). The temperature of the column, of the evaporation area and of the detector were 222, 270 and 270°, respectively. Dried nitrogen served as a carrier gas and the flow-rate through the column was 60 ml/min. The flow-rate of the purging gas (nitrogen) was 35 ml/min.

The radioactivity of [1,2-$^3H_2$]-18-OH-DOC was measured on a Packard Tricarb liquid scintillation spectrometer, Model 2405.

Sample preparation for gas chromatography

(1) Five ml of plasma was kept at —20°, then thawed. A known amount

of [1,2-$^3H_2$]-18-OH-DOC (65,000 dpm) was added in a manner similar to that for the 18-OH-DOC standard sample in water. The plasma was extracted three times with dichloromethane (10 ml each portion). The combined extracts were washed with 2 ml of 0.1 *M* sodium hydroxide then 2 ml of water, and evaporated to dryness.

(2) The dry residue was dissolved in dichloromethane, applied to the paper together with the standards and separated chromatographically in the system Bush 3 [9,10]: light petroleum (b.p. 30—50°)—benzene—methanol—water (667:333:800:200) for 4 h at room temperature. Depending on the location of the reference compound detected under short-wave UV irradiation (254 nm) the corresponding area was eluted three times with methanol—dichloromethane (1:1). The $R_{\text{deoxycorticosterone}}$ of the reference compounds were: deoxycorticosterone 1.0; corticosterone 0.32; 18-OH-DOC 0.25; aldosterone, 0.06; and cortisol, 0.04.

(3) The extract was evaporated in vacuum and oxidized as described in ref. 8.

(4) The evaporated oxidation products were dissolved in a small volume of methanol—dichloromethane (1:1), applied to a Silufol foil and developed in the solvent system benzene—acetone (3:1) up to the height of 14 cm. The R_F of the reference compounds were: corticosterone, 0.07; 18-OH-DOC, 0.07 and 18-OH-DOC γ-lactone, 0.29. The 18-OH-DOC γ-lactone was detected under UV irradiation (254 nm), the corresponding areas were scraped off, moistened with water and extracted three times with 1 ml benzene.One-tenth of each sample was taken for measuring tritium radioactivity to determine the losses at this stage. The residue was evaporated under nitrogen.

(5) Benzene (10 μl) and 10 μl heptafluorobutyric acid were added to the dry sample and the mixture was heated to 60° for 30 min. The reaction mixture was evaporated in a nitrogen atmosphere and the evaporate was dissolved in 10—30 μl benzene with a known amount of canrenone as internal standard. A 3- to 4-μl portion of this solution was injected into the gas chromatograph. Retention times of 18-OH-DOC and canrenone derivatives were 6 and 8.5 min, respectively. Peak areas were measured by planimetry.

RESULTS AND DISCUSSION

For typical chromatograms of plasma samples from the vena cava inferior and from the adrenal vein see Fig. 2. To estimate losses during the extraction, paper chromatography, oxidation and thin-layer chromatography, labelled 18-OH-DOC was added to six test-tubes with 5 ml of plasma, and aliquot portions were used for measuring the losses of tritium after each operation. The results are summarized in Table I. In 8 other samples the recovery of [1,2-$^3H_2$]-18-OH-DOC added to water was determined and the total recovery was found to be 54.29 ± 2.3%.

The response of the detector to the HFB derivative of the 18-OH-DOC γ-lactone as well as to the internal standard (canrenone HFB derivative) was linear within the range 0 to 10 ng. The lowest detectable amount was 0.06 ng. The precision of the method was examined by simultaneous treating of six 5-ml samples of plasma to which 100 ng of 18-OH-DOC were added.

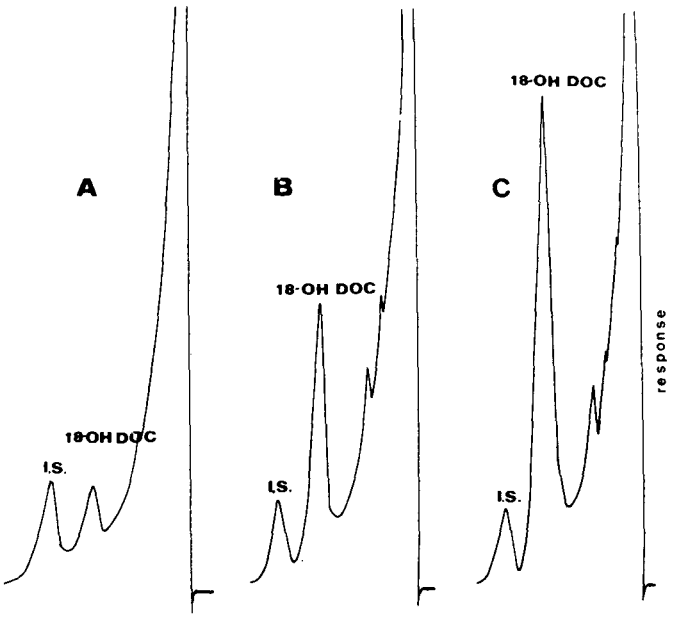

Fig.2. Gas chromatograms (for conditions see Experimental) of the plasma samples from the vena cava inferior below (A) and above (B) the orifice of the adrenal and renal veins, and from the left adrenal vein (C). I.S. = Internal standard; 18-OH-DOC = peaks corresponding to the 18-OH-DOC γ-lactone HFB derivative. The amounts found were: A, 0.55 ng; B, 2.18 ng; C, 6.15 ng.

TABLE I

PERCENTAGE OF [1,2-$^3H_2$]-18-OH-DOC RECOVERY IN INDIVIDUAL STEPS OF THE METHOD

Values are means of six samples ± standard error.

Recovery (%)			
Extraction	Paper chromatography	Oxidation + thin-layer chromatography	Total recovery (%)
70.62 ± 1.73	83.35 ± 2.29	78.06 ± 4.20	47.07 ± 3.09

In these samples the concentration of endogenous 18-OH-DOC was simultaneously determined before addition of the standard. After subtraction of the standard the average value was 98.54 ± 4.08 ng.

In a series of control experiments we found that most compounds interfering in the gas chromatography of 18-OH-DOC (cortisol, deoxycorticosterone, aldosterone) were separated by paper chromatography. However, corticosterone was separated only partially in both Bush 3 and Bush 1 systems. We chose the system Bush 3 because it does not produce tailing of the cortico-

sterone spot. Total separation is possible on thin-layer chromatography only after oxidation of 18-OH-DOC to its γ-lactone.

The basic pre-requisite is the absolute purity of all solvents and glassware used. This prevents formation of multipolar 18-OH—DOC derivatives whose mobilities are different [2]. Roy et al. [3] demonstrated the formation of C-20 ketals in alcoholic solvents, and particularly in the presence of acid impurities. This can be prevented by removing impurities or by their neutralization. Dominguez [2] previously described the existence of two interconvertible forms of 18-OH-DOC with different chromatographic mobilities.

Estimation of 18-OH-DOC in plasma from the adrenal veins in our 18 patients with low-renin hypertensions revealed a clinical importance of determining this mineralocorticoid for the differential diagnosis of some types of arterial hypertension. Together with measurement of plasma concentration of aldosterone, the estimate was able to contribute significantly to the differentiation of morphological changes of the adrenals in mineralocorticoid-dependent hypertensions [11]. In aldosterone-producing adenoma of the adrenal cortex the concentration of 18-OH-DOC in the adrenal venous blood was on average 3.6 times higher on the side of the adenoma (average value 5.681 μg/100 ml on the side of the adenoma and 1.261 μg/100 ml on the intact side). In idiopathic hyperaldosteronism with bilateral hyperplasia of the adrenals, the 18-OH-DOC concentration, similarly to that of aldosterone, was increased symmetrically on both sides (average concentrations 8.962 μg/100 ml on the left and 7.108 μg/100 ml on the right side). In low-renin essential hypertensions, 18-OH-DOC concentration was high in both adrenal veins (10.889 μg/100 ml on the left and 7.146 μg/100 ml on the right side), whereas aldosterone concentrations were low. In Cushing's syndrome, 18-OH-DOC concentrations in the adrenal venous blood were variable (0.116—6.828 μg/100 ml). For details see our previous communication [11]. Synthetic ACTH increased the 18-OH-DOC concentration several times. Our findings of 18-OH-DOC concentrations in the adrenal venous blood were in the range reported by Melby et al. [1].

The determination of 18-OH-DOC in the peripheral venous blood is important for the investigation of the possible etio-pathogenic participation of this mineralocorticoid in the initiation and in the course of certain types of arterial hypertension, particularly in patients with suppressed renin [12—14]. The 18-OH-DOC values in peripheral venous blood of our patients with hypertension varied in the range of 0.069—0.360 μg/100 ml and are somewhat higher than those presented by Mason and Fraser [8] for control persons when a similar technique was used. The administration of ACTH raised the peripheral 18-OH-DOC concentration in a similar manner to that in the adrenal veins.

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Biomedical Applications

CHROMBIO. 097

Note

Gas chromatographic detection of N-methyl-2-phenylethylamine: a new component of human urine

G.P. REYNOLDS* and D.O. GRAY

Department of Botany and Biochemistry, Westfield College (University of London), Kidderpore Avenue, London NW3 7ST (Great Britain)

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2-Phenylethylamine (PE) may well play an important role in normal [1,2] and abnormal [2,3] brain function. Thus the novel detection of its biologically active N-methyl derivative in human urine is of considerable interest. In this note we report the identification of this compound by gas chromatography (GC) and the confirmation of its presence using GC—mass spectrometry.

METHODS

The phenylethylamines were first concentrated by a solvent extraction procedure that has been published in detail elsewhere [4]. Briefly, they were extracted from NaCl-saturated, alkaline urine with diethyl ether and back extracted into acid. This was taken to dryness, made alkaline and re-extracted with diethyl ether. Benzylamine was added to the urine before extraction to serve as an internal standard.

The extracted phenylethylamines were reacted with trifluoroacetic anhydride and separated by GC as their trifluoroacetyl (TFA) derivatives [4]. Samples (2 μl) of a hexane solution of the derivatives, corresponding to 1—2 ml of urine, were chromatographed on a 6 ft × 4 mm I.D. column of 3% QF-1 on Diatomite C 'Q' (100—120 mesh) at 140°. The carrier gas was nitrogen (50 ml/min) and a hydrogen/air flame ionization detector was used.

*To whom correspondence should be addressed. Present address: Department of Chemical Pathology, Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, Goldhawk Road, London W6 OXG, Great Britain.

RESULTS AND DISCUSSION

Chromatograms of the phenylethylamines from two healthy individuals are shown in Fig. 1. Both urine samples had initially been hydrolysed with acid (pH 1) for 20 min at 100° to release conjugated amines. Peak 3 is coincident with that of the TFA derivative of authentic N-methyl-PE. Fig. 2 shows the mass spectrum of peak 3; the major ions at *m/e* 104 and 140 are consistent with a compound containing phenylethyl and N-methyl-trifluoroacetamide moieties, respectively. Comparison with the mass spectrum of genuine N-methyl-PE-TFA confirms the presence of N-methyl-PE in hydrolysed human urine and shows that, for the sample investigated, its derivative is the major component of peak 3. There were no cases of a peak being detected which co-chromatographed with, but could not be identified as, this compound.

Thus N-methyl-PE excretion can be estimated from the height of peak 3 although accurate quantification, using the ratio of the peak heights of the N-methyl-PE and the benzylamine (internal standard) derivatives, is made difficult due to the low concentrations of the former compound. However, significant quantities (0.2—1.0 μg/24 h) have been demonstrated in eleven out of fifteen hydrolysed normal urine samples, but in no case did excretion exceed 1 μg/24 h. Reproducibility of the method was approximately ± 0.2

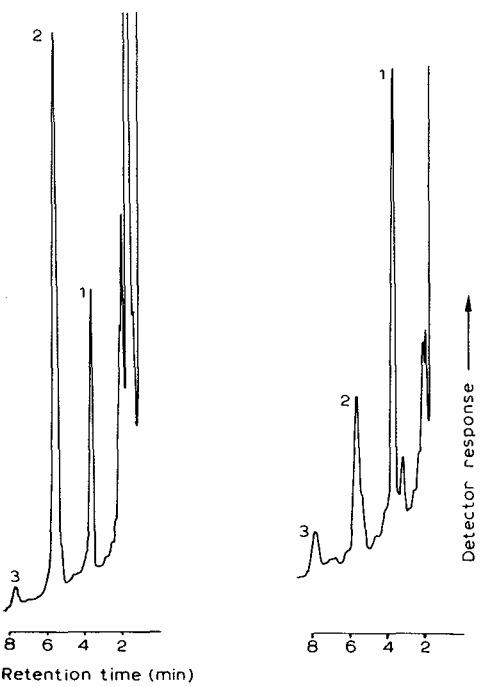

Fig.1. GC traces of hydrolysed urine extracts from normal individuals with peaks corresponding to benzylamine internal standard (1), PE (2) and N-methyl-PE (3). Gas chromatograph: Pye Unicam 104 Series 2; nitrogen flow-rate 50 ml/min; oven temperature, 140°. Column: 6 ft × 4 mm I.D. Diatomite C 'Q' (100—120 mesh) coated with 3% QF-1. Detector: hydrogen/air flame ionization.

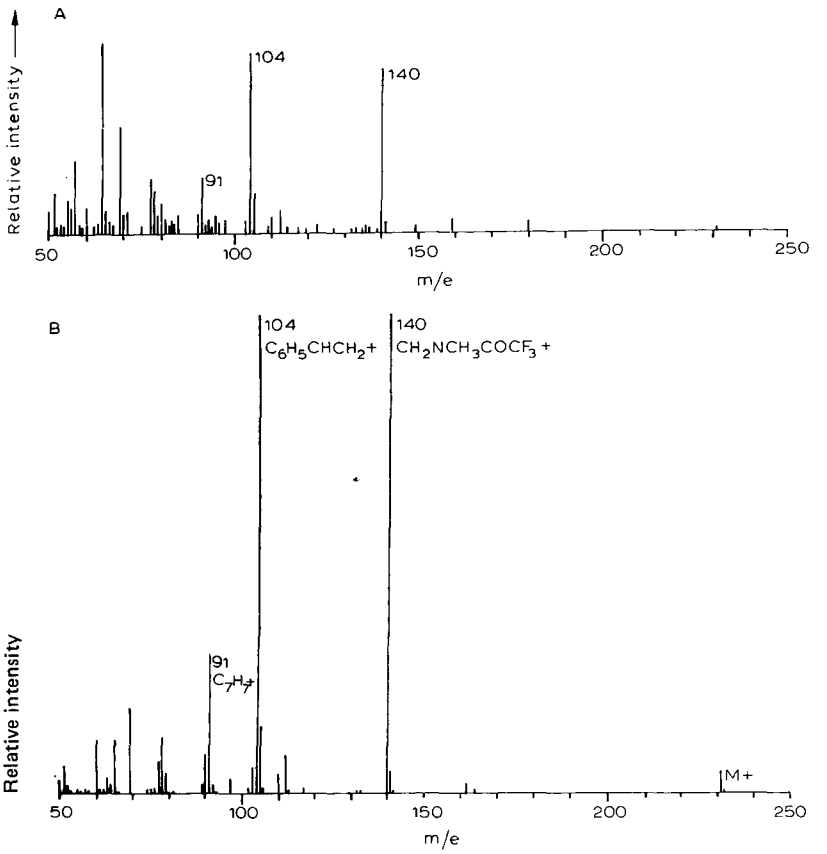

Fig.2. Mass spectra of peak 3 (A) and authentic N-methyl-PE-TFA (B). Mass spectrometer: LKB 9000S GC—MS. Column: 9 ft × 4 mm I.D. Diatomite C 'Q' (100—120 mesh) coated with 3% QF-1. Conditions: helium carrier gas flow-rate, 30 ml/min; oven temperature, 160°.

μg/24 h standard deviation, while variations in recovery were compensated for by the presence of the internal standard [4].

The compound is normally found only as an acid-labile conjugate. However, the corresponding GC peak has been obtained from a single unhydrolysed urine sample excreted by a normal individual who also exhibited a transient and anomalously large increase in urinary PE [5]. A peak corresponding to N-methyl-PE-TFA was also observed in some extracts of unhydrolysed urine from phenylketonuric patients, who normally excrete increased amounts of PE [5].

Urinary N-methyl-PE may perhaps be synthesized endogenously; a mammalian enzyme which methylates PE has already been described [6]. However, its variable excretion rate and presence as a conjugate could suggest that it is at least partially dietary in origin [7]. N-methyl-PE is known to occur in vegetable foodstuffs [8] in quantities up to 6.6 ppm, but it has not prev-

iously been reported in the animal kingdom. Nevertheless, the compound justifies some attention not only as a potential endogenous metabolite of PE, but also as a biologically active agent with sympathomimetic effects on mammalian blood pressure and heart rate [8, 9].

The procedure described here has been shown to be specific if only semi-quantitative. Minor changes to the method, such as using electron-capture GC detection (which is generally highly sensitive towards perfluoroacyl derivatives), should overcome this and permit the accurate quantification of such PE derivatives in a wide range of physiological samples.

ACKNOWLEDGEMENTS

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Biomedical Applications

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Note

High-speed liquid chromatographic determination of putrescine, spermidine and spermine in human urine

TOKISHI HAYASHI, TERUYUKI SUGIURA, SATOSHI KAWAI and TAKEO OHNO

Gifu College of Pharmacy, Mitahora, Gifu (Japan)

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Interest in aliphatic diamines and polyamines was stimulated by the reports of Russell et al. [1, 2] that these amines are present at elevated concentrations in the urine of patients with cancer. The compounds under consideration are putrescine, spermidine and spermine.

In a previous paper [3], we established a high-speed liquid chromatographic method for the determination of polyamines, based on the formation of the tosylated derivatives. It was found, however, that co-existing compounds in urine interfered in this method. This paper describes a procedure for pretreatment of the urine consisting in the hydrolysis of conjugated polyamines and the purification of the polyamines. A gradient elution technique for the tosylated polyamines is also described.

EXPERIMENTAL

Apparatus

This work was carried out in a Du Pont 840 liquid chromatograph equipped with an ultraviolet absorption detector (254 nm) and a high-pressure pump (Model KWU 32H Minimicro pump, Kyowa Seimitsu). The separation was carried out with a 1 m × 2.1 mm I.D. column of Zipax Permaphase ETH (30—50 μm) purchased from Shimadzu Seisakusho (Kyoto, Japan). Gradient elution was carried out in the apparatus shown in Fig. 1. Other experimental details are given in the legends to the figures.

Reagents

Putrescine dihydrochloride and spermidine phosphate were obtained from Tokyo Organic Chemicals (Tokyo, Japan), and spermine phosphate, 1,10-diaminodecane and *p*-toluenesulfonyl chloride (Ts-Cl) from Wako Chemicals

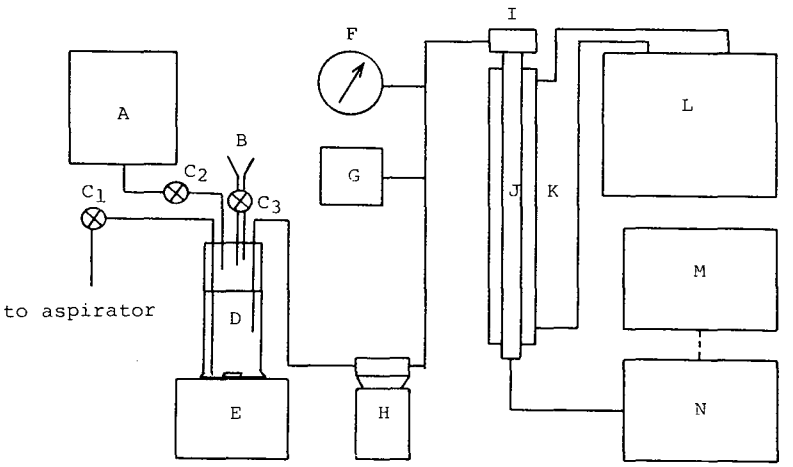

Fig. 1. Schematic diagram of gradient system. A, reservoir (75% acetonitrile); B, funnel; C, cock; D, mixing vessel; E, magnetic stirrer; F, pressure gauge (60—70 kg/cm^2, flow-rate 0.6 ml/min); G, damper; H, pump; I, injection port; J, column (1-m Zipax Permaphase ETH); K, column jacket; L, constant-temperature circulator (40°); M, recorder; N, UV detector (254nm). The gradient was prepared in the following manner. 1. About 25 ml of 25% aqueous acetonitrile solution is placed in the mixing vessel (D) from the funnel (B). 2. Aqueous acetonitrile, 25% solution, is pumped through the column. 3. When 25% aqueous acetonitrile solution is decreased to 20 ml, the pump is stopped and the sample is injected. 4. The cock C_3 is closed and the cock C_2 is opened so as to connect the mixing vessel with reservoir in which 75% aqueous acetonitrile solution is placed. The pump is then started. 5. When the separation comes to end, the pump is stopped. 6. The cock C_2 is closed and the cocks C_1 and C_3 are opened. The mixing vessel is washed with 25% aqueous acetonitrile solution before next run.

(Osaka, Japan). Acetone, *n*-hexane and methanol were redistilled before use. The other organic solvents and reagents used were of reagent grade.

Procedure

Three milliliters of urine are mixed with 4 ml of hydrochloric acid in a screw-cap tube and hydrolyzed for 3 h at 120° in an autoclave. The hydrolyzate is transferred to a 10-ml centrifuging tube, diluted to 10 ml with water and centrifuged (1000 *g*, 5 min). Then 8.0 ml of the supernatant are taken and evaporated to dryness on a rotary evaporator. The dried residue is dissolved in 10 ml of water, applied on a 10 × 0.5 cm column of 50—100 mesh Amberlite IRA-410 (OH^-), and the column is washed with water. The first 20 ml of eluate are collected. To this eluate, 10 ml of 0.1 *N* hydrochloric acid are added and the solution is applied on to a 5 × 0.5 cm column of 50—100 mesh Dowex 50W-X8 (H^+). After the column has been washed with 30 ml of 1 *N* hydrochloric acid, the polyamines adsorbed on the column are eluted with 6 *N* hydrochloric acid. The first 10 ml of eluate are collected. A 100-μl portion of the aqueous internal standard (1,10-diaminodecane) solution is added to this eluate and the mixture is evaporated to dryness on a rotary evaporator. The residue is dissolved in 1 ml of water. To this solution, 1 ml of 0.5 *M* sodium hydrogen carbonate and 20 mg of Ts-Cl dissolved in 2 ml

of acetone are added. The mixture is then warmed in a water-bath at about 70° for 1 h. The mixture is cooled, then 10 ml of a 1 *N* sodium hydroxide solution are added. The mixture is washed with four 5-ml volumes of *n*-hexane, and after the addition of 15 ml of 1 *N* hydrochloric acid, the tosylated polyamines are extracted with 10 ml carbon tetrachloride. The organic phase is dried on sodium sulfate, and carbon tetrachloride is evaporated to dryness on a rotary evaporator. The residue is redissolved in a few drops of methanol, and 10 μl of the resulting solution are subjected to the high-speed liquid chromatograph.

RESULTS AND DISCUSSION

For the pre-purification of the polyamines from a hydrolyzed urine sample, an Amberlite IRA-410 (OH^-) column (50—100 mesh, 10 × 0.5 cm) and Dowex 50W-X8 (H^+) column (50—100 mesh, 5 × 0.5 cm) were used.

Fig. 2 is an elution graph of the polyamines from the Dowex 50W-X8 column with 6 *N* hydrochloric acid. But 1,10-diaminodecane (I.S.) adsorbed on the column was not eluted with 6 *N* hydrochloric acid. These amines could not be eluted with hydrochloric acid diluted below 1 *N*.

Fig. 3A shows the effect of the pre-purification of a hydrolyzed urine sample from a patient with cancer, with the Dowex 50W-X8 column. The chromatogram shown in Fig. 3B was obtained by passing the hydrolyzed urine sample through the Amberlite IRA-410 column before application to the Dowex 50W-X8 column. Unfortunately, a poor separation of the tosyl-

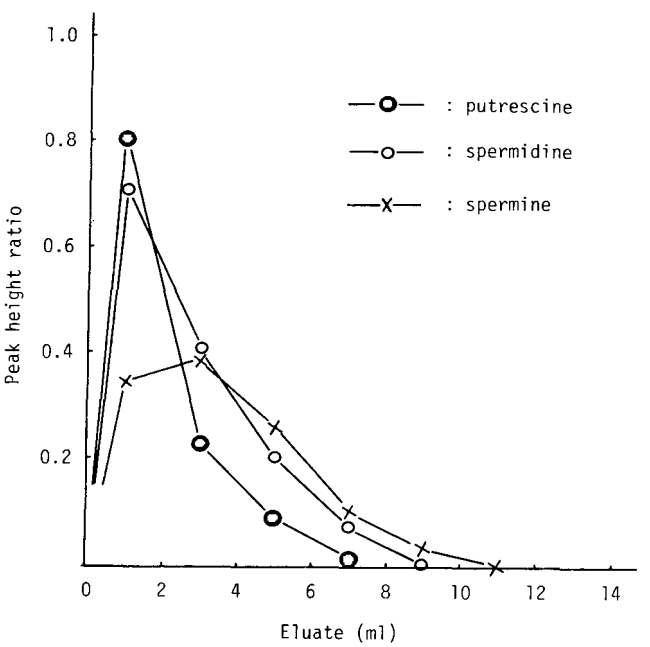

Fig. 2. Elution graph of the polyamines from Dowex 50W-X8 column (5 × 0.5 cm) with 6 *N* HCl.

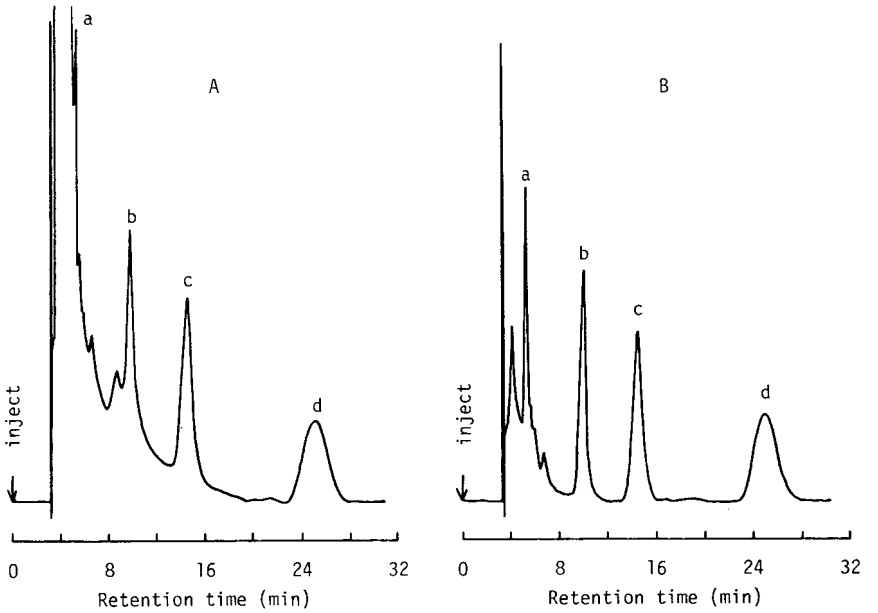

Fig. 3. Effect of pre-purification of a hydrolyzed urine sample on the liquid chromatogram. Operating conditions: column, 1-m Zipax Permaphase ETH (particle size, 30—50 μm); mobile phase, 40% acetonitrile; column temperature, 35°; flow-rate, 0.40 ml/min (pressure, 50 kg/cm^2); detector, UV photometer (254 nm). Peaks: a = Ts-putrescine; b = Ts-spermidine; c = Ts-1,10-diaminodecane; d = Ts-spermine.

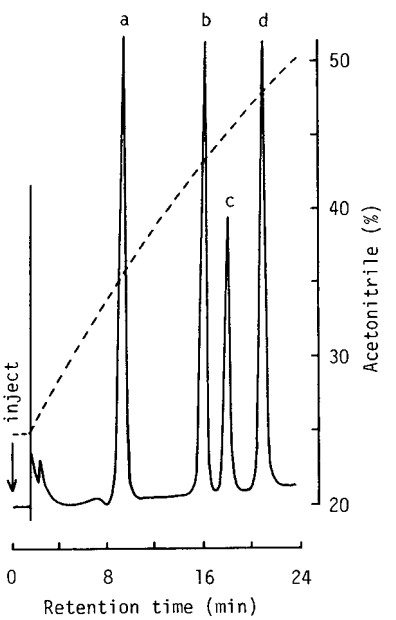

Fig. 4. Gradient elution of Ts-polyamines and Ts-1,10-diaminodecane. For operating conditions, see legend to Fig. 1. Peaks: a = Ts-putrescine; b = Ts-spermidine; c = Ts-1,10-diaminodecane; d = Ts-spermine.

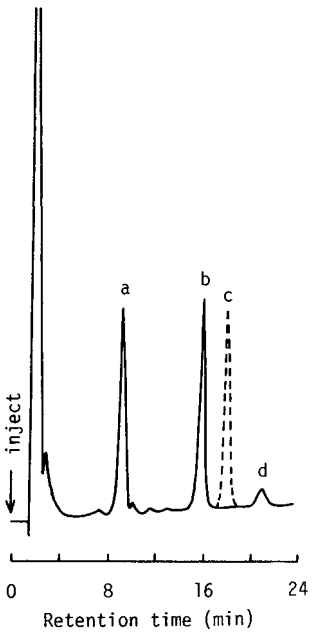

Fig. 5. Chromatogram obtained for the determination of polyamines in the urine from a patient with cancer. For operating conditions, see legend to Fig. 1. Peaks: a = Ts-putrescine; b = Ts-spermidine; c = Ts-1,10-diaminodecane; d = Ts-spermine.

TABLE I

RELATIONSHIP BETWEEN THE EXTENT OF HYDROLYSIS OF CONJUGATED POLYAMINES AND HYDROLYSIS TIME

Results are expressed in peak height ratios. Pu = putrescine; Spd = spermidine, and Sp = spermine.

Sample		Hydrolysis time (h) at 120°					Hydrolysis time (h) at 110°			
		1	2	3	5	15	1	2	15	20
A	Pu	1.73	1.81	1.82	1.82	1.85				
	Spd	0.46	0.51	0.50	0.50	0.51				
	Sp	—	—	—	—	—				
B	Pu	7.96	9.60	9.72	9.72	10.12	6.00	7.88	9.76	9.92
	Spd	18.48	21.24	21.36	21.44	21.40	16.80	19.48	21.64	21.32
	Sp	1.48	1.73	1.74	1.68	1.64	1.26	1.61	1.72	1.73
C	Pu	2.12	2.40	2.85	2.66	2.67				
	Spd	1.29	1.36	1.43	1.51	1.48				
	Sp	0.07	0.09	0.09	0.09	0.08				
D	Pu	1.74	1.77	1.80	1.79	1.82	1.04	1.47	1.80	1.82
	Spd	0.82	0.90	0.94	0.88	0.95	0.54	0.68	0.94	0.93
	Sp	0.11	0.17	0.17	0.17	0.17	0.10	0.13	0.17	0.17

ated polyamines from other compounds present in the urine sample was occasionally observed. The gradient elution technique for the tosylated polyamines was tried by using the apparatus shown in Fig. 1. Fig. 4 shows a typical chromatogram of tosylated polyamines and the gradient curve of the acetonitrile concentration in the eluent. The chromatogram shown in Fig. 5 was obtained for the determination of polyamines in the urine from a patient with cancer.

In most methods reported, conjugated polyamines have been hydrolyzed by the procedures of Marton et al. [4] with some modifications. We have investigated the hydrolysis conditions of conjugated polyamines in urine. Table I shows that the conjugated polyamines could be hydrolyzed for 3 h under our conditions.

To check the precision of this method, seven 3-ml portions of the same urine sample, supplemented each with 10 μg each of putrescine, spermidine and spermine were analyzed by the over-all procedure. The mean recoveries were 90.7% (S.D. 3.5%), 87.4% (S.D. 3.2%) and 67.0% (S.D. 5.2%), respectively.

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Note

Thin-layer chromatographic method for the separation of conjugated deoxycholic acid from conjugated chenodeoxycholic acid

SATINDRA K. GOSWAMI and CHARLES F. FREY

Department of Surgery, Veterans Administration Hospital, Martinez, Calif. 94553 (U.S.A.)

(Received April 1st, 1977)

The quantitation of individual conjugated bile acids in biological fluids depends largely on the separation of the bile acids by thin-layer chromatography (TLC) before being analysed by enzymatic [1] or gas—liquid chromatographic [2] methods. Gas—liquid chromatographic separation of bile acids usually requires derivatization and had not been successfully achieved with bile salts [3]. Various solvent systems have been proposed for the separation of conjugated bile acids [4—14], but to our knowledge, no solvent system has been able to separate the conjugated dihydroxy isomers (glycochenodeoxycholic and glycodeoxycholic acids) and the corresponding taurine conjugates (taurochenodeoxycholic and taurodeoxycholic acids) by TLC. Attempts have been made to quantify these conjugated dihydroxy isomers by sophisticated differential colorimetric techniques [15, 16] or enzymatic or alkaline hydrolysis [17] of the unresolved isomeric mixtures from a TLC plate followed by chromatographic separation of the hydrolysate with the appropriate solvent system and enzymatic assay. The colorimetric method is tedious and the disadvantages of hydrolysis and TLC procedures are the destruction of conjugates and the loss of time required for hydrolysis and re-chromatography. Recently, a combination of 3α-hydroxysteroid dehydrogenase and 7α-hydroxysteroid dehydrogenase have been used for quantifying the components of binary mixtures of dihydroxy conjugates [18]. High-pressure liquid chromatography has been able to resolve partially the tauro-dihydroxy conjugates [19]. The solvent system described here clearly separates the isomeric conjugated dihydroxy bile acids along with other conjugated bile acids on the same plate. The quantitative analysis of individual bile acids can be carried out by scraping off the appropriate portion of the bile acid from the TLC plate and enzymatic assay by 3α-hydroxysteroid dehydrogenase.

MATERIAL AND METHODS

Glycocholic, taurocholic, glycodeoxycholic, taurodeoxycholic, glycochenodeoxycholic, taurochenodeoxycholic, cholic, chenodeoxycholic and deoxycholic acids were purchased from P-L Biochemicals (Milwaukee, Wisc., U.S.A.), glycolithocholic and taurolithocholic acids from Calbiochem (San Diego, Calif., U.S.A.), and cholesterol from Sigma (St. Louis, Mo., U.S.A.). All solvents were Baker analyzed reagent grade (J.T. Baker, Phillipsburg, N.J., U.S.A.). Methanolic solutions of bile acids and cholesterol were applied as streaks with a Hamilton microliter syringe on 20 × 20 cm pre-coated TLC silica gel 60 plates of 0.25 mm thickness (E. Merck, Darmstadt, G.F.R., distributed by Curtin Scientific, Brisbane, Calif., U.S.A., catalogue No. 5763). The plate was marked at 7 cm and 15 cm from the starting line. Samples (10—15 μg) of bile acids and cholesterol and methanolic solution of hamster gallbladder bile were applied 1.5 cm above the bottom edge of the plate, allowed to dry with cold air and placed in a rectangular glass tank (29 × 9 × 25.5 cm). The solvent was chloroform—isopropand—isobutand—acetic acid—water (30:20:10:2:1) and the plate was developed at room temperature (23—25°). First two successive runs up to 15 cm and the next four successive runs up to 7 cm were allowed. After each run, the plate was dried with cold air by means of a hair drier. Finally the plate was removed from the chromatographic chamber, dried in hot air, sprayed with copper—molybdenum spray reagent [20] and heated for 15 min in an oven at 70—80° in order to make the components visible. Each bile acid can be identified by its characteristic color which is very distinct amongst each others.

DISCUSSION

The positions of different bile acids (conjugated and free) and cholesterol on a TLC plate after its development with the solvent system chloroform—isopropanol—isobutanol—acetic acid—water (30:20:10:2:1) is shown in Fig. 1. It is evident from the figure that the conjugated isomeric mixtures have been clearly separated. The free bile acids and cholesterol run well ahead of the conjugated bile acids. The R_F values of different conjugated bile acids are shown in

TABLE I

R_F VALUES OF CONJUGATED BILE ACIDS ON SILICA GEL

Solvent system: chloroform—isopropanol—isobutanol—acetic acid—water (30:20:10:2:1).

Bile acid	R_F	
	Glyco-	Tauro-
Cholic acid	0.32	0.09
Deoxycholic acid	0.55	0.22
Chenodeoxycholic acid	0.61	0.25
Lithocholic acid	0.83	0.39

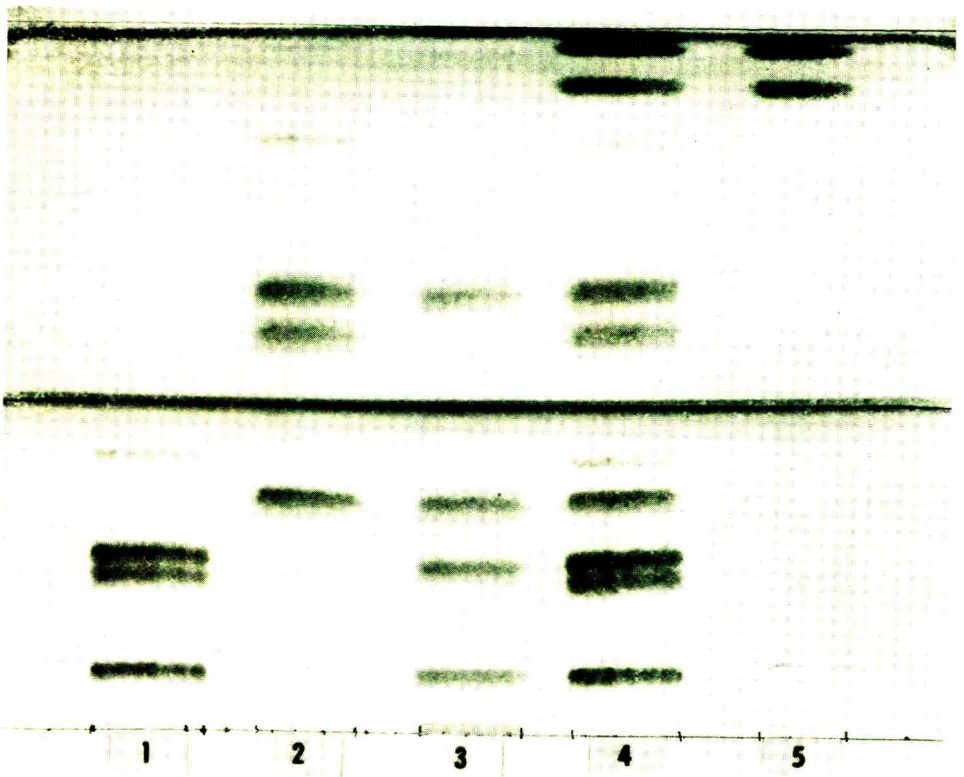

Fig. 1. Thin-layer chromatogram developed in chloroform—isopropanol—isobutanol—acetic acid—water (30:20:10:2:1) and sprayed with copper—molybdenum spray reagent [20]. In ascending order: 1 = taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid and taurolithocholic acid; 2 = glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid and glycolithocholic acid; 3 = methanolic solution of hamster gallbladder bile; 4 = mixture of 1, 2 and 5; 5 = cholic acid, deoxycholic and chenodeoxycholic acid and cholesterol.

Table I. Published methods of chromatography generally fail to separate the taurine- and glycine-conjugated dihydroxy bile acids. This method is therefore unique, i.e. the only method to date to separate the isomeric conjugated dihydroxy bile acids, glycodeoxycholic from glycochenodeoxycholic acid and taurodeoxycholic from taurochenodeoxycholic acid. The advantage of this method is the direct separation of the conjugates in biological samples. It also does not require any hydrolysis of the conjugated dihydroxy isomers (enzymatic or alkaline), solvent extraction or some other laborious procedure. Each individual bile acid can be scraped off the plates and assayed enzymatically by conventional steroid dehydrogenase method [21]. The method when applied will be helpful clinically in understanding bile acid metabolism and the diagnosis of the hepatic and gastrointestinal states [22].

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Note

Screening of plasma lipids by thin-layer chromatography with flame ionization detection on chromarods

D. VANDAMME, V. BLATON and H. PEETERS

Simon Stevin Instituut, Jerusalemstraat 34, B-8000, Brugge (Belgium)

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Flame ionization detectors (FIDs) are widely used in gas—liquid chromatography (GLC) and are now applied to other types of chromatography. Column chromatographic elution can be monitored continuously by an FID on a moving wire system [1]. Cotgreave and Lynes [2] fed a vapourised sample from a narrow chromatoplate to the FID. Further approaches in this area were conceived by Padley [3] and Szakasits et al. [4]. The idea of Padley to use a rod as a support instead of a plate was further developed by Okumura and Kadono [5]. The latter developed a sintered thin-layer chromatographic (TLC) rod (chromarod) to which silica gel powder or alumina is fused using fine glass powder as a binding agent.

In Japan several analytical procedures using the TLC—FID combination have been developed. Kawai et al. [6], Nakano et al. [7] and Ueda et al. [8] have analysed serum lipids by this technique. Phospholipids have been analysed by Tokunaga et al. [9], Ishii and Yoshioka [10] analysed phospholipids with respect to the sphingomyelin—lecithin ratio in amniotic fluid. Tanaka et al. [11] described the technique for triglycerid analysis in view of the degree of unsaturation.

This paper describes a method for the separation and identification of lipids by a TLC—FID combination in view of the development of a screening method for plasma lipids.

EXPERIMENTAL

The equipment used is the Iatroscan TH-10 (Iatron Lab). A differential and integral curve is recorded by the Omniscribe TM-recorder (Houston Instruments).

Lipids extracted from plasma [12] or standard mixtures are separated on

silicagel—chromarods in a 20 min run in light petroleum—diethyl ether (85:15, v/v). In a more polar solvent system, chloroform—methanol—water (80:35:5), the plasma phospholipids can be fractionated within 30 min.

The chromarods are activated in a preliminary step by passing them through the FID just prior to use. For nonpolar lipid separation the lipid extract of 0.25 ml plasma is redissolved in 0.1 ml chloroform and 1 μl is applied to the chromarod. For phospholipids 0.5 ml of plasma is redissolved in 0.1 ml chloroform and 1 μl is applied. After the run, scanning is performed under the following conditions: hydrogen pressure, 0.7 kg/cm^2; air flow-rate, 1400 ml/min; scanning speed, 32 sec/rod.

RESULTS AND DISCUSSION

Two non-polar plasma lipid profiles obtained are shown in Fig. 1. Similar separations of plasma neutral lipids by TLC—FID were already described [6—8]. The relative distribution of the peak areas is obtained either by triangulation or by measurement of the appropriate step height of the integral trace. The reproducibility of the method on a single sample is given in Table I. Both results are in good agreement. In some cases of hyperlipidemia, however, the integral curve may show an inflection in the trace, due to incomplete separation of the components, so that it becomes difficult to separate the steps but qualitative screening of the hyperlipidemia by the TLC—FID method is always possible (Fig.2). When the separation is performed in a more polar solvent the plasma sphingomyelin—phosphatidylcholine (S—PC) ratio can be calculated as demonstrated in Fig.3.

In general the most important requirement of this method is the selectivity

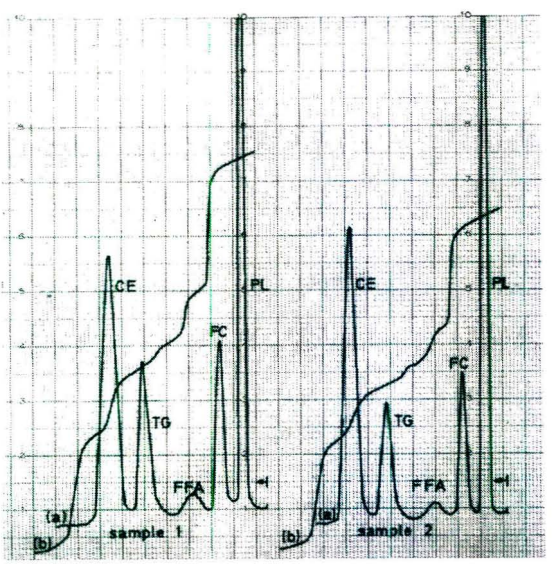

Fig.1. TLC—FID of plasma lipids. CE = Cholesterol esters; TG = triglycerides; FFA = free fatty acids; FC = free cholesterol; PL = phospholipids. (a), Differential curve; (b), integral curve.

TABLE I

REPRODUCIBILITY OF A PLASMA LIPID SCREENING BY TLC—FID ON CHROMARODS, MEASURED BY TRIANGULATION AND INTEGRATION

Compound	Triangulation	Integration
Cholesterol esters	33.3 ± 1.95	35.0 ± 1.02
Triglycerides	11.6 ± 1.08	11.6 ± 2.51
Free fatty acids	2.8 ± 1.24	2.4 ± 0.64
Free cholesterol	14.9 ± 1.22	14.8 ± 0.64
Phospholipids	37.4 ± 1.76	36.2 ± 2.05

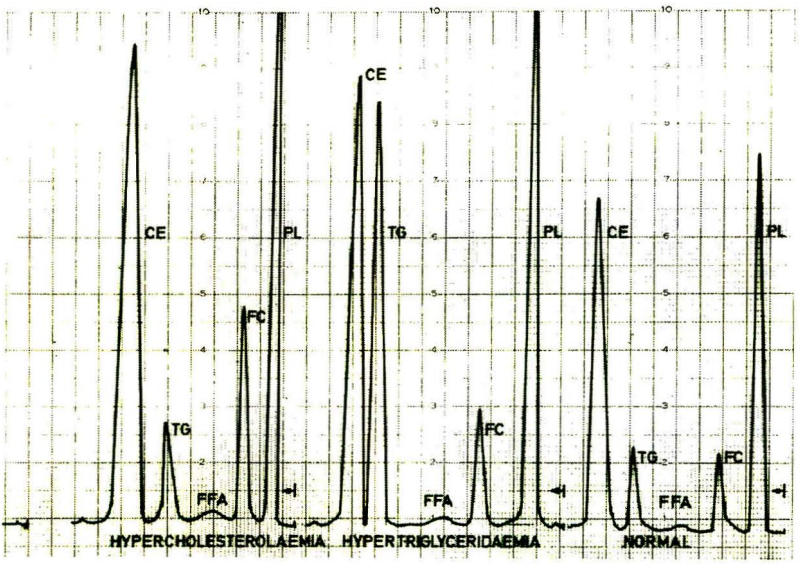

Fig.2. Screening of plasma hyperlipidemia by TLC—FID.

and the purity of the extraction since the FID is not a specific but a general detector. As a first step towards a quantitative evaluation, a mixture of lipid standards has been analysed in a concentration of 4 mg/ml for each lipid. The purity and homogeneity of the standards applied were checked by classical TLC methods. The results obtained by the FID indicate that the relative responses of the individual lipids are different. Especially the triglycerides give a lower response so that a quantitative analysis requires the introduction of correction factors. At any rate the relationship of peak area response to sample concentration remains linear within the examined concentration range of 2—16mg/ml of the lipid standards. A comparison of lipid profiles obtained by the TLC—FID technique and those obtained by classical chemical analysis is underway in order to establish practical correction factors.

In conclusion the TLC—FID technique is a rapid and simple screening procedure applicable to epidemiological studies such as the screening of hyperlipidemia in newborn or to prevention studies of atherosclerosis in adults.

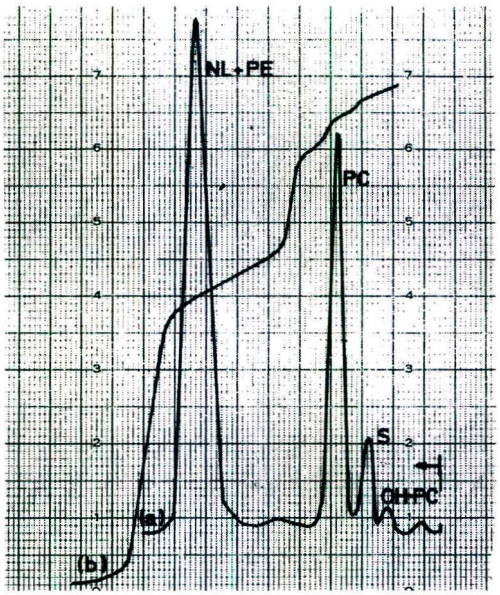

Fig.3. TLC-FID of plasma phospholipids. OH-PC = lysophosphatidylcholine; S = sphingomyelin; PC = phosphatidylcholine; PE = phosphatidylethanolamine; NL = neutral lipids. (a), Differential curve; (b), integral curve.

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Note

Gas chromatographic method for the quantitative determination of tris(hydroxymethyl)aminomethane in plasma

ABRAM HULSHOFF* and HARRY B. KOSTENBAUDER

College of Pharmacy, University of Kentucky, Lexington, Ky, 40506 (U.S.A.)

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Tris(hydroxymethyl)aminoethane (THAM) is frequently used in the clinic as an alkalinizing agent. Pediatricians use THAM for the treatment of respiratory failure (respiratory distress syndrome) and certain other conditions accompanied by a low plasma pH and a large negative base excess [1]. Although THAM is a comparatively non-toxic agent, there are some dangers connected to the administration of large doses [2, 3]. A pharmacokinetic study of THAM in (often premature) infants would therefore be helpful in establishing a safe dosage regimen. A spectrophotometric method for the determination of THAM in plasma has been reported [4]. However, this method is not sensitive enough if the plasma samples are small (50—100 μl); nor are other analytical methods reported in the literature suitable [5—9]. In the proposed method the three hydroxyl groups and the primary amine group of the THAM molecule are benzoylated and the benzoylated product is extracted with an organic solvent mixture; after concentration by evaporation the organic phase is analyzed by gas chromatography. 1,2,6-Hexanetriol was found to be an appropriate internal standard.

MATERIALS AND METHODS

THAM (primary standard) was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). 1,2,6-Hexanetriol (HEX) was obtained from Carbide and Carbon Chemicals (New York, N.Y., U.S.A.) All of the other solvents and reagents used were of analytical grade.

*Present address: Farmaceutisch Laboratorium van de Rijksuniversiteit, Catharijnesingel 60, Utrecht, The Netherlands.

Gas chromatography

A Varian Aerograph Model 2700 gas chromatograph equipped with a flame ionization detector was used. The glass column (3 ft. × ¼ in. O.D.) was packed with 1% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The packed column was conditioned overnight at 300° (40 ml/min nitrogen flow), and silylated at 250° by injecting five portions of 10-μl Silyl 8 (Pierce, Rockford, Ill., U.S.A.). The operating conditions were: injection port temperature, 290°; column temperature, 255°; detector temperature, 290°; carrier gas (nitrogen) flow-rate, 40 ml/min; hydrogen flow-rate, 35 ml/min; air flow-rate, 350 ml/min.

Preparation of reference compounds

The benzoylated products of THAM (B_4-THAM) and of HEX (B_3-HEX) were prepared using the method as described by Bighley et al. [10] for the preparation of the reaction product of pentaerythritol with *p*-methoxybenzoyl chloride.

B_4-THAM.

B_4-THAM was prepared by reacting 0.025 mole THAM in 50 ml pyridine with 0.10 mole benzoyl chloride at room temperature for 16 h. The reaction mixture was poured into 200 ml of ice-water and extracted with two successive 200-ml portions of ethyl acetate. The combined ethyl acetate extract was washed successively with 5% sodium bicarbonate, 10% hydrochloric acid, water and saturated sodium chloride solution, and then dried over anhydrous sodium sulphate. The ethyl acetate extract was reduced to dryness in vacuo. The oily residue was washed with warm hexane; after cooling the mixture the hexane was decanted and the residue dried at room temperature under a nitrogen flow. Upon standing the oil turned into a white, crystalline solid (yield 96%), m.p. 109—111°; the nuclear magnetic resonance (C^2HCl_3) spectrum was consistent with the expected structure of B_4-THAM, δ 7.1—8.0 (m, 21H, aromatic and amino), 4.8—5.1 (s, 6H, CH_2O); single spot at R_F = 0.66 on a 0.25-mm silica gel GF_{254} plate (New England Nuclear, Boston, Mass., U.S.A.) developed with benzene—methanol (9:1).

Analysis. Calculated for $C_{32}H_{27}O_7N$: C, 71.50; H, 5.06; O, 20.83; N, 2.61. Found: C, 71.27; H, 4.98; O, 20.91; N, 2.55.

B_3-HEX.

B_3-HEX was prepared by reacting 0.025 mole HEX with 0.075 mole benzoyl chloride and following the procedure as described for the preparation of B_4-THAM. After several days the oily product turned into a white crystalline solid (yield, 96%); m.p. 55—57°; the nuclear magnetic resonance (C^2HCl_3) spectrum was consistent with the expected structure of B_3-HEX, δ 7.1—8.2 (m, 15H, aromatic), 5.2—5.8 (m, 1H, CHO), 4.1—4.7 (m, 4H, CH_2O), 1.4—2.2 (m, 6H, CH_2); single spot at R_F = 0.76 (chromatographic system, see under B_4-THAM).

Analysis. Calculated for $C_{27}H_{26}O_6$: C, 72.63; H, 5.87; O, 21.50. Found: C, 72.61; H, 5.79; O, 21.34.

Procedure

A 100-μl volume of plasma in a centrifuge tube was mixed with 2 ml 0.003% HEX in water and 0.8 ml 10% trichloroacetic acid and centrifuged at 5000 *g* for 15 min. The supernatant was decanted into a 15-ml tube closed with a PTFE-lined screw cap; 0.8 ml 10% sodium hydroxide solution and 200 μl benzoyl chloride were added, and the contents of the tube were mixed for 2.5 min with a vortex-type mixer. A 2-ml volume of hexane—chloroform (3:2.05) was added and the mixture was vortexed for 1.5 min. After addition of 0.6 ml 10% sodium hydroxide solution and mixing for 15 sec the tube was centrifuged for 3 min (5000 *g*). The organic (upper) phase was transferred to a weighed centrifuge tube and the hexane and chloroform were evaporated under a nitrogen stream at 40°. The residue, consisting mainly of benzoyl chloride (15—30 mg) and the benzoylated products, was mixed with 60—150 μl hexane—chloroform (3:2.05). A 2.5-μl portion of the resulting solution, containing approximately 20% benzoyl chloride, was injected into the gas chromatograph.

Calibration curve

Pooled plasma samples (100 μl) were spiked with 0.5—10.0 μg THAM and treated as indicated under *Procedure*. Following chromatography the heights of the peak corresponding to B_4-THAM and to B_3-HEX were measured. The peak-height ratio was plotted against the amount of THAM in the sample.

RESULTS AND DISCUSSION

THAM has very low oil—water partition coefficients in organic solvent—water systems and can not be extracted as such from the plasma. A derivatization method similar to that described by Bighley et al. [10] was therefore developed. HEX was selected as an internal standard because it also is derivatized to yield a product with increased oil—water partition coefficient.

Fig. 1 shows typical chromatograms obtained with blank plasma, plasma to which THAM and HEX were added, and plasma analyzed as described under *Procedure* from a patient treated with THAM. The reaction between THAM and benzoyl chloride was virtually complete under the experimental conditions; the yield of B_4-THAM did not change when the reaction mixture was shaken for 30 min or 1 h at 40°. The yield of B_3-HEX was slightly decreased after shaking for 1 h at 40°. Precipitation of the plasma proteins with trichloroacetic acid was necessary because without such treatment a plasma peak appeared in the chromatogram, interfering with the B_3-HEX peak.

After mixing the reaction mixture for 2.5 min the contents of the tube were acidic and alkali had to be added in order to prevent the extraction of disturbingly large amounts of benzoic acid into the organic phase. The amount of benzoyl chloride used in the reaction proved to be critical. After completion of the reaction procedure the organic phase must contain a slight excess of benzoyl chloride, which is needed to prevent degradation of the benzoylated products; however, too much benzoyl chloride would result in a large final volume, because the injected sample should not contain more than 20% ben-

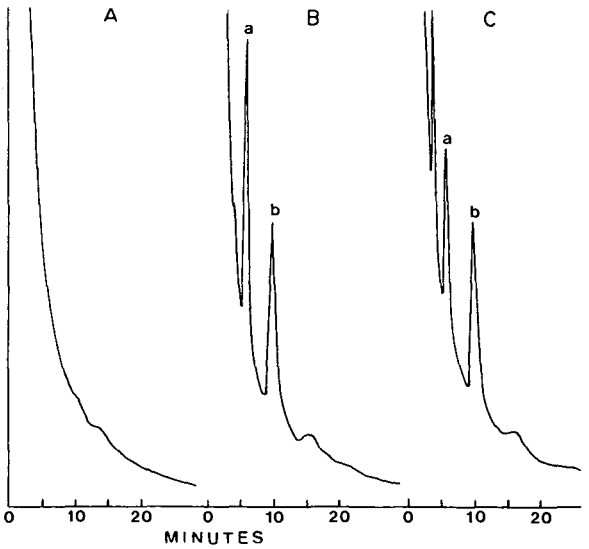

Fig.1. Chromatograms obtained from an extract of blank plasma (A), an extract of blank plasma with THAM (a) and HEX (b) (B), and an extract of plasma from a patient who received THAM (a) to which HEX (b) has been added (C). Attenuation, $128 \circ 10^{-12}$

zoyl chloride, higher concentrations yielding too broad a solvent front. The concentration of benzoyl chloride in the injected sample influenced the peak-height ratio of the B_4-THAM and the B_3-HEX peaks, without a concomitant change in the retention times. The ratio (B_4-THAM/B_3-HEX) increased with increasing benzoyl chloride concentrations (0—10%) in the injected solution. At higher concentrations of benzoyl chloride (10—20%) the peak-height ratio remained fairly constant. This change in the peak-height ratio was mainly the result of a decrease in the height of the B_3-HEX peak, possibly caused by an on column decomposition of a part of the injected B_3-HEX. The B_4-THAM peak was much less affected, and was somewhat higher in the presence of benzoyl chloride, owing to a decrease in the extent of tailing.

Before injection of the samples two 5-μl injections were done with a concentrated solution of B_4-THAM (10 mg/ml) and B_3-HEX (20 mg/ml) in hexane—chloroform (3:2.05), containing 20% benzoyl chloride. The time between injection of the samples was 30 minutes.

The recovery of the compounds from plasma as compared with water was 89% (coefficient of variation 8%, n = 7) for THAM and 62% (coefficient of variation, 12%, n = 7) for HEX. After a single extraction of the reaction mixture with hexane—chloroform (3:2.05), no B_4-THAM and B_3-HEX could be detected in the aqueous phase. The peak-height ratios at different concentrations and the standard deviations are presented in Table I.

The sensitivity of the method was sufficient for the determination of 0.3—0.5 μg THAM in 100 μl plasma. The plasma levels of an infant (boy, 2860 g) who had received an intravenous bolus dose of 109 mg THAM was found to be 430 μg after 30 min and 86 μg/ml after 4.5 h. The method appears to be quite sufficiently sensitive for the quantitative determination of therapeutic levels of THAM in small amounts of plasma.

TABLE I

PEAK-HEIGHT RATIO OF B_4-THAM TO B_3-HEX, STANDARD DEVIATION AND COEFFICIENT OF VARIATION OBTAINED WITH PLASMA SAMPLES (100 μl) CONTAINING 5—100 μg/ml THAM

Concentration (μg/ml)	Peak-height ratio (mean)	Number of determinations	Standard deviation	Coefficient of variation (%)
5	0.091	6	0.021	23.5
25	0.488	6	0.042	8.6
50	0.973	6	0.065	6.7
75	1.465	6	0.088	6.0
100	2.008	6	0.090	4.5

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CHROMBIO. 098

Note

Gas—liquid chromatographic determination of procetofenic acid in human plasma and urine

J.P. DESAGER

Laboratoire de Pharmacothérapie FART 53.49, Université Catholique de Louvain, 53, Avenue E. Mounier, B-1200 Brussels (Belgium)

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Procetofenic acid or 2[4'-(*p*-chlorobenzoyl)phenoxy]-2-methylpropionic acid (LF 153) is the active metabolite of a new hypolipidemic drug, procetofene (LF 178—isopropyl ester of procetofenic acid). This compound is chemically related to the well known hypolipidemic drug, clofibrate or ethyl (*p*-chlorophenoxy)-2-methylpropionate, in which the *para*-chlorine atom is spaced by a benzoyl group. In the body, clofibrate is also completely transformed in an active metabolite, clofibric acid [1].

No methods suitable for the determination of this new compound have been reported as yet. An enzymic hydrolysis of the conjugates was performed prior to the urine analysis. The method described in this paper was developed for the determination of the active circulating metabolite, procetofenic acid, in human plasma and hydrolyzed urine down to the level of 100 ng/ml in 0.5-ml samples. The compound was determined as its methyl ester and the parent drug, procetofene, was used as the internal standard.

EXPERIMENTAL AND RESULTS

Reagents

The solvents used were of analytical grade. Ethereal diazomethane was prepared with Diazald (Aldrich, Milwaukee, Wisc., U.S.A.) in the Diazald-kit instrumentation set and stored at −20°. Procetofene and procetofenic acid were of analytical purity. β-Glucuronidase—aryl sulphatase preparations in water (B grade) were obtained from Calbiochem (San Diego, Calif., U.S.A.).

Quantitative analysis of procetofenic acid in human plasma and urine

Gas—liquid chromatography. A Varian Model 1445 gas chromatograph

equipped with an electron-capture detector (^{3}H Sc) was used. The column was a 5 ft × 2 mm I.D. coiled glass tube packed with 4% SE-30 on Gas-Chrom Q, 80—100 mesh (Applied Science Labs, State College, Pa., U.S.A.) and was conditioned at 270° for 48 h (nitrogen flow-rate 30 ml/min). The instrument settings were: column temperature, 230°; injection port temperature, 250°; detector temperature, 270°; ^{3}H Sc foil temperature, 240°; and carrier gas (ultra-pure nitrogen) flow-rate, 45 ml/min. Under these conditions, the retention time was 4.25 min for the methyl ester of procetofenic acid and 5.25 min for the internal standard, procetofene.

Enzymic hydrolysis of conjugates in urine. A 0.5-ml volume of urine and 4.5 ml of 0.2 *M* sodium acetate buffer pH 5 were incubated with the β-glucuronidase—aryl sulphatase preparation (10.000 units) at 37° for 24 h.

Extraction procedure. To a 60-ml stoppered tube were added successively 0.5 ml of plasma or hydrolyzed urine, 1 ml of distilled water, 2 ml of 3 *M* hydrochloric acid, 10 ml of diethyl ether and 1 ml of the internal standard solution (procetofene, 5 μg/ml in heptane). After continuous manual extraction for 30 sec and separation of the two layers, 8 ml of the upper layer were transferred to another tube. To this extract, 2 ml of ethereal diazomethane were added and the mixture was allowed to stand for 15 min. Evaporation on a Rotavapor at 40° afforded the residue for the gas chromatographic analysis. Before injection, solubilization was effected with 1 ml of *n*-hexane and 2 μl were injected into the gas chromatograph (duplicate injections).

Quantitation. One calibration graph for plasma and one calibration graph for urine were obtained by assaying respectively drug-free plasma or urine containing known amounts of procetofenic acid and plotting the ratio of peak heights (methylated procetofenic acid to internal standard) against the amount of procetofenic acid added. The graph covered the range 100 ng/ml—6 μg/ml of procetofenic acid in plasma with a slope of 1.32. For urine the slope was quite similar, 1.35.

The peak height ratio of procetofenic acid to internal standard was calculated for each sample and the amount of procetofenic acid present was determined by reference to each calibration graph. The reproducibility and accuracy were found to be 4 ± 1%. The sensitivity was about 100 ng/ml and the recovery was 96.5 ± 1% for both plasma and urine.

Specificity. In all of the plasma samples investigated, a small peak was found at the retention time of the internal standard (Fig. 1). This constant interference was small in contrast with the height of the internal standard peak. The urine chromatograms were free from interfering peaks.

Application. The above method was performed on plasma and urine samples from one healthy volunteer who was given a single oral dose of 300 mg of procetofene (Lipanthyl®, Laboratoires Fournier, Dijon, France) (Fig. 2 and Table I).

DISCUSSION

The method described for the quantitative analysis of procetofenic acid is based on the use of an internal standard with a similar chemical structure for human plasma and urine samples. Both dosages proved to be accurate and

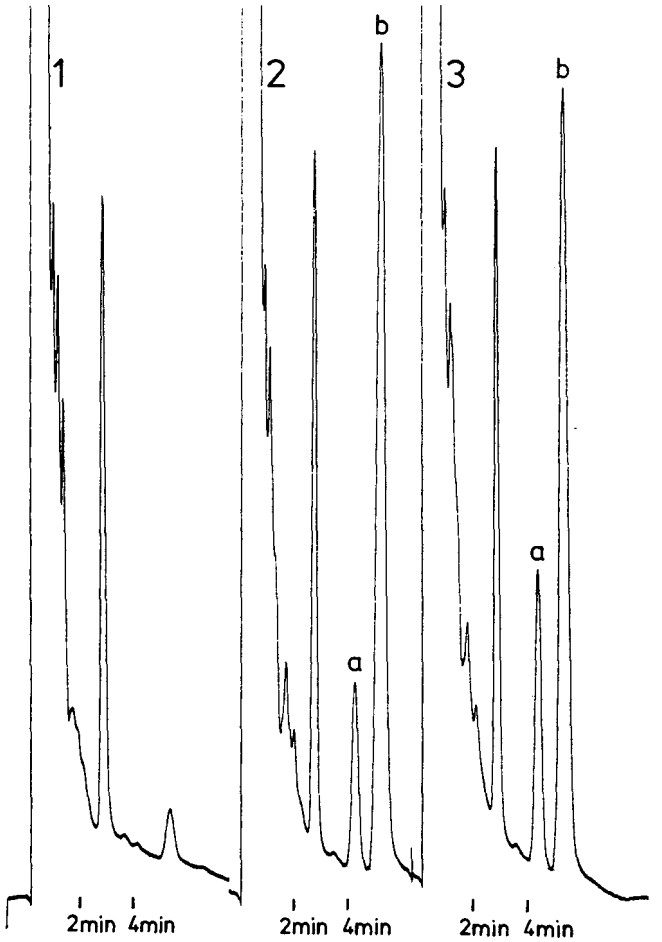

Fig.1. Chromatograms of a drug-free plasma extract (1), a plasma extract containing added procetofenic acid at a level of 2 μg/ml (2), and a plasma extract from a volunteer receiving procetofene (a= active metabolite; b= standard— (3).

easy to perform. A small but constant interfering peak in the plasma samples did not impair the results. To remain in the linear range, drug-free human plasma or hydrolyzed urine were used to dilute the samples in which a concentration above 6 μg/ml was found. No traces of procetofenic acid could be detected in drug-free samples (plasma and hydrolyzed urine) containing the internal standard. The stability of procetofene was so ascertained in our experimental conditions. Moreover, the parent drug was not found in the plasma and hydrolyzed urine samples as reported elsewhere [2].

The free fraction of procetofenic acid in human urine was estimated to be about 1 or 2%. The enzymic hydrolysis of the conjugates with a β-glucuronidase—aryl sulphatase preparation was prefered to the acid hydrolysis with hydrochloric acid. Between 25 and 35% of procetofenic acid was destroyed

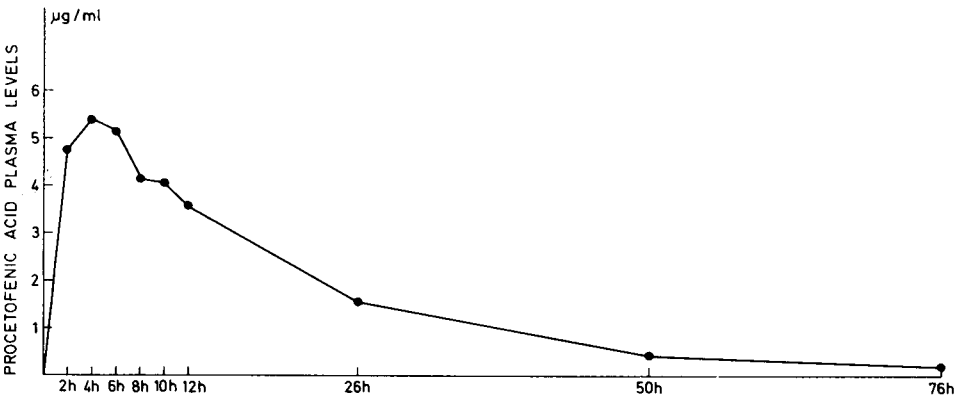

Fig.2. Graph illustrating the absorption and elimination from plasma of 300 mg of procetofene (Lipanthyl®) administered to one volunteer as a single oral dose.

TABLE I

LEVELS OF PROCETOFENIC ACID IN HYDROLYZED URINE FROM ONE HEALTHY VOLUNTEER RECEIVING 300 mg OF PROCETOFENE AS A SINGLE ORAL DOSE

Period of collection (h)	Procetofenic acid excreted (mg)
0—24	49.13
24—48	8.32
48—72	3.29
72—96	1.27

in the urine samples when they were hydrolyzed for 15 min at 100° with 3 *M* hydrochloric acid.

The sensitivity of the method made it possible to study the pharmacokinetic parameters of this new drug. If needed, it is possible to detect 10 ng/ml in the biological fluids.

The first results on a human volunteer showed that the drug is slowly cleared from the plasma, following a biexponential curve on a semi-logarithmic scale. The elimination half-life was about 24 h and 20.67% of the administered dose were recovered from the 96-h hydrolyzed urine collection. In a previous study [3] conducted with a radiochemical tracer in two healthy volunteers, a half-life of about 7 h was found and the existence of another metabolite of procetofene was suggested. However, the presence of this metabolite could not be confirmed [2]. In this study, the specificity of the method was checked using a coupled gas—liquid chromatographic—mass spectrometric system (LKB 9000; column, 3% OV-1 on Chromosorb W, 60—80 mesh; temperature 230°; helium flow-rate 30 ml/min; ionization potential, 70 eV). Only the mass spectra of the methyl ester of procetofenic acid was recorded during the gas—liquid chromatographic—mass spectrometric analysis of our samples.

In conclusion this method for the determination of procetofenic acid proved to be fast, specific and sensitive.

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Note

Determination of serum tolbutamide and chlorpropamide by high-performance liquid chromatography

R.E. HILL and J. CRECHIOLO

Department of Pathology, McMaster University, Hamilton, Ontario (Canada)

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Tolbutamide (1-butyl-3-(*p*-tolylsulphonyl)urea) and chlorpropamide (1-propyl-3-(*p*-chlorphenyl sulphonyl)urea) are two of the sulphonylurea derivatives used as oral hypoglycaemic agents for the treatment of diabetes mellitus [1, 2]. These compounds have been in use for a number of years and it is known that wide intra-individual variation is often present after similar therapeutic doses [3, 4] and that their therapeutic index is fairly low. Routine monitoring aimed at correlating serum levels with clinical effect or to estimate compliance is not widespread, however. One reason for this omission has been the absence of analytical techniques which meet the requirements of a routine monitoring service, i.e. that the assay involve the minimum number of operations on a small volume of sample, whilst producing accurate results with good long-term precision. Colorimetric [5, 6] and spectrophotometric [7, 8] procedures which are time-consuming, non-specific and lack sensitivity, clearly do not meet these standards. Gas chromatographic procedures have been reported [9—11]. However, tolbutamide and chlorpropamide are thermolabile and a derivatization step is required in the analysis. Moreover, the analysis depends upon on-column pyrolysis, followed by on-column methylation to the corresponding N,N-dimethylsulphonamide and it has been reported that reproducible chromatograms require a careful injection technique [11]. High-performance liquid chromatography (HPLC) of sulphonylureas in pharmaceutical preparations has been reported [12]. This present communication describes the application of this approach to the determination of therapeutic levels of tolbutamide and chlorpropamide in serum. The method meets the requirements of a routine assay outlined above. A single extraction step from a small volume of serum (200 μl) is followed by reversed-phase chromatography, without prior derivatization.

EXPERIMENTAL

Apparatus

The liquid chromatograph used was an ALC Model 202, with Model 6000A pump, U6K injector and Model 440 absorbance detector (Waters Assoc., Milford, Mass., U.S.A.).

Chromatographic Conditions

A stainless-steel column (30 cm × 4 mm I.D.) was packed with a stable reversed-phase stationary phase, consisting of porous silica beads (mean diameter 10 μm) coated with a chemically-bonded monolayer of octadecylsilane (μBondapack C-18, Waters Assoc.). The mobile phase was 1% acetic acid (adjusted to pH 5.5 with NaOH (2 *N*)–acetonitrile (72:28, v/v). The operating temperature was ambient and the flow-rate 2.2 ml/min with an operating pressure of 17.25 MPa (2500 p.s.i.). The column effluent was monitored continuously at 254 nm, with a full scale deflection of 0.1 *A*. A short acetonitrile wash (20 min at 1 ml/min) at the end of each analytical day was included to remove strongly retained solutes.

Reagents

All chemicals were reagent grade. Tolbutamide and chlorpropamide were donated by Hoechst Pharmaceuticals, Willowdale, Canada. 1-Isopentyl-3-(*p*-tolylsulphonyl)urea was purchased from Aldrich (Milwaukee, Wisc., U.S.A.). Solvents are routinely filtered through 0.45-μm filters (Millipore Corp., Bedford, Mass., U.S.A.) prior to use in the liquid chromatograph.

Standards

Tolbutamide (200 mg) and chlorpropamide (200 mg) were dissolved in absolute ethanol (10 ml). 1 ml of this solution was made up to 100 ml with plasma. This standard (200 mg/l) was serially diluted with plasma to prepare standards containing 100, 50 and 25 mg/l respectively. These preparations were divided into 1-ml aliquots and frozen (−20°). The internal standard, 1-isopentyl-3-(*p*-tolylsulphonyl)urea (5 mg), was dissolved in chloroform (10 ml). A 5-ml volume of this solution was made up to 1 l with chloroform and this solution served as the extraction solvent.

Extraction

Serum or plasma (200 μl) is added to a 50-ml glass tube fitted with a PTFE-lined screw-cap. Chloroform (10 ml) containing the internal standard is added, followed by sodium chloride (ca. 1 g). Extraction is for 5 min (Buchler Omnishaker), followed by centrifugation at 500 *g* for 2 min. The aqueous phase is removed by aspiration, the chloroform layer decanted into a disposable tube and taken to dryness by warming under a stream of dry nitrogen. The residue is dissolved in acetonitrile (ca. 40 μl) and 25 μl is injected into the liquid chromatograph. This procedure is followed for patient and standard samples. Standard curves are constructed by plotting the peak height ratios of each drug to the internal standard against the drug concentration in each standard. The level of drug in an unknown sample is derived from this curve.

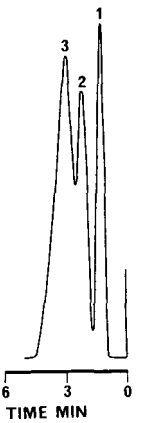
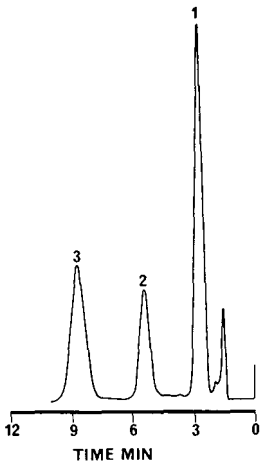


Fig. 1. Chromatogram of chlorpropamide (1), tolbutamide (2) and 1-isopentyl-3-(*p*-tolylsulphonyl)urea (3). Solvent system, acetonitrile—water (28:72, v/v).

RESULTS AND DISCUSSION

Acceptable and rapid separation of the sulphonylureas in this reversed-phase system is not only a function of the amount of organic modifier in the solvent, but also depends on the effective pH and ionic strength of the aqueous component. A simple acetonitrile—water system (28:72, v/v) with an effective pH of 5.5 does not resolve the solutes [k'(chlorpropamide) = ca. 0; k'(tolbutamide) = 0.8; and k'(internal standard) = 1.4] (Fig. 1). Since the sulphonylureas are weak acids (pk_a(tolbutamide) = 5.4 [13]), their capacity factors are increased by lowering the effective pH of this solvent [12], and the system acetonitrile—1% acetic acid (28:72, v/v) (effective pH = 2.9) resolves the three solute peaks [k'(chlorpropamide) = 3.3; k'(tolbutamide) = 4.8; and k'(internal standard) = 8.5]. However, the increase in overall elution time is inappropriate for a routine assay procedure. A return to pH 5.5 by titration with NaOH (see Experimental) produces a solvent with the same effective pH as the acetonitrile—water but with an effective ionic strength (ca. 0.14) which is infinitely larger. In reversed-phase chromatography this solvent is weaker than the acetonitrile—water and unlike this system effects excellent separation of the sulphonylureas in the minimum time.

A chromatogram of a plasma standard (100 mg/l) is shown in Fig. 2. The chromatography is complete within 10 min, with baseline separation between the three solutes [k'(chlorpropamide) = 0.8; k'(tolbutamide) = 2.6; and k'(internal standard) = 5.0]; other than unretained material no endogenous peaks are present. Fig. 3 shows the chromatogram from a patient on tolbutamide therapy with a found plasma level of 79 ± 5 (1 S.D.) mg/l. Analysis of the standards and plasma blank showed the relationship between the plasma concentration of both drugs and the peak height ratios of each drug to the internal standard to be linear between 0 and 200 mg/l. This range encom-



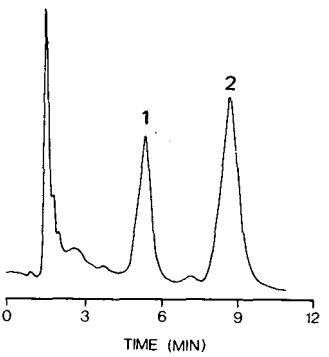


Fig. 2. Chromatogram of a plasma extract: chlorpropamide (1), tolbutamide (2) and 1-isopentyl-3-(*p*-tolylsulphonyl)urea (3). Solvent system, acetonitrile—1% acetic acid (28:72, v/v, adjusted to pH 5.5 as described in the text).

Fig. 3. Chromatogram of a patient plasma extract: tolbutamide (1), 1-isopentyl-3-(*p*-tolylsulphonyl)urea (2). Solvent system, acetonitrile—1% acetic acid (28:72, v/v, adjusted to pH 5.5 as described in the text).

passes the therapeutic ranges for tolbutamide (53—96 mg/l) [14] and chlorpropamide (30—140 mg/l) [14]. The regression equations are $y = 0.004 + 0.01\ x$, $r = 0.9993$ for tolbutamide and $y = -0.04 + 0.03\ x$, $r = 0.9994$ for chlorpropamide (y = peak height ratio drug/internal standard and x = drug concentration). The limits of sensitivity are 6 mg/l for tolbutamide and 7 mg/l for chlorpropamide. The extraction procedure yields greater than 95% recovery for each drug. A pool sample containing each drug (100 mg/l) was processed to determine the accuracy and precision of the method. The between batch variations are 5.4%, mean = 98.0 ± 5.3 (1 S.D.) (n = 30) for chlorpropamide and 6.6%, mean = 102.5 ± 6.4 (1 S.D.) (n = 30) for tolbutamide.

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Note

Determination of plasma α-tocopherol by high-performance liquid chromatography

B. NILSSON, B. JOHANSSON, L. JANSSON and L. HOLMBERG

Department of Clinical Chemistry and Department of Pediatrics, University of Lund, Malmö General Hospital, Malmö (Sweden)

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Vitamin E, a fat-soluble vitamin, is chemically composed of four forms of tocopherol (α, β, γ, δ). α-Tocopherol is the most abundant member of the series (ca. 12—36 μmole/l plasma) in human serum and is also biologically the most active form. A large number of methods [1—3] exists for the determination of tocopherols in plasma samples but most of these are based on indirect measurement of vitamin E. In such methods carotenoids often interfere with the analysis [3].

In this paper we describe a fast accurate method to determine α-tocopherol in plasma by high-performance liquid chromatography. To our knowledge this technique has not been used for plasma samples. The method has been applied to plasma from healthy individuals as well as to plasma from pre-term newborn infants. Detection of vitamin E deficiency in the plasma of such infants may explain anaemia [4]. The results of this latter study will be reported elsewhere.

EXPERIMENTAL

Materials

n-Hexane (analytical grade, redistilled once before use), diisopropyl ether and isopropanol (spectroscopic grade) were purchased from BDH (Poole, Great Britain). α-Tocopherol was obtained from Merck (Darmstadt, G.F.R.) and the mixture of β-, γ- and δ-tocopherol was a generous gift from AB Ferrosan (Malmo, Sweden). α-Tocopheryl acetate was of purum quality from Fluka (Buchs, Switzerland).

Apparatus

A Waters Model ALC/GPC 204 liquid chromatograph equipped with a U6K loop injector and a Model 440 UV spectrophotometer was used. The outlet of the injector was connected to a stainless-steel column (60 cm × 2 mm I.D.) packed with Corasil I (purchased from Waters Assoc., Milford, Mass., U.S.A.). The column was eluted with *n*-hexane—diisopropylether (96:4) at a flow rate of 60 ml/h. The eluent was de-gassed by ultrasonication for 15 min before use. The absorbance at 280 nm was monitored at a chart speed of 0.5 cm/min. Peak areas were automatically obtained by means of a Varian CDS 111 chromatography data system.

RESULTS AND DISCUSSION

Preparation of standard solutions

Eight standard mixtures of α-tocopherol and α-tocopheryl acetate in *n*-hexane were prepared and analyzed by high-performance liquid chromatography. The solutions were stored under nitrogen in a refrigerator when not in use. The plot of peak area ratios *vs.* concentration ratios for the components in the calibration mixtures shows a good linearity (correlation coefficient, r = 0. 9986) for α-tocopherol concentrations in the range 6—60 μmole/l.

Preparation of samples

Human blood collected in EDTA tubes was immediately centrifuged and the plasma fraction was pipetted off. In a typical experiment 500 μl 99.5% ethanol, containing 26.6 μmole/l α-tocopheryl acetate, were added to 500 μl plasma. Five-ml conical centrifuge tubes were used. After the addition of 500 μl *n*-hexane the tube was stoppered and the sample was carefully cyclo-mixed. After centrifugation for 10 min at 30000 *g*, 15 μl of the organic layer was directly injected into the column. The smallest volume of plasma that was extracted, was 100 μl.

Chromatogram

As shown in Fig. 1, α-tocopherol (d) and the internal reference compound (c) are well separated (retention times 5.1 min and 3.3 min, respectively) but there are other peaks in the chromatogram that are not completely resolved. To identify some of these peaks, vitamin K_1, vitamin A, β-carotene and the β-, γ- and δ-tocopherols were injected separately and as a mixture. These compounds were also added one by one to a sample. The compounds which are extractable with *n*-hexane are indicated in the chromatogram (a—e). Unfortunately, γ-tocopherol, which has been determined from plasma samples (after silylation of the phenol group) by gas chromatography [5], could not be completely separated from other compounds in our system. Perhaps the use of a fluorescence detector instead of a UV detector, as used by Van Niekerk [6] and by Abe and co-workers [7,8] in their studies on free tocopherols in plant extracts, would have improved the method. To make sure that no peaks in our system were hidden under the α-tocopherol peak, the sample was recycled several times. No separation into further peaks was observed.

Another mobile system was also tested in which isopropanol (0.15%) was

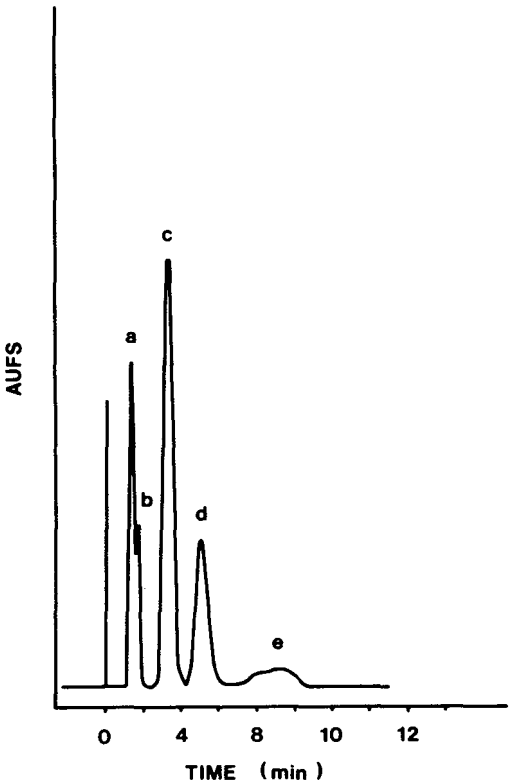

Fig.1. High-performance liquid chromatogram of α-tocopherol (d) in serum Peaks: a, β-carotene; b, vitamin K; c, α-tocopheryl acetate. The broad peak, e, was found to be due to both β- and γ-tocopherol and some other unidentified compounds. Experimental conditions are given in the text.

exchanged for diisopropyl ether (4%). The results of the α-tocopherol determination in this system compared to the one used above agreed within 2.4%. The concentration of α-tocopherol in plasma from fourteen healthy individuals varied between 17.0 and 39.9 μmole/l. This range is in good agreement with that found in the gas chromatographic study [5].

The precision and reproducibility of the method was tested by injection of the same plasma sample three times a day over a period of three days. Small variations in retention times were noticed but this did not disturb the analysis. The mean value of α-tocopherol was found to be 39.9 μmole/l, with an S.D. of 2.31 μmole/l, giving a coefficient of variation of 6.0%.

The lower limit of detection of a sample (5 μl injected) was about 6 μmole/l. The limit could be lowered if α-tocopherol was added to the sample in order to reach the linear range of the method. The recovery of added α-tocopherol to the sample was 100%. The minimum detectable amount of pure α-tocopherol was found to be 8.4 pmole, which value corresponds to twice the noise level.

The method described here is both fast and easy to apply and is suitable for small sample volumes (100 μl). It can be recommended as a complement

to the widely used spectrophotometric method [3] in which compounds such as the carotenoids often interfere with the analysis.

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Book Review

Advances in mass spectrometry in biochemistry and medicine, Vol. II (Proc. 3rd Int. Symp., June, 1975), edited by A. Frigerio, Spectrum Publications/ Halsted (Wiley), New York, 1977, XXII + 609 pp., price £35.00, US$60.00, ISBN 0-470-99039-2.

This volume contains 48 of the papers presented at the symposium organised by Dr. A. Frigerio (Mario Negri Institute, Milan) and held at Alghero, Sardinia, in June, 1975. In addition there are a Foreword by Dr. J. Throck Watson, a subject index and a list of the 151 contributors (in which the addresses ascribed to J. and K. Sjövall should be interchanged).

Most of the papers are based on combined gas chromatography—mass spectrometry (GC—MS), which is largely effected with short (0.5—3 m) packed columns, affording rapid and sensitive analyses but comparatively low separation efficiency. The greatly improved resolving power available with open-tubular columns is exemplified by analyses of tobacco smoke (Chapman and Compson), and especially by the studies, by Delaforge et al., of safrole metabolism in the rat and in cultured rat liver cells.

Applications of deuterium labelling are well represented. Deuterated analogues of compounds to be analysed are used as internal standards for quantitative determinations of drugs such as clonidine (Draffan et al.) and natural metabolites, exemplified by prostaglandins (Watson et al.), steroids (Siekmann et al.) and Krebs cycle acids (Lee and Pollitt). Administration of specifically deuterated compounds allows elucidation of the nature, sequence and rate of metabolic transformations, and this method has been applied, inter alia, to aniline mustard (Cox et al.), pregnane derivatives (Baillie et al.) and homovanillic acid (Sjöquist and Anggard). The stereoselective transfer of deuterium in vivo from chiral ethanol-d_1 to reduced metabolites is reported by Cronholm and Fors. The isopropylidene ketones, pulegone and elemenone, undergo facile exchange of the three *cis*-methyl protons during GC on a deuterated Carbowax column (Talman et al.).

Interesting metabolic pathways reported are the conversion of mephenesin into N-acetyl-β-(*o*-tosyloxy)alanine (Kuhara et al.) and of an N-methyl group into an N-[3-oxobutyl] group (De Ridder).

Other noteworthy contributions involving GC—MS of biological samples include the extension of the automated procedures of Sweeley and co-workers to quantitative analysis; quantitative metabolic "profiling" of urinary organic

acids (Thompson et al.); studies of microsomal metabolites by computerised GC—MS (Thenot et al.); analyses of prostaglandins in femtogram amounts (Wilson et al.); and the identification of epoxide metabolites of dibenzocycloheptene drugs (Belvedere et al.). Peptide sequencing by GC—MS is discussed by Priddle and by Nau, who describes the convenient use of electron capture GC to determine optimal hydrolytic conditions.

Direct probe sampling is used by Marino for N-dansylpeptides, by Durden et al. for N-dansyl derivatives of tetrahydroisoquinoline alkaloids, and by Jackson's group in a substantial paper on electron impact and field desorption spectra of bile pigments.

An important technical development reported by Boettger and co-workers is the electro-optical multichannel ion detection system, which is of great potential value in clinical analysis. An improved mass spectrometer for respiratory gas analysis is also described (Nishi et al.).

Other papers cover a wide range of topics including comparative anion and cation MS, biomedical applications of field ionisation MS, detection of inorganic hydrides, and analyses of pollutants in air and water. One paper reports on the mass spectra of sterol methyl ethers, regrettably without citing the prior work of Idler and of Narayanan; it also depicts an incorrect structure for stigmasterol. With this exception, the standard of the papers in this volume is generally high, and the book is well produced, with few typographical errors. The compilation should be of interest to a wide range of research workers in the area of biomedical MS.

Glasgow (Great Britain) C.J.W. BROOKS

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Book Review

Porphyrins in human diseases (Proc. 1st Int. Porphyrin Meeting, Freiburg i.Br., May 1—4, 1975), edited by M. Doss, S. Karger, Basel, 1976, XX + 512 pp., 108 figs., 143 tables, price DM 192.00, US $ 74.00, ISBN 3—8055—2259—2.

Although disorders of porphyrin biosynthesis (porphyrias) are relatively rare by comparison with many other genetically related diseases, interest in the chemical, biochemical, and clinical aspects of porphyrins has shown a remarkably rapid (and worldwide) growth in the past few years. This is because of the vital role that haemoproteins play in the vast majority of living organisms. The low incidence of porphyrias is not surprising because major defects in such an important biosynthetic pathway are likely to be lethal and thus major hereditary deficiencies will not be transmitted from generation to generation. However, porphyrias may also be caused (or exacerbated) by lead, chlorinated hydrocarbons, or certain types of drugs (including some of those in contraceptive pills) and there is evidence that the incidence of porphyrias of this type is on the increase.

The Freiburg meeting in 1975 was centered around the clinical biochemistry of the porphyrins including both diagnostic aspects and treatment. Some 75 papers were presented by contributors from various parts of the world, with specialist interests ranging from biochemistry to clinical medicine. The main themes included the effects of drugs on porphyrin biosynthesis, regulation of hepatic haeme metabolism, enzymes of the haeme pathway biochemistry of the porphyrias diagnosis and therapy of porphyrias, experimental porphyrias, pharmacology and neurochemistry of porphyrin precursors (ALA and PBG) and the analytical biochemistry of the porphyrias.

Chromatographic methods have played a very important if not essential role in most of the work described. The patterns of porphyrin excretion determined in this way have helped to define the types of porphyrias exhibited by different patients and whether the disease is of genetic origin, or induced by environmental pollutants. Indeed, the last session of the conference was largely devoted to this topic. Paper chromatography was originally the most common technique, but it has now been superceded by thin-layer chromatography, as this is the most rapid and convenient *qualitative* method now available. However, two of the papers in the final section of the book are concerned with the use of high-pressure liquid chromatography for the identification of porphyrin excretion patterns, and this newer technique seems likely to be of increasing value because of its speed, specifity, and resolving power

and the ease with which *quantitative* studies can be made.

The importance of chromatography in studying the metabolism of drugs in environmental pollutants which may cause porphyrias is also recognised, but clearly much more work is required to determine whether or not the original substance or a metabolite is the primary cause of the porphyria. Combined gas chromatography—mass spectrometry has an important role to play in this work as well as in structure determination of porphyrins and their oxidation products.

As a whole the book provides a useful summary of most of the current areas of interest, and will provide a helpful work of reference for most clinical workers in the field. It should also act as a guide to the many as yet unsolved biochemical and clinical aspects of the porphyrias, and it highlights the need for still more refined chromatographic procedures, for example in the separation of porphyrin isomers.

Cardiff (Great Britain) A.H. JACKSON

Fourier Transform N.M.R. Spectroscopy

by **DEREK SHAW,** Varian Associates Ltd., Walton-on-Thames

1976. xviii+358 pages. US $49.75/Dfl. 129.00. ISBN 0-444-41466-5

Nuclear magnetic resonance spectroscopy has grown into a major spectroscopic technique during the past twenty years. This development has had profound effects on organic chemistry and, more recently, biochemistry. In the last few years, NMR itself has undergone a revolutionary change in technique following the realisation in 1966 that pulse excitation followed by Fourier transformation could considerably increase the achievable sensitivity. The increase in sensitivity has especially catalysed the growth of Carbon-13 NMR.

This work is the first to be written using the Fourier approach throughout and will form a suitable text book for students of NMR. Older books based on swept techniques do not provide a suitable basis for understanding and using Fourier NMR spectrometers.

The present book is orientated towards technique rather than applications. The basic theory of NMR is combined with Fourier theory in a unified approach which differs from that taken in other works on high resolution NMR. The middle part of the book is concerned with the practical aspects of Fourier NMR, both instrumental and experimental. The final chapters deal briefly with the general applications of NMR but concentrate strongly on those areas where Fourier NMR can give information not available by conventional techniques.

CONTENTS: Preface. Acknowledgement. Definition of symbols. Chapters: 1. Introduction. 2. Principles of Magnetic Resonance. 3. The Mathematics of Fourier N.M.R. 4. Excitation Techniques in N.M.R. 5. Pulsed N.M.R. 6. Instrumentation. 7. Experimental Techniques. 8. The N.M.R. Spectrum. 9. Multiple Resonance. 10. Relaxation.

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.
4 R. H. Doremus, B. W. Roberts and D. Turnbull (Editors), *Growth and Preparation of Crystals, Proc. Int. Conf. Crystal Growth, Coopertown, N.Y., August 27–29, 1958*, Wiley, New York, 1958.

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