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CHROMBIO. 1707

IDENTIFICATION OF LONG CHAIN DICARBOXYLIC ACIDS IN THE SERUM OF TWO PATIENTS WITH REYE'S SYNDROME

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(Received February 10th, 1983)

SUMMARY

Sera from two patients with Reye's Syndrome were analysed by computerized capillary gas chromatography—mass spectrometry profiling techniques. The most striking abnormalities were the accumulation of long chain dicarboxylic acids. Four saturated dicarboxylic acids (dodecanedioic, tetradecanedioic, hexadecanedioic, and octadecanedioic), and six unsaturated long chain dicarboxylic acids (dodecenedioic, tetradecenedioic, tetradecadienedioic, hexadecenedioic, octadecadienedioic, and octadecenedioic) were identified. The C_{14} and C_{18} dicarboxylic acids have never been reported for Reye's Syndrome or any other dicarboxylic acidemias. The data might reflect marked increase of extramitochondrial ω-oxidation of long chain fatty acids or impaired metabolism of ω-dicarboxylic acids formed in Reye's patients.

INTRODUCTION

Reye's Syndrome, first reported as an acute life-threatening disease in 1963 [1] is characterized by rapidly developing encephalopathy and fatty infiltration (microvesicular steatosis) of the liver and kidney. A viral prodrome, followed by vomiting and acute encephalopathy without focal neurological signs or jaundice suggests Reye's Syndrome. While its etiology and pathogenesis remain obscure, swollen, injured mitochondria with disorganized cristae represent the major subcellular abnormality [2].

Blood and urine samples from healthy individuals contain very small quantities of short chain aliphatic dicarboxylic acids (C_6—C_8). Longer chain dicarboxylic acids (C_6—C_{14}) have been noted in only a small number of pathologic conditions generally termed the dicarboxylic acidurias [3—9]. Among the more prevalent disease processes wherein short chain dicarboxylic acidurias have been described are diabetic ketoacidosis and neonatal lactic acidosis

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associated with hypoglycemia. Other rare dicarboxylic acidurias include systemic carnitine deficiency [6], suberylglycinuria [7], hypoglycin toxicity (Jamaican Vomiting Sickness) [8], ketotic dicarboxylic aciduria [9], and non-ketotic dicarboxylic aciduria [10]. It is interesting to note that certain dicarboxylic acidurias have features that resemble Reye's Syndrome (i.e., encephalopathy and fatty degeneration of the liver).

Recently, two groups reported the presence of medium chain dicarboxylic acids in Reye's Syndrome (adipic, suberic and sebacic acids) [11, 12]. We now confirm the finding of suberic and sebacic acids and report the discovery of several long chain dicarboxylic acids from the sera of two cases of Reye's Syndrome. These long chain dicarboxylic acids have not been previously reported in Reye's Syndrome. Some of them have also not been reported in any other cases of dicarboxylic acidurias.

Case presentations

Patient 1. J.M., a 7-year-old girl admitted to the Children's Hospital, Columbus with encephalopathy and liver failure, was clinically diagnosed as having Reye's Syndrome (stage I) [13]. Prior to admission she had a prodrome of chicken pox and had taken aspirin, 5 g/day for four days. Initial serum levels of glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were 859 and 1476 units, respectively. Serum ammonia was at 165 μg/dl and glucose at 70 mg/dl. Amino acid pattern appeared normal with the exception of lysine and glutamine which were slightly elevated. She was alert and much improved after 72 h of intensive supportive treatment. Upon discharge from the hospital after 7 days, she was asymptomatic.

Patient 2. T.R., a 6-year-old girl admitted to the Children's Hospital, Columbus because of the acute onset of severe encephalopathy and liver failure was diagnosed as having Reye's Syndrome (stage II). Prior to admission, she had an acute upper respiratory illness. While treatment with aspirin was initiated, the quantity remains uncertain. Initial levels of SGOT and SGPT were 155 and 248 units, respectively. However, these values reached over 1000 units toward the end of the illness. Initial serum levels of ammonia and glucose were 495 μg/dl and 159 mg/dl, respectively. A salicylate level determined by the Trinder test was 12 mg/dl. Liver biopsy showed mitochondria swelling and fatty degeneration compatible with Reye's Syndrome. Despite intensive supportive efforts, her illness rapidly progressed to stage V and she died seven days after admission.

Two ml of whole blood were obtained from each of the above patients shortly after admission. Serum samples were frozen immediately at −20°C until thawed for processing. The clinical protocol used in this study was approved by our university human investigation committee.

MATERIALS AND METHODS

Reagents

The organic solvents used for extraction were either of nanograde quality and purchased from Mallinckrodt (St. Louis, MO, U.S.A.) or Omnisolv glass-distilled from MCB Manufacturing Chemists (Cincinnati, OH, U.S.A.). Tri-

methylsilylating agent, N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.). Authentic organic acids were obtained from Sigma (St. Louis, MO, U.S.A.), Analabs (North Haven, CT, U.S.A.), and Applied Science Labs. (State College, PA, U.S.A.).

Extraction of samples

Serum samples from Patients 1 and 2, each of 0.8 ml, were extracted, derivatized and analyzed according to procedures slightly modified from those described previously [14]. To each sample, an internal standard (phenyl-d_5-mandelic acid) was added to obtain a concentration of 1.5 μg/ml. Three volumes of preextracted saturated sodium borate buffer were added both for salting effect and for adjusting the pH to a value of 8.5—9.0. Five ml of dichloromethane were added, and the mixture was hand-shaken vigorously for 15 sec in a separatory funnel. The emulsion-like content was drained into a pyrex tube, and centrifuged at 1400 *g* for 10 min. The upper aqueous layer was transferred with a pasteur pipette into the separatory funnel to be reextracted two more times with dichloromethane to remove neutral and basic components. The pH of the aqueous fraction was adjusted to 1.0 by adding 3 *N* hydrochloric acid. The acidified aqueous fraction was then extracted three times with 5 ml of dichloromethane. Each extraction was followed by centrifugation at 1400 *g* for 10 min. The pooled dichloromethane extracts containing organic acids were concentrated at 40°C to a volume of about 1 ml with a rotary evaporator. The extract was then transferred to a small reaction vial and blown dry with purified dry nitrogen at 35—40°C.

Gas chromatographic—mass spectrometric analysis of samples

One μl of triethylamine and 20 μl of BSTFA were added to the dried acidic extracts. The cap was immediately secured and the vial was vortexed for 15 sec. The sealed vial was then heated at 70°C for 30 min. The sample was allowed to cool prior to gas chromatographic—mass spectrometric (GC—MS) analysis.

Samples were analysed with a quadrupole mass spectrometer (Hewlett-Packard 5985 GC—MS system) equipped with a 5840A HP gas chromatograph and a 21MX E-series computer. A bonded-phase fused silica capillary column (30 m × 0.32 mm, DB-1, 1 μm thickness, J & W Scientific Co.) was used, with helium as carrier gas and column head pressure at 1.5 bar (23 p.s.i.). The GC—MS analyses were performed under the following conditions: The temperature of the injection port, the GC—MS interface and the ion source were set at 320°C, 320°C and 200°C, respectively. A splitless mode of injection was used with a sample size of 3 μl. The temperature for GC analyses was programmed from 70°C, with a delay of 5 min, and then increased to 310°C at 3°C/min. Mass spectral data were acquired utilizing electron impact at 70 eV and an electron multiplier voltage of 3000 V.

RESULTS

The GC—MS profile of trimethylsilylated derivatives of long chain fatty acids from Reye's Patients 1 and 2 are presented in Figs. 1 and 2, respectively. The

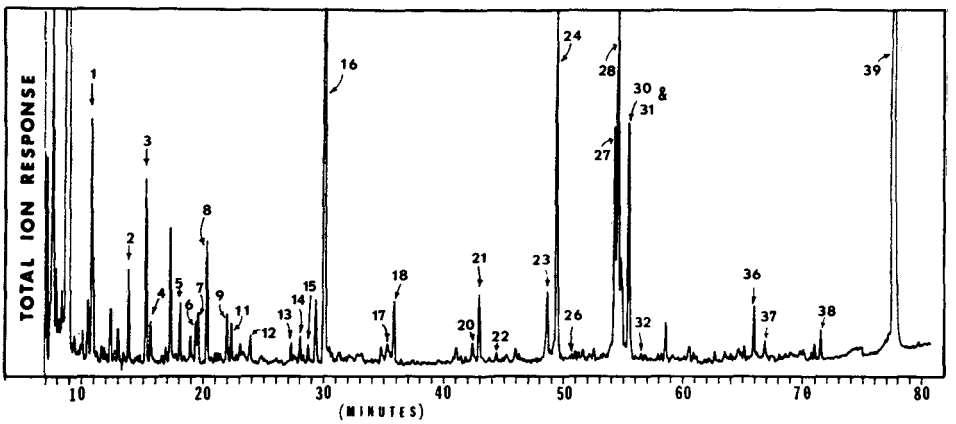

Fig. 1. GC—MS profile of trimethylsilylated serum long chain fatty acids from Patient 1. The ordinate represents total ion response with the most intense component normalized as 100%. The abscissa represents the time axis. GC—MS conditions are described in the text. The numbered peaks are explained in Table I.

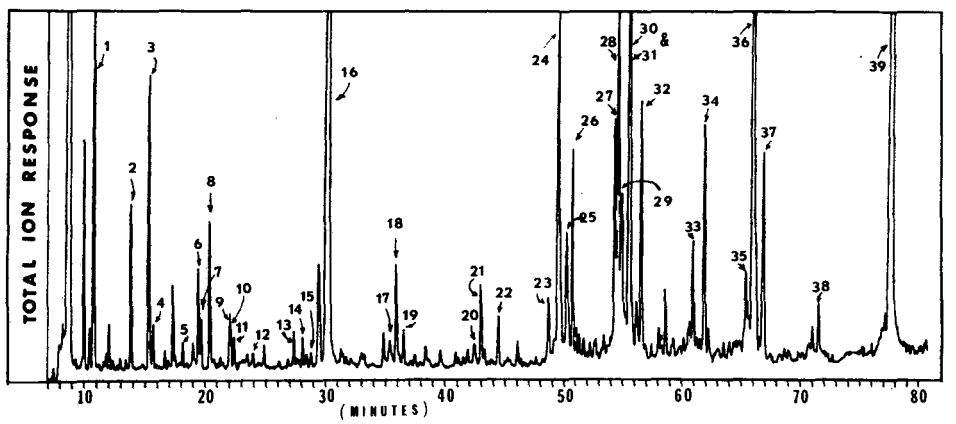

Fig. 2. GC—MS profile of trimethylsilylated serum long chain fatty acids from Patient 2.

identification of the major peaks in these profiles is provided in Table I. Each profile represents a continuous plot of the total ion current on the ordinate, normalized by the computer to 100% for the most abundant component. The abscissa represents the time axis. Mass spectral data acquisition was delayed for 7 min to vent off derivatizing agents.

The serum profiles determined for both Patient 1 and Patient 2 contain the normal, short chain hydroxy- and keto-acids, as well as fatty acid routinely observed in our GC—MS laboratory. These include lactic, 2-hydroxy-butyric, 3-hydroxy-butyric, 2-hydroxy-isovaleric, 2-ketoisocaproic, 2-ketovaleric, 2-keto-3-methylvaleric, 2-ketocaproic, nonanoic, decenoic, decanoic, lauroleic, lauric, myristoleic, myristic, palmitoleic, palmitic, linoleic, oleic, and stearic acids. In addition the profile of patients 1 and 2 contain salicylic acids at concentrations of 9 mg/dl and 12 mg/dl, respectively.

However, the fatty acid profile from Patient 1 (Stage I Reye's Syndrome)

TABLE I

IDENTIFICATION OF MAJOR PEAKS IN THE GC—MS PROFILES OF TMS DERIVATIVES OF LONG CHAIN FATTY ACIDS FROM REYE'S PATIENTS 1 AND 2

Peak No.	Compound	Patient 1	Patient 2
1	Lactic-di-TMS	+	+
2	2-OH-Butyric-di-TMS	+	+
3	3-OH-Butyric-di-TMS	+	+
4	2-OH-Isovaleric-di-TMS	+	+
5	Benzoic-TMS	+	+
6	2-Ketoisocaproic-di-TMS	+	+
7	2-Ketovaleric-di-TMS	+	+
8	Phosphoric-tri-TMS	+	+
9	2-Keto-3-methyl-valeric-di-TMS	+	+
10	Glutaric-di-TMS	ND*	+
11	2-Ketocaproic-di-TMS	+	+
12	Nonanoic-TMS	+	+
13	Decenoic-TMS	+	+
14	Decanoic-TMS	+	+
15	Phenyl-d_5-mandelic-di-TMS (I.S.)**	+	+
16	Salicylic-di-TMS	+	+
17	Lauroleic-TMS	+	+
18	Lauric-TMS	+	+
19	Suberic-di-TMS	ND	+
20	Myristoleic-TMS	+	+
21	Myristic-TMS	+	+
22	Sebacic-di-TMS	+	+
23	Palmitoleic-TMS	+	+
24	Palmitic-TMS	+	+
25	Dodecenedioic-di-TMS	ND	+
26	Dodecanedioic-di-TMS	+	+
27	Linoleic-TMS	+	+
28	Oleic-TMS	+	+
29	Tetradecadienedioic-di-TMS	ND	+
30	Stearic-TMS	+	+
31	Tetradecenedioic-di-TMS	+	+
32	Tetradecanedioic-di-TMS	+	+
33	Hexadecenedioic-di-TMS	ND	+
34	Hexadecanedioic-di-TMS	ND	+
35	Octadecadienedioic-di-TMS	ND	+
36	Octadecenedioic-di-TMS	+	+
37	Octadecanedioic-di-TMS	+	+
38	Cholestadiene	+	+
39	Cholesterol	+	+

*ND = not detected.
**I.S. = internal standard.

also contains six dicarboxylic acids [i.e. sebacic ($C_{10:0}$), dodecanedioic ($C_{12:0}$), tetradecenedioic ($C_{14:1}$), tetradecanedioic ($C_{14:0}$), octadecenedioic ($C_{18:1}$), and octadecanedioic ($C_{18:0}$) acids]. Interestingly, the profile of Patient 2, who was more seriously ill with Reye's Syndrome upon admission, included long chain saturated and unsaturated ω-dicarboxylic acids that are not normally observed in the serum of healthy individuals. These are: suberic ($C_{8:0}$), sebacic, dodecenedioic ($C_{12:1}$), dodecanedioic ($C_{12:0}$), tetradecadienedioic ($C_{14:2}$), tetradecenedioic, tetradecanedioic, hexadecenedioic ($C_{16:1}$), hexadecanedioic ($C_{16:0}$), octadecadienedioic ($C_{18:2}$), octadecenedioic, and octadecanedioic acids. These saturated compounds were confirmed by comparison of the MS data and GC retention time obtained with those of authentic compounds. The unsaturated long chain dicarboxylic acids were identified by their mass spectral patterns in comparison with their saturated analogues, but await the synthesis of the authentic compounds for MS confirmation.

The profile from Patient 2 who died of Reye's Syndrome differs from that of Patient 1 who recovered from the disease in the following aspects: (A) Patient 2 had a much higher level of sebacic, dodecanedioic, tetradecenedioic,

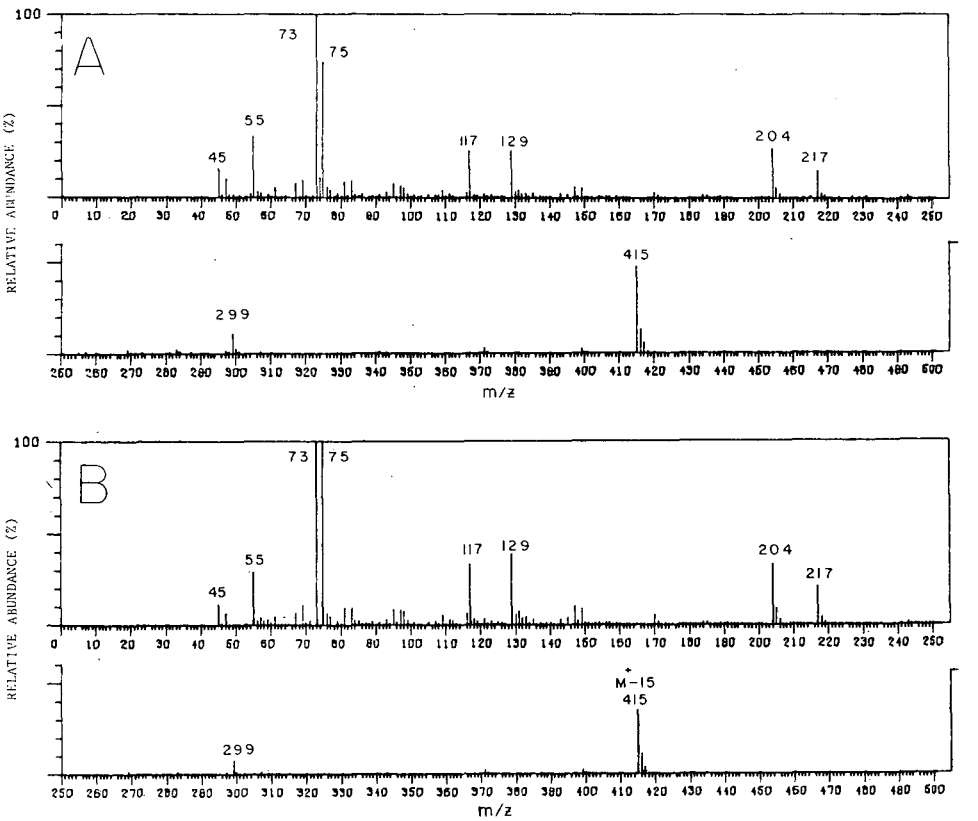

Fig. 3. Mass spectrum of the trimethylsilyl derivative of hexadecanedioic acid obtained from the serum of Patient 2 is presented in the upper panel (A). The mass spectrum of the derivatized authentic hexadecanedioic acid is presented in the lower panel (B).

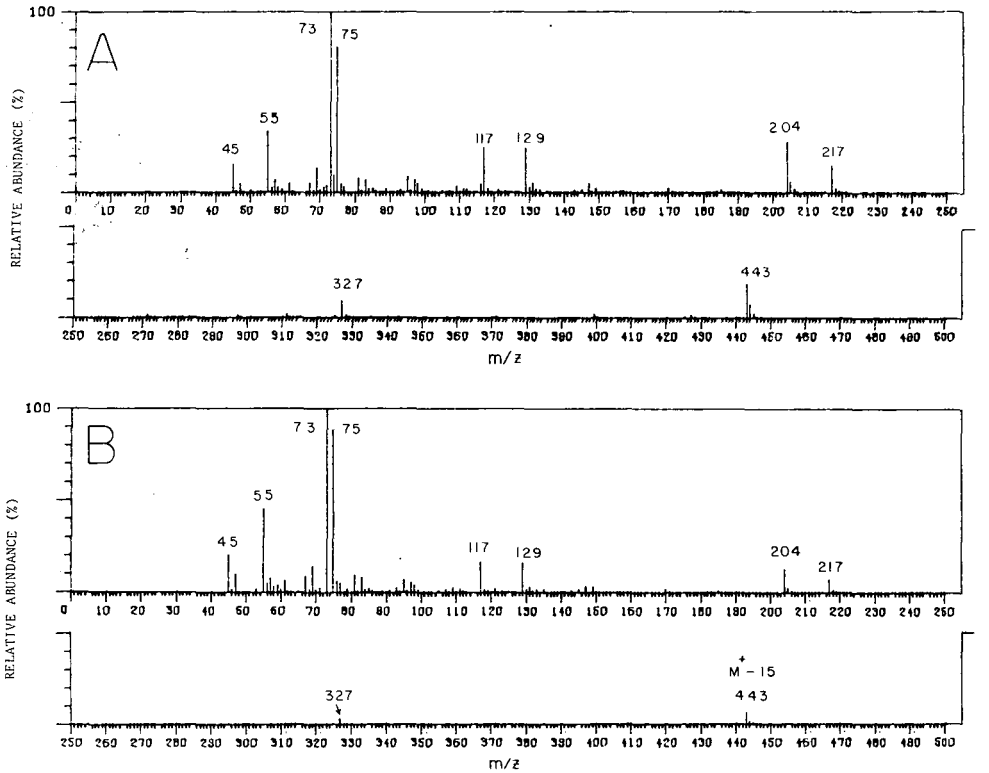

Fig. 4. Mass spectrum of the trimethylsilyl derivative of octadecanedioic acid obtained from the serum of Patient 2 is presented in the upper panel (A). The mass spectrum of the derivatized, authentic octadecanedioic acid is presented in the lower panel (B).

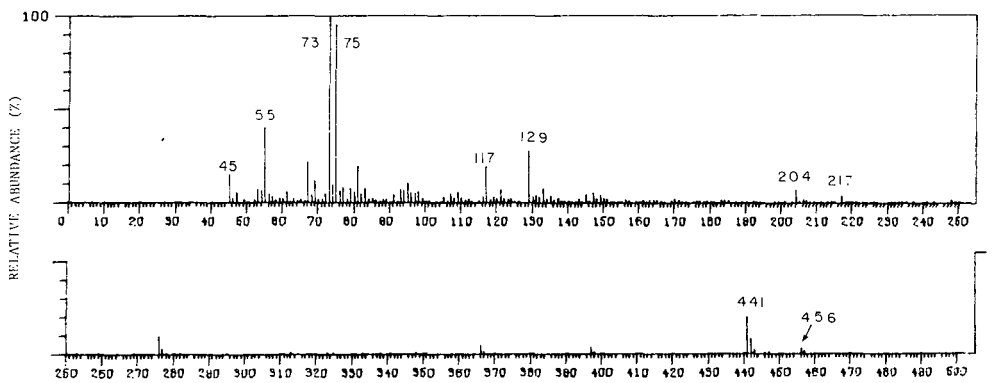

Fig. 5. Mass spectrum of trimethylsilyl derivative of octadecenedioic acid obtained from the serum of Patient 2.

tetradecanedioic, octadecenedioic, and octadecanedioic acids than Patient 1. (B) Patient 2 had certain long chain dicarboxylic acids not detected in Patient 1, namely, dodecenedioic, tetradecadienedioic, hexadecenedioic, hexadecanedioic, and octadecadienedioic acids.

Since hexadecanedioic and octadecanedioic acids have not been previously

TABLE II

MASS SPECTRAL ION IDENTIFICATION (*m/z*)

Fragment	Ion $C_{16:0}$ (Fig. 3)	Ion $C_{18:0}$ (Fig. 4)	Ion $C_{18:1}$ (Fig. 5)
M^+	(430)*	(458)*	456
$M^+ - 15$ ($-CH_3$)	415	443	441
$M^+ - 59$ ($-CO_2$-CH_3)	—	—	397
$M^+ - 90$ (— TMS-OH)	—	—	366
$M^+ - 131$ ($CH_2COOTMS$)	299	327	—
$M^+ - 180$ (— 2 TMS-OH)	—	—	276
$TMS\text{-}O\text{-}C(OTMS)^+\text{=}CH_2$	204	204	204
$TMS\text{-}O\text{-}C(=\overset{+}{O}TMS)\text{-}CH\text{=}CH_2$	217	217	217
$H_2C\text{=}C(\overset{+}{O}H)\text{-}OSi(CH_3)_2$	117	117	117
$[(CH_3)_2SiOH]^+$	75	75	75
$[TMS]^+$	73	73	73

*Very weak intensity.

found in human serum or urine, the mass spectra of the trimethylsilyl derivatives of these compounds, together with those of authentic compounds, are presented in Figs. 3 and 4, respectively. In each of these figures, the upper panel, A, represents the spectrum of the compound detected in the serum sample, and the lower panel, B, represents the spectrum of the authentic sample. To illustrate the mass spectra of trimethylsilylated long chain unsaturated dicarboxylic acids, we have presented the mass spectrum of octadecenedioic acid in Fig. 5. Significant ions are explained in Table II.

DISCUSSION

Although an abnormal accumulation of several monocarboxylic acids [15] and certain small to medium chain dicarboxylic acids have been reported in Reye's Syndrome, the long chain dicarboxylic acids described herein were not noted previously (peak Nos. 25, 26, 29, and 31—37 in Table I).

Recent technical improvements [16, 17] (use of long, specially coated, open tubular fused silica capillary columns in place of short, packed, large bore chromatographic columns) and the use of a less polar, but more selective extraction solvent (dichloromethane instead of either ethyl acetate or diethyl

ether) aided significantly in the separation and identification of these dicarboxylic acids.

It is generally recognized that the hepatic mitochondrial damage readily apparent in Reye's Syndrome would inhibit both β-oxidation and oxidative phosphorylation processes. Normal β-oxidation is a well defined cyclic process that sequentially cleaves fatty acid chains by two carbon units to yield cellular energy. Since this capacity for β-oxidation is markedly impaired in Reye's Syndrome, extramitochondrial ω-oxidation may be stimulated with the enhanced production of several long chain dicarboxylic acids that we have noted in these two patients. Alternatively, this could be due to impaired metabolism of dicarboxylic acids in Reye's patients. Also, ω-oxidation is not dependent upon carnitine. Carnitine deficiency cannot be ruled out in these two patients with Reye's Syndrome. However, elevated concentrations of pimelic and heptenedioic acids associated with carnitine deficiency were not observed in these patients [6].

It will be important to determine whether this finding is either specific for Reye's Syndrome or is a common, but heretofore unrecognized concomitant to severe mitochondrial disease. The toxicity of these long chain saturated and unsaturated dicarboxylic acids in humans is not known. Mortensen and Gregersen [18] fed C_8—C_{16} dicarboxylic acids to three groups of rats: unstarved, starved and diabetic rats. Only the group of rats which were starved for 48 h and then fed with hexadecanedioic acid died within 24 h. This probably indicates that hexadecanedioic acid is toxic to rats in a starved state. The study of Mortensen and Gregersen [18] could be construed to indicate that the nutritional status of the organism affects the severity of the toxic influence of hexadecanedioic acid on the organism. The toxicity of these long chain saturated and unsaturated dicarboxylic acids to Reye's Syndrome patients with vomiting and poor nutritional status remains to be determined.

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CHROMBIO. 1702

GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF THE LOWER VOLATILE ALCOHOLS IN RAT BLOOD AND IN HUMAN STOOL SPECIMENS ON A FUSED SILICA CAPILLARY COLUMN

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SUMMARY

A method is described for the simultaneous quantitation of the lower volatile alcohols in stool specimens and rat blood. The addition of potassium carbonate to the assay mixture markedly increased the sensitivity in the detection of these compounds. The method is shown to be simple and reproducible and is suitable for following the metabolism of ethanol in human stool specimens.

INTRODUCTION

We have been investigating the possibility that the metabolism of the colonic flora may influence the host's response to ethanol. Of particular interest has been the detection of 2-propen-1-ol (allyl alcohol)* during the metabolism of ethanol by human stool specimens [1]. Numerous gas chromatographic (GC) methods have been described for the measurement of alcohols in the blood and in other biological tissues. However, these methods were primarily adapted to the analysis of blood ethanol [2—5] and/or its metabolite, acetaldehyde [6—8], which precluded the determination of other volatile alcohols that may be present at much lower concentrations than ethanol. Volatile alcohols have been observed in the urine of diabetic patients [9, 10] and in some blood samples [11—13]. The quantitation of the aliphatic alcohols in serum and urine of these subjects was recently accomplished by GC—mass fragmentography [10]. The availability of such sophisticated instrumentation is not universal. Therefore, this paper describes a simple GC method for simul-

*The common names of some of the alcohols will be used in the text.

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taneous quantitation of the lower alcohols in stool specimens and rat blood in the presence of large quantities of ethanol.

EXPERIMENTAL

Reagents and chemicals

Standard solutions were prepared from dehydrated reagent-grade ethanol (U.S. Industrial Chemical Co., Tuscola, IL, U.S.A.), *n*-butanol (Mallinckrodt, St. Louis, MO, U.S.A.), *n*-propanol, *n*-pentanol, 2-propen-1-ol and 3-methyl-1-butanol (all from Fisher Scientific, Fair Lawn, NJ, U.S.A.). The internal standard was prepared from an aqueous stock solution containing 2000 nmol/ml of reagent grade 3-methyl-1-butanol (isoamyl alcohol)*.

INSTRUMENTATION

A Hewlett-Packard (HP) 5880A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a flame ionization detector, a column compensation unit, a cryogenic attachment, and a terminal was used. A fused silica capillary column (50 m × 0.2—0.21 mm I.D.) coated with Carbowax 20M (HP) was used. The chromatograph was operated isothermally at 20°C for 6 min, programmed at 5°C/min to 40°C followed by a 10°C/min increase to 90°C and maintained at this temperature for a further 5 min. The chromatograph oven was cooled with liquid carbon dioxide.

The instrument used for the mass spectral analysis was a HP 5992B GC—mass spectrometric (MS) unit with a 50-m fused silica capillary column as above. The separation was carried out isothermally at 20°C for 6 min, programmed at 10°C/min to 90°C and kept at this temperature for an additional 10 min. The oven was cooled with liquid carbon dioxide. The spectra were obtained at 23 eV.

Quantitation

Isoamyl alcohol was used as the internal standard since no endogenous isoamyl alcohol could be detected in the rat blood or in the human stool specimens and no isoamyl alcohol was detected during the anaerobic metabolism of ethanol by stool specimens. External standards for the volatile alcohol mixtures were prepared in distilled water in various concentrations from 250 to 2500 nmol/ml. The retention time of each of the alcohols was verified by injecting aliquots of each standard directly into the gas chromatograph. They were further verified by GC—MS by their abundance *m/e* peaks of 31, 31, 57, 56, 55, and 55 respectively for the alcohols as they were eluted from the GC column (see Fig. 2). The peak areas for each standard were automatically quantitated with the HP programmable GC terminal (5880A).

For recovery studies, rat blood was diluted 1:1 with cold physiological saline, then with an equal volume of isoamyl alcohol as internal standard (2000 nmol/ml). Stool samples were prepared by thoroughly mixing 1 g of the stool specimen (wet weight) with 2 ml of 0.1 *M* phosphate buffer in saline (pH 7.4),

*The common names of some of the alcohols will be used in the text.

followed by equal portions of the isoamyl alcohol internal standard as above.

For GC analyses, a 0.2-ml aliquot of the above sample was added to a 9-ml septum bottle containing 200 mg of anhydrous potassium carbonate (previously dried overnight at 100°C), the bottle was immediately sealed with a rubber stopper, heated in a water bath with constant stirring for 20 min at 70 ± 2°C. A 0.2-ml aliquot of gas was withdrawn through the stopper with a 1.0-ml gas-tight A-2 Pressure-Lok syringe (Precision Sampling, Baton Rouge, LA, U.S.A.) and injected directly into the gas chromatograph.

Incubation of the stool specimens with ethanol was carried out anaerobically in a 50-ml rubber stoppered Erlenmeyer flask. The stool sample in 0.1 *M* phosphate buffer in saline (pH 7.4) was deaerated in the presence of carbon dioxide and then incubated with 0.1% (v/v) ethanol. Aliquots of the reaction mixture were taken at various time intervals and assayed as above on the gas chromatograph.

RESULTS AND DISCUSSION

Very low concentrations of alcohols in biological fluids may be difficult to quantitate by GC because of the incomplete four-variable recovery of these compounds from the gas phase [13]. The vapor pressures of the alcohols can, however, be substantially increased by the addition of different salts to the alcoholic solutions [3, 14]. Anhydrous potassium carbonate was selected as the salt of choice in this present method because of its greater ability to increase the vapor pressure of these alcohols [3]. As indicated in Fig, 1. the release of the various alcohols into the vapor phase was 2—10 times greater in the presence of the salt than in its absence. The strong affinity of water for each alcohol apparently limits the release of these alcohols into the vapor phase [15].

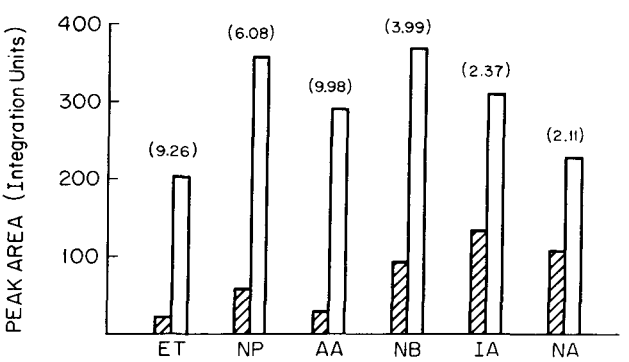

Fig. 1. Recovery of alcohols from aqueous solutions in the presence (□) and absence (▨) of potassium carbonate. Concentration of each alcohol at 1000 nmol/ml. ET = ethanol; NP = *n*-propanol; AA = 2-propen-1-ol (allyl alcohol); NA = *n*-pentanol (*n*-amyl alcohol). Values in parentheses denote the recovery enhancement factor in the presence of potassium carbonate.

The retention times of the alcohols were sufficiently separated to allow good baseline resolution (see Fig. 2). Reproducibility of the method was measured from 22 separate determinations of the external standard alcohol mixture (con-

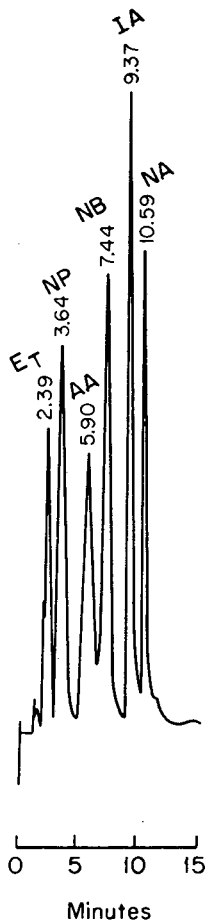

Fig. 2. Gas chromatogram of a standard alcohol mixture. Concentration of each alcohol at 1000 nmol/ml in aqueous solution. Abbreviations as in Fig. 1. Numbers at each peak denote the retention times of the individual alcohols.

taining 1000 nmol/ml of each) over a period of 3—4 weeks. The standard error of mean ranged from 1.3 to 2.8% for these alcohols.

Standard curves (Fig. 3), prepared using 250—2500 nmol/ml of the alcohols in aqueous solutions as a mixture, yielded straight lines through the origin when peak area units were plotted against the concentrations of the alcohols. For the detection of ethanol, this represents a 5- to 10-fold increase in sensitivity as compared to those previously reported by a GC method [7, 8].

As seen in Table I, the recovery of known amounts of ethanol added to rat blood was 104.8 ± 3.0% (mean ± S.E.M.). Trace amounts of ethanol only were detected in the normal rat blood. Consequently, no corrections for endogenous ethanol content were made in the recovery studies (Table I). In absence of potassium carbonate, however, the recovery of ethanol from the rat blood was less than 30%. Concentrations below 1000 nmol/ml of ethanol were barely detectable and could not be quantitated.

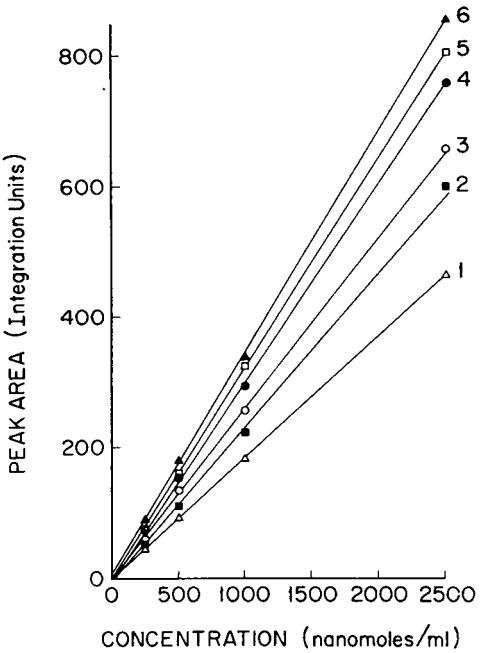

Fig. 3. Plot of peak area vs. concentration of the lower volatile alcohols in aqueous solutions. 1 = Ethanol; 2 = *n*-pentanol (*n*-amyl alcohol); 3 = 2-propen-1-ol (allyl alcohol); 4 = 3-methyl-1-butanol (isoamyl alcohol); 5 = *n*-propanol; 6 = *n*-butanol.

TABLE I

RECOVERY OF ETHANOL ADDED TO RAT BLOOD

Isoamyl alcohol was used as the internal standard.

Amount added (nmol)	Amount recovered			
	With potassium carbonate		Without potassium carbonate	
	nmol ± S.E.M.	% ± S.E.M.	nmol ± S.E.M.	% ± S.E.M.
543	591 ± 16	108.6 ± 2.9	trace	0
1085	1094 ± 35	100.8 ± 3.2	287 ± 24	26.3 ± 2.1
10,850	11,144 ± 277	102.6 ± 2.5	2954 ± 159	27.2 ± 1.4
21,700	23,221 ± 706	107.1 ± 3.2	6615 ± 242	30.4 ± 1.4

The possibility that the presence of other alcohols in the mixture would affect the release of any of the alcohols at various concentrations from stool specimens was also tested. The endogenous content of the volatile alcohols in stool specimens from 21 normal human subjects were as follows: ethanol, 21—549 nmol/g (wet weight) with a mean of 134 ± 31 (S.E.M.); *n*-propanol, 0—330 nmol/g with a mean of 37 ± 15; *n*-butanol, 0—86 nmol/g with a mean of 16 ± 4; and *n*-amyl alcohol, 0—13 nmol/g with a mean of 1.3 ± 0.6. Trace amounts of isobutanol and allyl alcohol were observed in few samples. How-

TABLE II

RECOVERY OF THE LOWER VOLATILE ALCOHOLS ADDED TO HUMAN STOOL SPECIMENS

Isoamyl alcohol was used as the internal standard. Alcohols were added to the stool specimens as a single mixture.

Amount added (nmol)	Amount recovered			
	Ethanol		*n*-Propanol	
	nmol ± S.E.M.	% ± S.E.M.	nmol ± S.E.M.	% ± S.E.M.
250	259 ± 4.6	103.8 ± 1.8	269 ± 4.8	107.9 ± 1.9
500	500 ± 11	100.0 ± 2.2	472 ± 8	94.6 ± 1.5
1000	952 ± 18	95.2 ± 1.8	996 ± 22	99.6 ± 2.2
2000	1948 ± 32	97.3 ± 1.5	1955 ± 32	97.6 ± 1.6

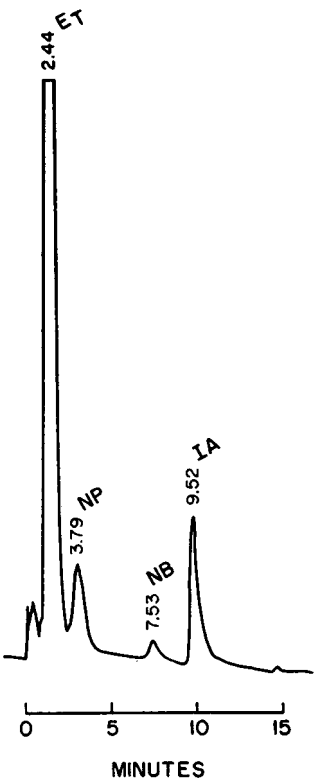

Fig. 4. Gas chromatogram of a typical run during the anaerobic metabolism of ethanol by a normal human stool specimen. Abbreviations as in Fig. 1.

ever, only trace amounts of ethanol were detected in the specimens used in the recovery studies (Table II). Therefore, corrections for the endogenous content of these alcohols in the stool specimens are not included in Table II. As shown

2-Propen-1-ol (allyl alcohol)		*n*-Butanol		*n*-Pentanol (*n*-amyl alcohol)	
nmol ± S.E.M.	% ± S.E.M.	nmol ± S.E.M.	% ± S.E.M.	nmol ± S.E.M.	% ± S.E.M.
240 ± 5.3	96.1 ± 2.1	254 ± 4.7	101.5 ± 1.9	258 ± 3.6	103.1 ± 1.4
487 ± 9	96.2 ± 2.1	491 ± 7	98.3 ± 1.5	498 ± 6	99.2 ± 1.3
1039 ± 24	103.9 ± 2.4	1101 ± 16	110.1 ± 1.9	998 ± 15	98.8 ± 1.5
1922 ± 39	96.1 ± 2.0	1856 ± 24	92.9 ± 1.2	1893 ± 39	94.7 ± 2.0

in Table II, the presence of these alcohols as a mixture in human stool specimens did not interfere with the recovery of each of the individual alcohols. The apparent recovery for these volatile alcohols ranged from 98.1 to 100.7% with standard error of mean of 1.6—2.2%. Although not shown, similar recoveries were observed when varying amounts of the alcoholic mixture were added to rat blood. Excessive amounts of ethanol did not interfere with the quantitation of other alcohols. A typical chromatogram obtained during an anaerobic metabolism of ethanol by a stool specimen shows that *n*-propanol is adequately separated form a large excess of ethanol to allow quantitation (Fig. 4). In this instance, the amount of ethanol (approximately 40 μmol/ml) was 32 times as much as *n*-propanol.

During the anaerobic metabolism of ethanol by a normal human stool specimen, *n*-propanol, *n*-butanol and *n*-amyl alcohol were formed (Fig. 5). Although not shown here, allyl alcohol was also noted in lesser amounts in some specimens. Trace amounts of isobutanol were detected in few samples.

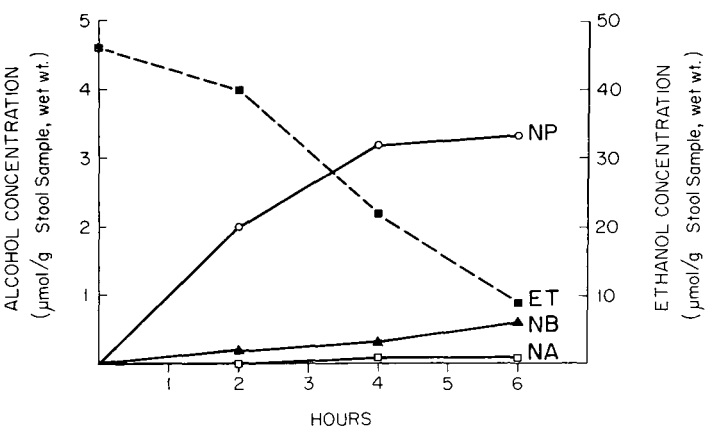

Fig. 5. Formation of various alcohols during the anaerobic metabolism of ethanol by a normal human stool specimen. Abbreviations as in Fig. 1.

Isobutanol elutes prior to allyl alcohol and can interfere with its determination. However, the amount of isobutanol found in these samples was not significant to hinder the quantitation of allyl alcohol. The identity of the formation of these alcohols was further confirmed by GC—MS. Implications of these findings are discussed elsewhere [1].

The advantages of the method reported here are its simplicity, reproducibility and accuracy. Utilizing this method, simultaneous quantitation of the lower volatile alcohols is now attainable in the presence of a large excess of ethanol.

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GAS—LIQUID CHROMATOGRAPHY OF FREE AMINO ACIDS IN THE CYTOSOL OF MAMMALIAN ATRIUM AND VENTRICLE OF THE HEART

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SUMMARY

N-Trifluoroacetyl *n*-butyl ester derivatives of amino acids were studied. The investigations, carried out on random slaughter-house material, were based on the results of histological and biochemical differences between the atrium and ventricle of the mammalian heart muscle. The data indicate a high level of glutamic acid, and a predominance of tyrosine in the atrium and phenylalanine in the ventricle. The results provide evidence for metabolic differences between the atrium and the ventricle of the heart. This finding may have some significance in heart muscle defects.

INTRODUCTION

Gehrke and Lamkin [1] developed the basis of amino acid derivatization which led to the development of methods for the investigation of volatile derivatives. Amino acids require derivatization of the functional groups before their analysis so that they can be separated by gas—liquid chromatography (GLC). A detailed review of this problem was published by Hušek and Macek [2].

In our investigations we used the technique of Gehrke and co-workers [3, 4], who found that separation on two columns was adequate for quantitative amino acid determinations. The work of Casagrande [5], Raulin et al. [6], Amico et al. [7], Adams [8] and Pellizzari et al. [9] was also very helpful with regard to the analysis of non-protein amino acids. The function and role of amino acids in metabolic processes were considered in detail by Meister [10].

In our previous work [11—13] we separated amino acids in mammalian tissues by GLC, and determined the amino acid composition of the tissues. The aim of this work was to indicate the quantitative difference in the amino

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acid composition in the atrium and ventricle of mammalian heart muscle. We based this investigation on results of histological pictures of muscular cells of the atrium and ventricle, from which we concluded that contractile cells of the atria differed from those of the ventricle in the following respects: (1) a smaller diameter (5—6 μm) and shorter length; (2) a less developed T system, or a lack of it; (3) the presence of grains of diameter 300—400 μm, which showed ATPase activity; (4) polar localization of mitochondria; (5) cytoplasm containing a greater number of thick, flattened vessicles; and (6) a well developed Golgi apparatus.

The results of biochemical investigations carried out by Rumyantsev [14, 15] with experimentally administered [^{3}H] thymidine indicate that the number of isotope impulses and mitotically divided cell nuclei in myocytes of the atrium of rats affected by acute left ventricular infarct is greater than that in the perinecrotic ventricular myocardium. In this work we intended to demonstrate that the quantitative amino acid composition of the atrial muscle differs from that of the ventricular muscle.

Literature data provide valuable information on the participation of the amino acid pool of the heart muscle in myogenesis and regeneration of muscular fibres [16, 17], hypertrophy of myocardium [18—20] and protein synthesis in sarcolemma [21, 22]. The important role of alanine [23, 24] in processes of glucogenesis, taurine [25] in the increased influx of amino acids, creatine in energetic processes [26, 27] and oxidation of leucine [28] in enzymatic changes in the heart muscle has been emphasized. The speed, sensitivity and high precision of GLC were advantageous in finding significant differences in the quantitative amino acid composition of the heart muscle of random animal material obtained from the slaughter-house at Bytom.

EXPERIMENTAL

Animals

The sample material was taken from pigs, sheep and cows at the time of slaughter. The purpose of such a choice was to investigate material from animals subjected to different feeding conditions. From each animal examined about 20 g of tissue were taken.

Apparatus

The following equipment was used: glass homogenizer (25 ml capacity) with a PTFE piston, K-24 and VAC 601 centrifuges produced by Janetzki Heinz (Ilmenau, G.D.R.), ion-exchange columns (150 × 12 mm I.D. and 300 × 15 mm I.D.), lyophilizing apparatus produced by VEB MLW Labortechnik (Engelsdorf, G.D.R.), reaction vessels of our own design equipped with PTFE-lined screw-caps, an ultrasonic cleaner produced by Bronson, Smith Kline Co. (Shelton, CN, U.S.A.), oil-baths with a thermoregulator, an evaporator produced by Pierce (Rockford, IL, U.S.A.), a gas chromatograph (Varian 3700) with a CDS 111 C data analyzer and A 25 recorder (Varian, Palo Alto, CA, U.S.A.), 10-μl syringes from Hamilton (Reno, NV, U.S.A.), and 1000- and 100-μl automatic pipettes produced by Eppendorf (Hamburg, G.F.R.).

Reagents

Ammonia solution (7 *N*), hydrogen chloride gas and Dowex 50W-X8 (H^+) (100—200 mesh) were obtained from Fluka (Buchs, Switzerland), pure picric acid from P.O.Ch. (Gliwice, Poland), *n*-butanol Seq. grade and trifluoroacetic anhydride (TFAA) from Pierce and standard amino acids from BDH (Poole, Great Britain), Mann Research Labs. (New York, NY, U.S.A.), Merck (Darmstadt, G.F.R.) and Pierce. A calibration mixture was prepared using Pierce Amino Acid Standard Physiological A/N.

Columns

Chromatographic packings for the analysis of amino acids as their N-trifluoroacetyl (TFA) *n*-butyl ester derivatives were as follows. Column A packing was 0.65% EGA-PS on 80—100 mesh Chromosorb W AW (Supelco, Bellefonte, PA, U.S.A.). Column B packing was 2% OV-17 + 1% OV-210 on 100—120 mesh Supelcoport (Supelco) for the separation only of His, Arg, Trp and Cys.

Preparation of amino acids, homogenization, centrifugation and ultracentrifugation

Tissues were homogenized in the ratio of 1:5 in 1% picric acid at 4°C. The homogenate was centrifuged at 600 *g* for 15 min and the supernatant was then decanted and ultracentrifuged at 105,000 *g* for 60 min. Cell hyaloplasm was obtained from the supernatant. The deproteinized supernatant containing an excess of picric acid was introduced into the ion-exchange column.

Ion exchange

In the following ion-exchange procedure a 60-fold excess of resin capacity to exchangeable ions placed in the column was maintained, i.e., 6 g of Dowex 50W-X8 (100—200 mesh) for 25 ml of the examined supernatant. The procedure was carried out according to Zumwalt et al. [29].

Lyophilization

A 55-ml volume of the eluate and washings was collected and quickly frozen in liquid nitrogen. The sample was placed in a 1000-ml condenser, and after lyophilization, the dry sediment was retained and transferred to an esterfication vessel.

Derivatization

The reaction was carried out according to the method of Kaiser et al. [30].

RESULTS AND DISCUSSION

The results of the investigations shown in the chromatograms in Figs. 1 and 2 and the results of the qualitative and quantitative evaluations are shown in Tables I and II. We selected 25 amino acids from the calibration mixture used in our laboratory. The results obtained provided basic data for evaluation of the amino acid pool in the cytosol of mammalian atrium and ventricle of heart muscle.

Detailed evaluation of the results, common for atrium and ventricle, pre-

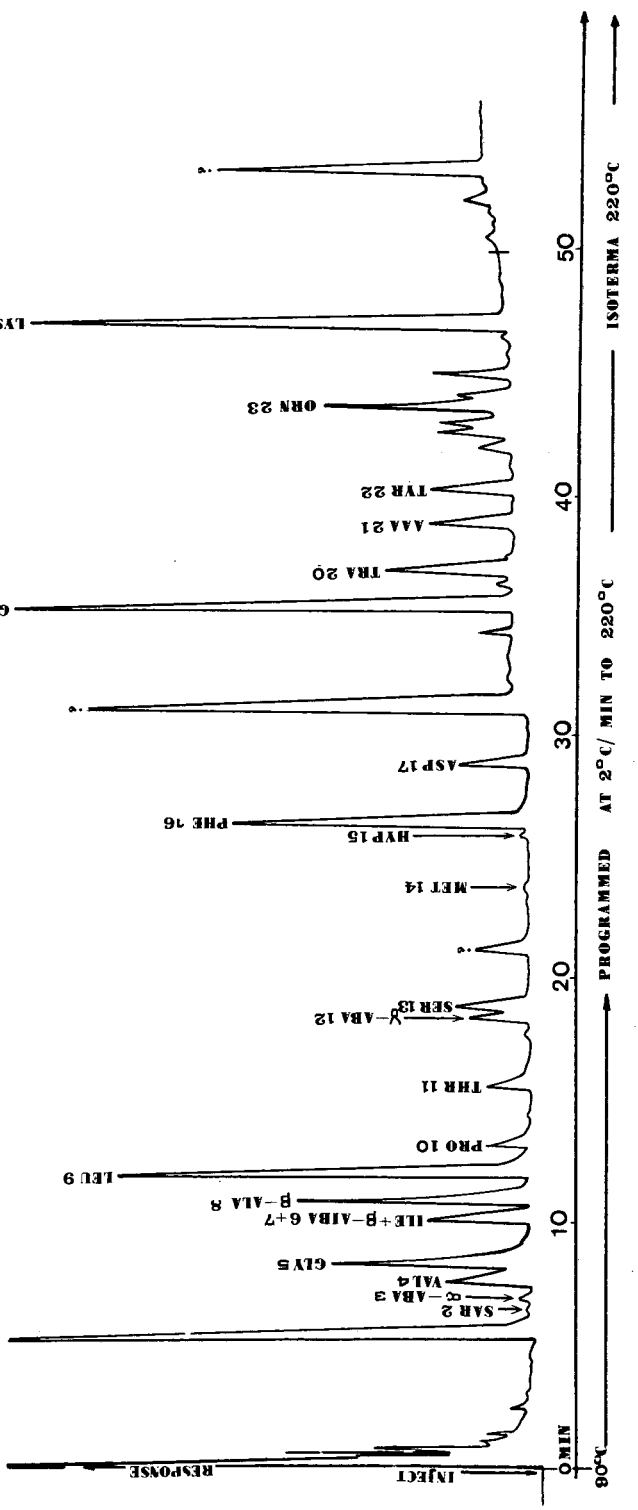

Fig. 1. Simultaneous GLC separation of N-TFA *n*-butyl esters of amino acids in the cytosol of the cow heart atrium. Sample injected: ca. 1 μl. Column: Pyrex (200 cm × 6.35 mm O.D. × 2 mm I.D.), filled with 0.65% EGA-PS on 80—100 mesh Chromosorb W AW (Supelco). Attenuation, 2×10^{-10} a.u.f.s.; initial temperature, 90°C; programming rate, 2°C/min; final temperature, 220°C, flow-rates, carrier gas (helium) 10 ml/min, hydrogen 30 ml/min and air 300 ml/min; internal standard tranexamic acid (peak 20, TRA). High disproportion in amino acid tissue levels makes the correct separation of high and low peaks difficult.

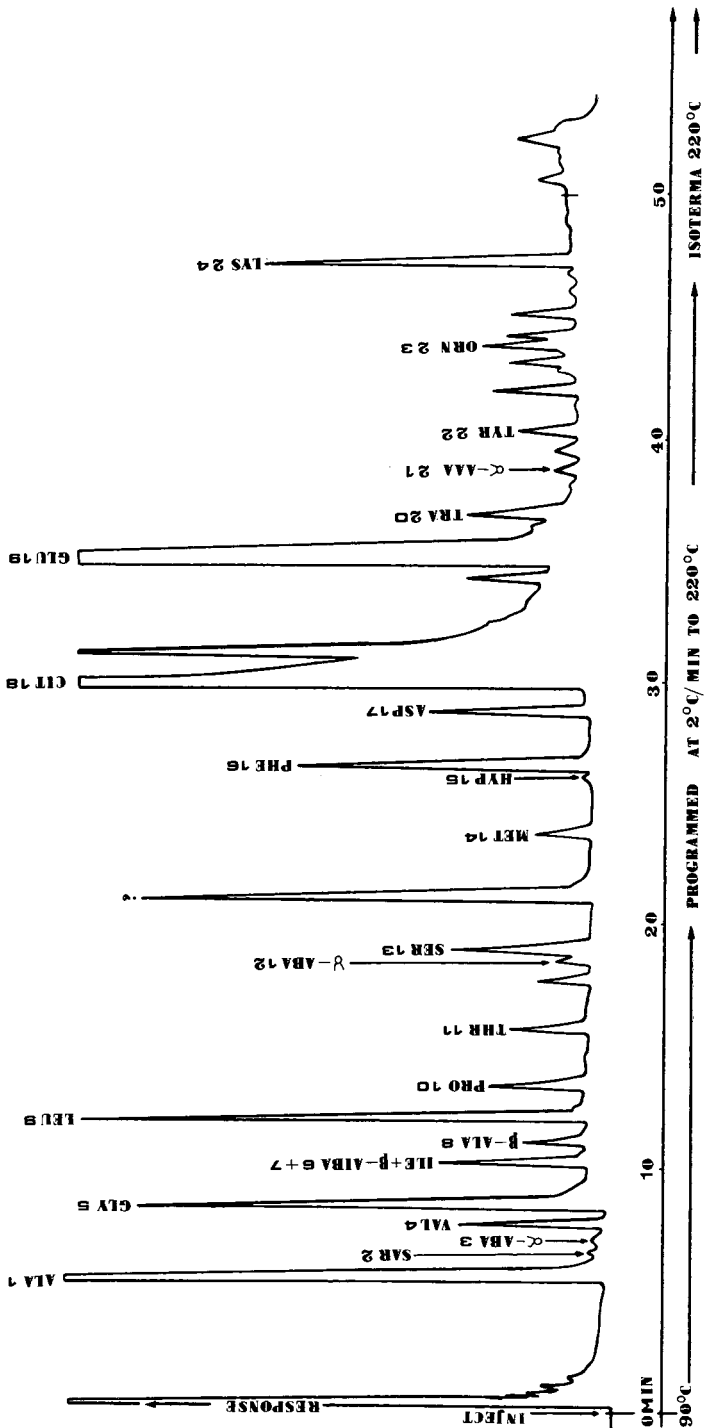

Fig. 2. Simultaneous GLC separation of *n*-butyl esters of amino acids in the cytosol of the cow heart ventricle. Column and conditions as in Fig. 1.

TABLE I

COMPARISON OF GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF ATRIAL AMINO ACIDS IN THE HEART MUSCLES OF MAMMALIANS

Abbreviations of non-protein amino acids: α-ABA = α-aminobutyric acid; SARC = sarcosine; α-AAA = α-aminoadipic acid. Values are expressed in mg of amino acid and represent the mean ± S.D. of five independent determinations.

No.	Amino acid	In atrium of cow (mg ± S.D.)	Amino acid	In atrium of pig (mg ± S.D.)	Amino acid	In atrium of sheep (mg ± S.D.)
1	βALA	0.585 ± 0.065	GLU	1.141 ± 0.074	GLU	4.325 ± 0.183
2	GLU	0.582 ± 0.029	ALA	0.500 ± 0.056	ALA	2.650 ± 0.092
3	LYS	0.525 ± 0.022	TYR	0.145 ± 0.038	SER	0.775 ± 0.090
4	ALA	0.515 ± 0.058	CYS	0.130 ± 0.000	GLY	0.610 ± 0.018
5	LEU	0.451 ± 0.059	PRO	0.128 ± 0.001	THR	0.184 ± 0.022
6	TYR	0.412 ± 0.108	GLY	0.121 ± 0.019	ASP	0.168 ± 0.007
7	PHE	0.390 ± 0.019	PHE	0.100 ± 0.019	βALA	0.155 ± 0.043
8	ARG	0.215 ± 0.005	ARG	0.090 ± 0.006	TYR	0.143 ± 0.018
9	GLY	0.209 ± 0.032	THR	0.088 ± 0.016	PRO	0.039 ± 0.014
10	VAL	0.148 ± 0.010	LEU	0.086 ± 0.008	LEU	0.136 ± 0.012
11	HIS	0.135 ± 0.025	LYS	0.082 ± 0.015	PHE	0.105 ± 0.020
12	ILE	0.105 ± 0.014	αAAA	0.058 ± 0.027	αAAA	0.096 ± 0.009
13	SER	0.105 ± 0.001	ORN	0.057 ± 0.009	VAL	0.080 ± 0.001
14	THR	0.077 ± 0.003	SER	0.056 ± 0.016	LYS	0.080 ± 0.002
15	γABA	0.070 ± 0.002	HPR	0.040 ± 0.005	HIS	0.070 ± 0.030
16	ASP	0.061 ± 0.009	βALA	0.040 ± 0.010	ORN	0.067 ± 0.008
17	CYS	0.050 ± 0.020	ASP	0.035 ± 0.019	HPR	0.060 ± 0.016
18	ORN	0.049 ± 0.001	ILE	0.029 ± 0.008	ILE	0.060 ± 0.034
19	PRO	0.047 ± 0.006	VAL	0.028 ± 0.003	CYS	0.055 ± 0.005
20	TRY	0.035 ± 0.025	MET	0.010 ± 0.003	γABA	0.050 ± 0.030
21	HPR	0.020 ± 0.005	TRY	0.010 ± 0.000	ARG	0.050 ± 0.020
22	MET	0.016 ± 0.002	γABA	0.009 ± 0.001	MET	0.040 ± 0.010
23	αABA	0.015 ± 0.003	αABA	0.003 ± 0.001	TRY	0.030 ± 0.000
24	αAAA	0.008 ± 0.001	SARC	0.001 ± 0.000	αABA	0.020 ± 0.010
25	SARC	0.003 ± 0.001	HIS	0.000	SARC	0.004 ± 0.000
	Total	4.934	Total	2.995	Total	10.154

sented in Tables I and II indicates a predominant role of glutamic acid and alanine, which constituted about 50% of the total amino acid pool. Some workers [31, 32] reported a particular function of the compounds during the fasting period connected with substantial energetic expenditure and insulin synthesis. The level of glutamic acid was actively regulated by alanine and glycine as a result of polyvalent allosteric anhibition of glutamine synthetase. Alanine is the product resulting from D-glucose degradation. Glycine synthesis was preceded by glycolysis and 3-phosphoglycerine acid production. Combination of alanine and glycine with glutamic acid suggests that the energy was possibly taken from processes of degradation of amino acids and sugars.

The position of glycine in Table I is important because it is the precursor of some biochemically important compounds such as purine, glutathione, creatine, phosphocreatine and tetrapyrrole, all of which take part in the synthesis of proteins and amino acids.

Successive positions in Table I are occupied by lysine, leucine, phenylalanine

TABLE II

COMPARISON OF GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF VENTRICLE AMINO ACIDS OF THE HEART MUSCLES OF MAMMALIANS

For abbreviations, see Table I. Values are expressed in mg of amino acid and represent the mean ± S.D. of five independent determinations.

No.	Amino acid	In ventricle of cow (mg ± S.D.)	Amino acid	In ventricle of pig (mg ± S.D.)	Amino acid	In ventricle of sheep (mg ± S.D.)
1	GLU	4.417 ± 0.035	GLU	1.430 ± 0.067	GLU	2.260 ± 0.183
2	ALA	1.625 ± 0.091	ALA	0.373 ± 0.033	ALA	1.460 ± 0.173
3	LEU	0.553 ± 0.024	LEU	0.154 ± 0.010	GLY	0.515 ± 0.056
4	GLY	0.485 ± 0.088	GLY	0.149 ± 0.018	LEU	0.465 ± 0.023
5	PHE	0.305 ± 0.019	LYS	0.146 ± 0.009	PHE	0.375 ± 0.023
6	LYS	0.288 ± 0.016	PHE	0.138 ± 0.011	SER	0.260 ± 0.096
7	βALA	0.259 ± 0.053	TYR	0.072 ± 0.005	βALA	0.240 ± 0.023
8	TYR	0.220 ± 0.046	TRY	0.065 ± 0.015	TYR	0.195 ± 0.015
9	ILE	0.195 ± 0.107	ORN	0.056 ± 0.003	TRY	0.165 ± 0.036
10	SER	0.189 ± 0.006	PRO	0.050 ± 0.002	ORN	0.140 ± 0.006
11	THR	0.189 ± 0.010	ARG	0.045 ± 0.015	LYS	0.135 ± 0.013
12	VAL	0.155 ± 0.033	SER	0.041 ± 0.003	ILE	0.115 ± 0.013
13	PRO	0.132 ± 0.002	THR	0.039 ± 0.001	VAL	0.105 ± 0.020
14	ARG	0.100 ± 0.000	ASP	0.037 ± 0.001	PRO	0.090 ± 0.006
15	MET	0.099 ± 0.036	ILE	0.036 ± 0.003	ASP	0.080 ± 0.003
16	ASP	0.097 ± 0.002	CYS	0.030 ± 0.000	THR	0.065 ± 0.016
17	ORN	0.093 ± 0.014	VAL	0.024 ± 0.013	αAAA	0.045 ± 0.005
18	CYS	0.050 ± 0.000	βALA	0.016 ± 0.004	CYS	0.035 ± 0.005
19	γABA	0.040 ± 0.016	αAAA	0.015 ± 0.006	MET	0.025 ± 0.005
20	αAAA	0.025 ± 0.001	MET	0.006 ± 0.002	ARG	0.015 ± 0.005
21	HPR	0.016 ± 0.010	γABA	0.005 ± 0.003	γABA	0.010 ± 0.000
22	TRY	0.015 ± 0.005	αABA	0.004 ± 0.002	αABA	0.010 ± 0.006
23	SARC	0.004 ± 0.000	HPR	0.003 ± 0.002	SARC	0.000
24	αABA	0.004 ± 0.000	SARC	0.001 ± 0.000	HPR	0.000
	Total	9.559	Total	2.940	Total	6.795

and tyrosine, which participate in many metabolic pathways and are endogenic amino acids. Degradation of these amino acids leads to the formation of ketogenic products and acetyl-CoA. Alanine, glycine, serine and threonine make up another group of amino acids, which produce an indirect metabolite, i.e. pyruvate, which has glucogenic properties utilized in the synthesis of sugar compounds.

The positions of endogenic amino acids (3—25 in Table I) indicate that there is a great differentiation in the nutritional demands of the animals investigated. The pH properties in Table I show that basic lysine occupies a particularly high position. The results in Table II for ventricular muscle show that the similarity between the investigated animals (position 1) provides support for the need for metabolic stability.

Evaluation of the tyrosine and phenylalanine levels indicates that there is 50% more phenylalanine in the ventricles than in the atrium. It may be concluded that the atrial pathway of furan, to which both amino acids belong, differs from the ventricular pathway. This may be caused by the activity of

phenylalanine hydroxylase, which produces tyrosine by means of hydroacylation. This enzyme is of special interest because of the precursor role of tyrosine in the synthesis of the following pharmacological and biochemical compounds: adrenaline, noradrenaline, melanine, tyrosine, mescaline, tyramine, morphine, codeine and papaverine.

Leucine was found to be more abundant in the ventricular muscle. This amino acid is formed during the condensation of ketoisovaleric acid (which is the precursor of valine) with the pyruvate acetyl-CoA derivative. Further transformations are similar to the synthesis of α-ketoglutaric acid in the tricarboxylic acid cycle. This process provides evidence for the need to supply the organism with sugar compounds necessary for basic metabolism in the ventricular muscle.

There are also high levels of isoleucine and valine in the ventricular muscle. These are produced from the pyruvate derivative, i.e. active acetaldehyde combined with thiamine pyrophosphate. Subsequently, α-acetyl-α-hydroxy acids are formed, and aminated by aminotransferase to isoleucine and valine. Degradation of glucogenic compounds and D-glucose is necessary for these changes to occur.

The comparison of the final results for the analysis of amino acids found in the atria and ventricals shows a high content of histidine, the precursor of histamine, a vasodilating compound.

The amino acid positions in the atrial muscle in Table I indicate different qualitative compositions of the amino acids in the atria investigated (from position 3). The sequence of amino acids may indicate that energetic processes in the atrial muscle are less intense than those found in the ventricular muscle. It is difficult to make a comparison between the amino acid pools in the atria and ventricles. The amino acid composition in the atria allows the conclusion that the dynamic metabolic changes result from different metabolic properties, enzymatic composition and permeability of cell membranes of atrial and ventricle heart muscle.

Analysis of the chromatograms indicates that special attention should be paid to the peaks found between serine and methionine and between aspartic acid and glutamic acid. Also the peaks occupying positions lower than serine are of great interest, as the area of unidentified peaks undergoes continuous changes depending on the kind of tissue. The solvents applied and the TFAA do not affect the area of the unidentified peaks. The remaining unidentified peaks marked with question marks require the application of gas chromatography—mass spectrometry for elucidation.

CONCLUSIONS

The atria and ventricles of mammalian heart have a high level of glutamic acid and alanine, amounting to about 50% of the amino acid pool of the cell cytosol, with a predominance of tyrosine in the cytosol of the atrium and phenylalanine in cytosol of the ventricle. A high level of these compounds is connected with continuous regulation of energetic processes in the actively working heart muscle.

Differences in the metabolism are apparent from the sequence of amino

acids indicated in Tables I and II. The positions of the amino acids indicate that the enzymatic composition in the atria differs from that in ventricles. These differences are connected with the consequences of an acute heart muscle infarct which divides the nuclei in atrial cells as well as causing necrosis of ventricular muscle wall. Existing morphological, biochemical and regenerative differences may indicate significant participation of embryogenetic processes in the development of the mammalian heart muscle.

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CHROMBIO. 1715

ANALYSIS OF PHOSPHOLIPIDS IN HUMAN SEMEN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive method for the separation of phospholipids by a high-performance liquid chromatographic procedure is described. The chromatographic separation was achieved on a 25-cm column packed with Bio-Sil HP-10 coupled with a pre-column packed with Si-100 Polyol. Phosphatidylinositol, phosphatidylglycerol, cardiolipin, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, lysophosphatidylethanolamine and lysophosphatidylcholine were completely separated and quantitated. The eluted phospholipids were monitored at 203 nm. The method was shown to be applicable to the analysis of phospholipids from human semen.

INTRODUCTION

Lipids are major constituents of all plant and animal tissues. Among several other lipid classes the phospholipids are of physiological importance, as they are components of biologically active membranes. In studies of lipid metabolism by human spermatozoa the phospholipids have to be extracted, separated and analysed [1].

Previous determinations of phospholipids were based on quantitative thin-layer chromatography (TLC). Multi-component mixtures from biological material have required development in two dimensions to achieve satisfactory resolution [2, 3]. High-performance liquid chromatography (HPLC) combines the separation capability of TLC with several advantages, with reductions in sample preparation, clean-up steps and time. HPLC methods using silica gel columns to separate a variety of phospholipids have been reported [4—6].

The method described here is a modification of earlier HPLC methods for the determination of the lecithin/sphingomyelin ratio in amniotic fluids [7, 8],

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and has been used to analyse phospholipids from human seminal plasma and spermatozoa.

EXPERIMENTAL

HPLC was performed with a Beckman (Munich, G.F.R.) Model 334 instrument. The chromatographic column (250 × 4 mm I.D.) was pre-packed with Bio-Sil HP-10 (10 μm) supplied by Bio-Rad (Munich, G.F.R.). The guard column (75 × 4.6 mm) was pre-packed with Si-100 Polyol, 30 μm (Serva, Heidelberg, G.F.R.) or Vydac-101 S I, 30—40 μm (Macherey, Nagel & Co., Düren, G.F.R.). Detection was effected with a Biotronik BT 3030 variable-wavelength UV detector (190—350 nm) (Biotronik, Frankfurt, G.F.R.) coupled to a Shimadzu C-R 1 A data processor (Shimadzu, Kyoto, Japan). The solvents were HPLC-grade acetonitrile and purified water. The phospholipids were obtained from Sigma (Munich, G.F.R.) and Applied Science Europe (Heidelberg, G.F.R.). Silica gel 60 F-254 thin-layer plates were purchased from Merck (Darmstadt, G.F.R.).

The chromatographic analysis was performed at ambient temperature. Mobile phase component A was acetonitrile—water (80:20) and component B was acetonitrile. The solvents were degassed prior to use. The chromatographic system was programmed for gradient elution using these two mobile phases described. A linear solvent gradient from 87.5 to 25% B between 3 and 15 min was used in all instances, delivering a gradient of water running from 2.5 to 15% water. The flow-rate was held constant at 1 ml/min. The effluent was monitored at 203 nm.

Standard solutions of phosphatidylinositol (PI), phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (S), lysophosphatidylethanolamine (LE) and lysophosphatidylcholine (LC) were prepared at concentrations between 25 and 500 μg/ml in chloroform—methanol (2:1). A 20-μl volume of each standard solution was injected in triplicate and the areas of the peaks averaged to produce calibration graphs for each of the phospholipids. TLC was performed by the method of Darin-Bennett et al. [9].

Phosphorus analyses were carried out according to Fiske and Subbarow [10] as modified by Gentner and Haasemann [11].

Spermatozoa and seminal plasma were separated and lipids were extracted as described by Darin-Bennett et al. [9]. The treatment of the lipid extracts to separate neutral lipids and phospholipids has been described previously [12].

The phospholipid-bound fatty acid methyl esters were prepared and determined by gas—liquid chromatography [13]. Plasmalogens were hydrolysed as described by Vishwanathan et al. [14]. After removing the solvent with a stream of dry nitrogen, the phospholipids were exposed to fumes of concentrated hydrochloric acid for 3 min. The excess of hydrochloric acid was removed with a stream of dry nitrogen and the samples were dissolved in chloroform—methanol (2:1).

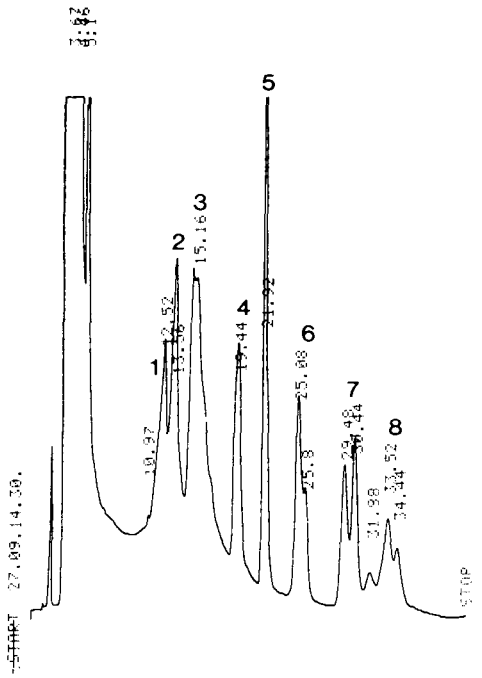

Fig. 1. Typical HPLC elution pattern for a standard mixture of eight phospholipids. Chromatographic conditions as given in the text. Peaks: 1 = CL; 2 = PI; 3 = PS; 4 = PE; 5 = LE; 6 = PC; 7 = S; 8 = LC.

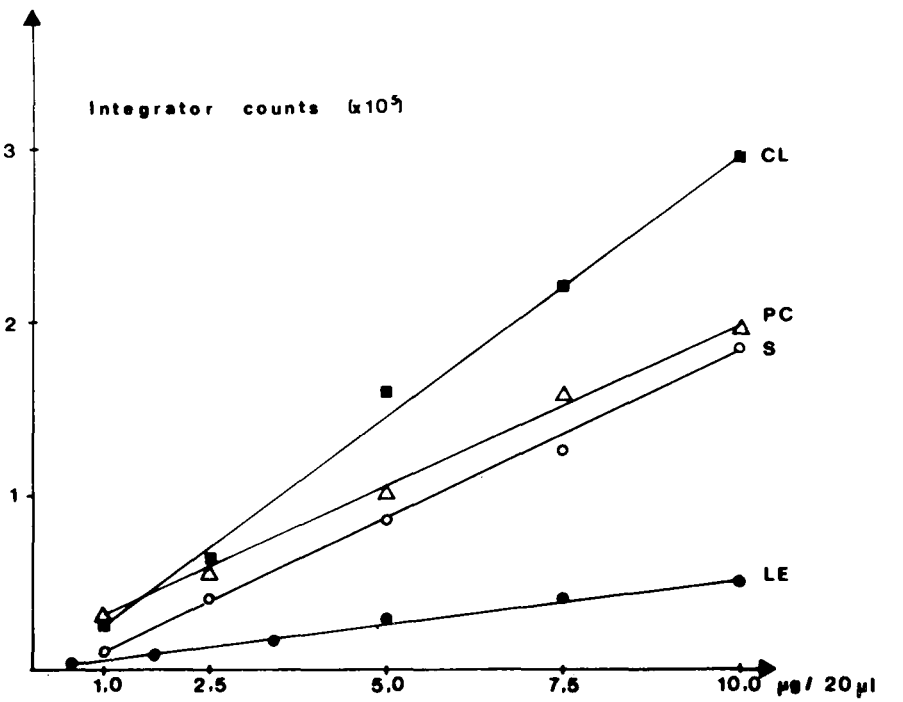

Fig. 2. Calibration graphs for CL, PC, S and LE. Each point represents the average of at least three determinations. Absorbance range: 0.08.

RESULTS

The HPLC analysis of a mixture of eight phospholipids is shown in Fig. 1. The analysis was completed in less than 41 min. PC and S appeared as a split peak in the chromatogram. The two species of S were characterized by analysing their fatty acid composition. The compositions of the two species were different. One contains long-chain fatty acids (> 20 carbon atoms) and the other fatty acids with 14—18 carbon atoms. In contrast, the two species of PC did not show any differences in their fatty acid patterns. Presumably this could account for species having an ether linkage instead of an ester linkage, as indicated by Do and Ramachandran [15] for PE.

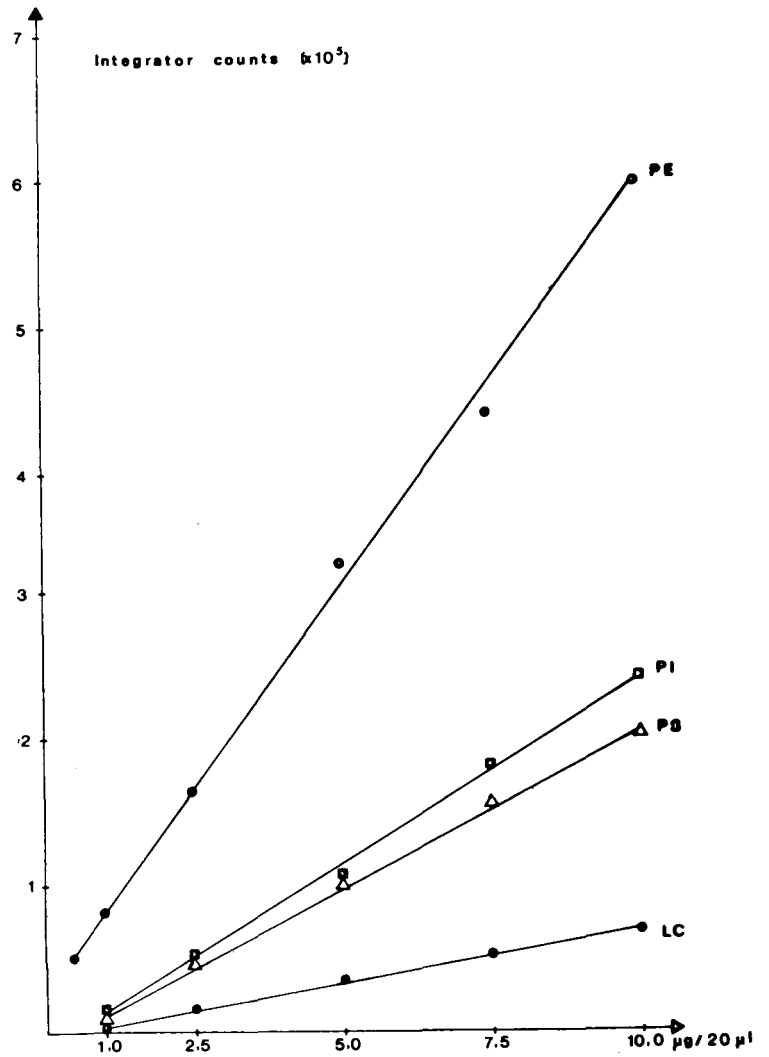

Fig. 3. Calibration graphs for PE, PI, PS and LC. Each point represents the average of at least three determinations. Absorbance range: 0.08.

PS was heterogeneous, producing broad split peaks in accordance with Briand et al. [5].

The primary difficulty was the separation of PI from PG. The use of a pre-column resulted in PG eluting prior to PI. PG and CL eluted together.

Figs. 2 and 3 show that a linear UV response, measured in terms of peak area, could be obtained for amounts of 0.5—10 μg of the different phospholipids. The linear regression correlation coefficients for each of the phospholipid calibration graphs was better than 0.98. Phosphatidalcholine and phosphatidalethanolamine were determined by the decrease in the amounts of PC and PE and the increase in the amounts of LC and LE after exposure to hydrochloric acid fumes.

A representative chromatographic separation of phospholipids from human semen is depicted in Fig. 4.

Some semen samples were analysed by both TLC and HPLC. The correlation between the results of the two methods was good (Table I).

The sensitivity of HPLC is reported to be dependent on the degree of unsaturation in the phospholipids [4], and if the degree of unsaturation varies quantitation by UV absorption would not be accurate. To reinvestigate the correlation of unsaturation and UV absorbance we analysed two commercial PE samples with different fatty acid compositions, especially with respect to the content of the polyunsaturated fatty acids (Table II).

The remarkable result of this investigation was that equal concentrations of the different PE samples produce nearly identical UV absorbances. We

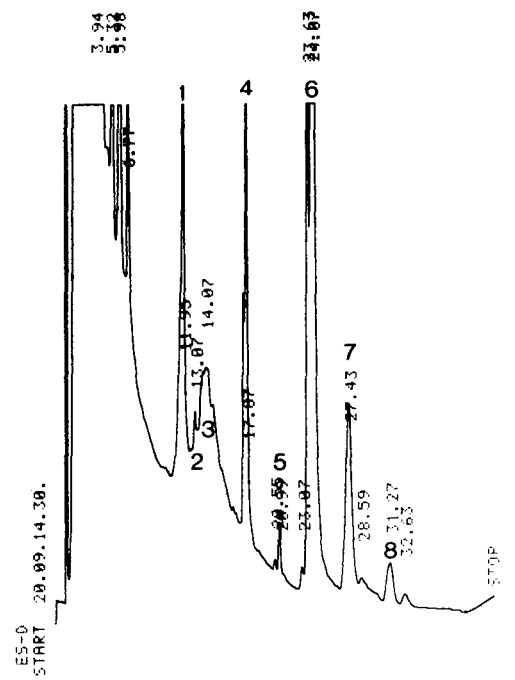

Fig. 4. HPLC trace for a spermatozoa lipid extract after exposure to hydrochloric acid fumes. Chromatographic conditions as given in the text. Peaks: 1 = CL; 2 = PI; 3 = PS; 4 = PE; 5 = PLE (phosphatidalethanolamine); 6 = PC; 7 = S; 8 = PLC (phosphatidalcholine).

TABLE I

CONSTITUENT PHOSPHOLIPIDS OF POOLED HUMAN SEMEN

Percentage of total phospholipids.

Sample	Method	PC	PE	PS	PI	PLC	PLE	S	Other phospho-lipids	Ref.
Sperma-tozoa	TLC	38.3	29.7	3.7		2.1	10.1	12.4	3.4	[1]
	TLC	28.8	21.6	6.6		2.7	9.4	21.4	9.5	[16]
	TLC	42.1	24.1	2.3	1.1	3.0	8.9	18.4	—	This work
	HPLC	48.9	18.3	3.6	2.0	1.8	8.7	16.6	—	This work
Seminal plasma	TLC	7.8	8.5	12.9		0.8	12.3	44.0	13.7	[1]
	TLC	17.6	11.0	1.1	4.8	1.3	14.6	49.6	—	This work
	HPLC	30.7	9.1	1.6	1.1	0.3	14.9	42.2	—	This work

TABLE II

FATTY ACID COMPOSITION OF TWO DIFFERENT PHOSPHATIDYLETHANOLAMINES

Results as % of the total peak area.

Fatty acid (carbon atoms/number of double bonds)	Bovine*	Pig*
13	1.34	
14	1.51	1.83
14/1		0.94
16	7.17	6.31
16/1	1.72	0.10
18	11.48	23.00
18/1	34.66	33.05
18/2	9.14	
18/3	0.57	
20	0.84	
20/1	4.26	
20/2	1.29	2.24
20/3	17.83	13.50
20/5		1.78
22	2.41	
22/1	0.49	
22/5		1.90
22/6	2.05	6.69
22/4	3.25	8.66
Integrator counts ($\times 10^5$)	23.66 ± 0.01**	25.83 ± 0.01**

*Mean values.
**20 μg.

therefore believe that the UV absorbance was not due only to the presence of carbon double bonds, but also to functional groups such as carbonyl, carboxyl, phosphate, amino and quaternary ammonium [17].

In conclusion, the results demonstrate the successful application of HPLC in the separation of naturally occurring phospholipids.

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CHROMBIO. 1730

QUANTITATIVE ANALYSIS OF AMINOPHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING SUCCINIMIDYL 2-NAPHTHOXYACETATE AS A FLUORESCENT LABEL

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SUMMARY

A simple and rapid high-performance liquid chromatographic procedure for the quantitative analysis of ethanolamine- and serine-containing phospholipids in tissue is described. The technique involves reaction of lipid extracts with succinimidyl 2-naphthoxyacetate to give fluorescent derivatives of aminophospholipids. Reaction products are separated by a silica gel column with gradient elution. The eluate is monitored by fluorescence detection at 228 nm (excitation) and 342 nm (emission). Ethanolamine and serine plasmalogens can be measured indirectly by converting their derivatives into lysophosphatidylethanolamine and lysophosphatidylserine derivatives with exposure to hydrogen chloride fumes. The method is highly sensitive and selective.

INTRODUCTION

Quantitative analysis of phospholipid composition in tissue extracts is frequently performed in biomedical research. A simple, rapid and accurate method for this analysis is highly desirable. Thin-layer chromatography (TLC) separates phospholipid classes but is tedious and requires additional assays for quantitation of fractions. Recently, several investigators [1—5] have developed high-performance liquid chromatographic (HPLC) methods for phospholipid analysis. These methods are adequate for the separation of many major and minor components. However, the use of ultraviolet (UV) detection does not allow direct quantitation of fractions, because the 200-nm range of phospholipid absorbance reflects the number of double bonds rather than the number of molecules [1]. HPLC with flame ionization detection [6] has been used to quantitate phospholipid fractions, but it is relatively insensitive.

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Ethanolamine- and serine-containing phospholipids can be easily converted into N-acyl derivatives prior to HPLC analysis. If the detection is aimed at the chromatophore introduced by derivatization, the peak area on the chromatogram reflects the amount of phospholipid eluted. This appears to be a convenient way of measuring the concentrations of different aminophospholipid classes. Thus Jungalwala et al. [7] have analyzed aminophospholipids as their biphenylcarbonyl derivatives by HPLC with UV detection at 280 nm. In order to improve the sensitivity we have previously converted aminophospholipids into Dns derivatives and monitored their HPLC separation with fluorescence detection [8]. In this report we describe the analysis by HPLC of aminophospholipids as their naphthyl derivatives. Compared with the analysis using Dns chloride [8], the method described here has the same sensitivity and specificity but offers the advantage of speed. The reaction time for making naphthyl derivatives is shorter and the HPLC using the new gradient elution program is twice as fast.

EXPERIMENTAL

Materials

Egg yolk phosphatidylethanolamine (PE), egg yolk lysophosphatidylethanolamine (LysoPE), bovine brain phosphatidylserine (PS), bovine brain lysophosphatidylserine (LysoPS) and N-succinimidyl 2-napththoxyacetate were purchased from Sigma (St. Louis, MO, U.S.A.). The purity of phospholipids was checked by TLC. All solvents were of reagent grade. [Dipalmitoyl-1-^{14}C]-phosphatidylethanolamine was purchased from New England Nuclear (Boston, MA, U.S.A.).

Rat brain lipid extract

Sprague—Dawley male rats weighing 150 g were used. Immediately after decapitation heads were placed in liquid nitrogen. A 1-g amount of tissue was removed from the frozen brain and homogenized in 30 ml of chloroform—methanol (2:1). After filtration the lipid extract was separated into two phases according to the procedure of Folch et al. [9]. An aliquot of the lower phase was used for derivatization.

Derivatization of aminophospholipids

An aliquot of lipid solution (phospholipid standards or the total lipid extract from rat brain), containing less than 3 μg of lipid phosphorus, was transferred to a 12 × 32 mm vial (Catalogue No. 223682; Wheaton Scientific, Millville, NJ, U.S.A.). The solvent was evaporated at 50°C under nitrogen. To the dried lipids 5 μl of triethylamine were added, followed by 45 μl of succinimidyl naphthoxyacetate (1 mg/ml in chloroform, freshly prepared). A 3-mole excess of the reagent over aminophospholipids was sufficient for derivative formation as indicated by the yield and recovery studies (see Results). Vials were tightly capped by aluminum seals with a crimper, and vortexed vigorously for 10 sec. They were shaken in the dark at room temperature for 2 h. After incubation, samples were either analyzed immediately or vials were stored at —20°C.

Phospholipid derivatives are stable for several days at this temperature. Because triethylamine might interfere with the PE peak on the chromatogram, just before HPLC analysis reaction products in the vial were evaporated to dryness under nitrogen at 50°C and redissolved in a small amount of chloroform. An aliquot was taken and injected onto the chromatograph.

HPLC analysis

We used a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 solvent delivery system combined with a Model 660 solvent programmer and a Model U6K injector. The chromatographic column was a 30 cm × 4 mm I.D. prepacked stainless-steel Mikro-Pak SI-10 column (Varian, Palo Alto, CA, U.S.A.), which contained silica gel, particle size 10 μm. The column was initially equilibrated with solvent A [dichloromethane—methanol—15 *M* ammonium hydroxide (90:11:1.5)]. The separation of phospholipid derivatives was carried out by programmed gradient elution as follows: flow-rate 1.5 ml/min, 5 min with linear gradient from 100% solvent A to 100% solvent B [dichloromethane—methanol—15 *M* ammonium hydroxide (70:20:5)], and 10 min with solvent B. The gradient program started at the time the sample was injected onto the chromatograph. Before the next analysis, the column was regenerated to its original polarity by equilibrating it with solvent A for 5 min or more. The column temperature was that of room temperature, 21°C.

Fluorescence detection

The column effluent was monitored by fluorescence detection with excitation and emission wavelengths of 228 and 342 nm, respectively. The slit width was 10 nm. We used a Perkin-Elmer Model MPF-44B fluorescence spectrophotometer equipped with an HPLC flow cell, part No. 063-0575. Chromatograms were recorded on a Model 057 x-y recorder. Peak areas were calculated by a Model 9874 digitizer interfaced with a Model 9830A calculator (Hewlett-Packard, Palo Alto, CA, U.S.A.). Uncorrected excitation and emission spectra of PE derivative were obtained by a stop-flow technique, i.e., spectra were scanned while the derivative was trapped in the flow cell by stopping the flow of eluent.

Hydrolysis of ethanolamine and serine plasmalogens

The total lipid extract was derivatized. The reaction mixture was dried under nitrogen. The open vial was then inverted and held over an open bottle of concentrated hydrochloric acid for 10 min. After flushing the vial with nitrogen, chloroform was added in an amount identical with that of the original sample. An aliquot was injected into the chromatograph for analysis.

RESULTS

Fluorescence spectra

Uncorrected spectra of PE derivative showed three peaks of excitation at 228 nm, 272 nm and 320 nm (Fig. 1). The emission maximum was 342 nm. Similar spectra were obtained from PS derivative. Either 228 nm or 272 nm can be used as the excitation wavelength for the fluorescence detection of amino-

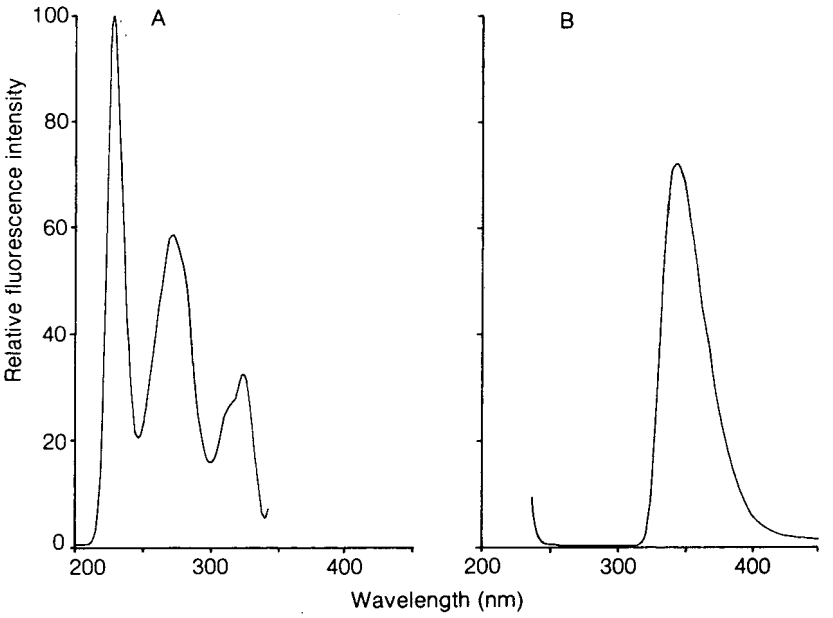


Fig. 1. Uncorrected excitation (A) and emission (B) spectra of phosphatidylethanolamine derivative in dichloromethane—methanol—15 *M* ammonium hydroxide (90:11:1.5).

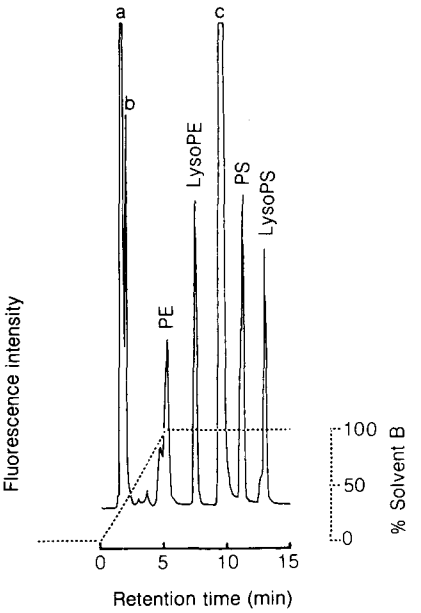
phospholipid derivatives. The excitaiton at 228 nm gave slightly greater response and was used in this study.

Gradient elution program

Several solvent mixtures containing dichloromethane—methanol—15 *M* ammonium hydroxide in various proportions were tested for their ability to elute derivatives of aminophospholipids. With isocratic elution it was not possible to separate all four derivatives rapidly, because the polarity of these compounds is quite different. The gradient elution program shown in Fig. 2 could efficiently separate all four derivatives in a single run within 15 min. Retention times of PE, LysoPE, PS and LysoPS derivatives were 5, 7.5, 11, and 13 min, respectively. Fluorescent peaks due to impurities of reagents and reaction by-products did not interfere with the analysis. For example, 2-naphtoxyacetic acid is located at peak c (Fig. 2). The identity of peaks on the chromatogram was established by injecting into the chromatograph separately the reaction product prepared from the individual phospholipid standard.

Derivatization conditions

Aminophospholipids reacted readily with succinimidyl 2-napthoxyacetate at room temperature. As shown in Fig. 3, the reaction time to reach a maximal and constant fluorescence response was within 1 h. In this present study, 2 h at room temperature was used as the standard condition. To evaluate the yield of derivatization and the recovery of HPLC analysis we determined the phosphorus content, with a micro colorimetric method [10], in the original phospholipid standard solution before derivatization and in the fluorescent



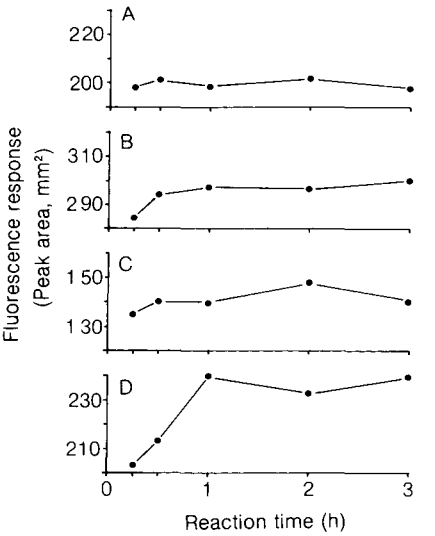


Fig. 2. Chromatogram of derivatives of phospholipid standards: phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LysoPE), phosphatidylserine (PS) and lysophosphatidylserine (LysoPS). Peaks a, b and c are due to reaction by-products and impurities of reagents. The aliquot contained 10 ng lipid phosphorus of each lipid. The elution was with a gradient of dichloromethane—methanol—15 *M* ammonium hydroxide from solvent A (90:11:1.5) to solvent B (70:20:5). The flow-rate was 1.5 ml/min.

Fig. 3. Influence of reaction time on derivatization of (A) phosphatidylethanolamine, (B) lysophosphatidylethanolamine, (C) phosphatidylserine and (D) lysophosphatidylserine. N-Succinimidyl 2-naphthoxyacetate was added to vials of phospholipid soluton which contained PE, LysoPE, PS and LysoPS. At 15 min, 30 min, 1 h, 2 h and 3 h after derivatization vials were open and analyzed by HPLC.

peak collected from HPLC. Recoveries of PE, LysoPE, PS and LysoPS were 95, 92.5, 93 and 81%, respectively (average of two determinations). The quantitative recovery of PE was also confirmed by counting the radioactivity in the PE derivative peak when a known amount of [dipalmitoyl-1-^{14}C]phosphatidylethanolamine (18,000 cpm) was derivatized and an aliquot was injected onto the chromatograph.

Standard curves

The fluorescence intensity was linear with respect to concentration (Fig. 4). Standard curves of PE and LysoPE, in terms of peak area per nmole of phospholipid, overlapped each other. Ethanolamine-containing phospholipids (PE and LysoPE) showed slightly greater fluorescence response per nmole of phospholipid than serine-containing phospholipids (PS and LysoPS). Although the amount of phospholipids injected in the experiment shown in Fig. 4 was in the nmole range, the present method is exquisitely sensitive and is suitable for measuring phospholipid quantities in the pmole range. The detection limit was 2 pmoles of phospholipid (60 pg of lipid phosphorus) which showed a signal-to-noise ratio of 2:1 on the chromatogram.

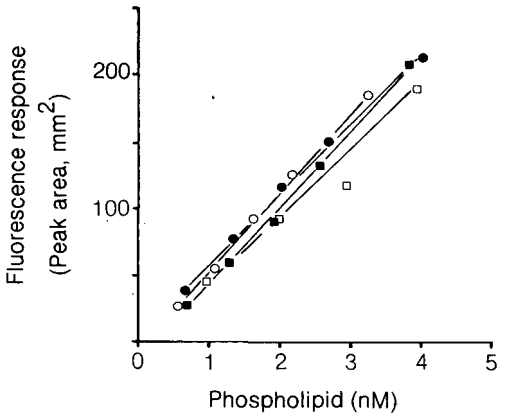


Fig. 4. Standard curves for four phospholipid derivatives: phosphatidylethanolamine (•), lysophosphatidylethanolamine (○), phosphatidylserine (▪) and lysophosphatidylserine (□). Known amounts of phospholipid standards were derivatized and injected for HPLC analysis under the same conditions as in Fig. 2. The fluorescence response in terms of peak area due to each phospholipid was measured.

Quantitative analysis of rat brain aminophospholipids

The usefulness of the present method for the quantitative analysis of aminophospholipids in tissue extracts is illustrated in Fig. 5 and Table I. The total

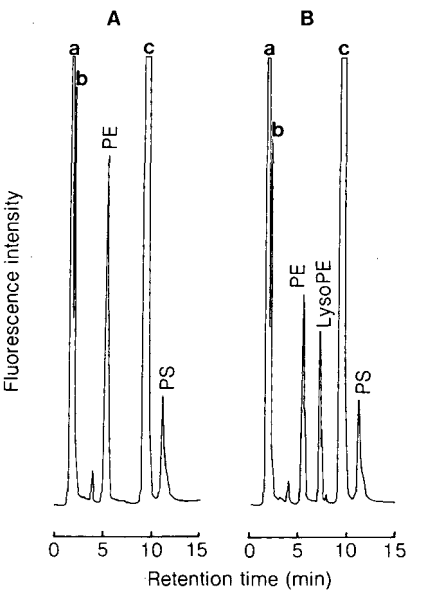


Fig. 5. HPLC analysis of derivatives of the total lipid extract of rat brain before (A) and after (B) exposure to hydrogen chloride fumes. The total lipid extract, containing 1 μg of lipid phosphorus, was derivatized as described in the Experimental section. An aliquot of the reaction mixture was injected into the chromatograph. Another aliquot was dried, exposed to hydrogen chloride fumes, redissolved in chloroform and then injected. The analysis was by gradient elution as described in Fig. 2. Peaks: PE = Phosphatidylethanolamine; lysoPE = lysophosphatidylethanolamine; PS = phosphatidylserine; a, b and c = reaction by-products and impurities of reagents.

TABLE I

PHOSPHOLIPID COMPOSITION OF RAT BRAIN

	Present analysis*	HPLC by Chen et al. [8]	HPLC by Chen and Kou [5]	HPLC by Jungalwala et al. [7]
Before exposure to HCl fumes				
PE	40.7 ± 0.5	43.6 ± 1.2	40.0 ± 2.8	41.6 ± 2.6
LysoPE	None detected		None	
PS	12.7 ± 0.2	17.1 ± 0.4		11.7 ± 1.6
LysoPS	None detected			
After exposure to HCl fumes				
PE	20.4 ± 1.8		19.2 ± 2.0	
LysoPE (derived from plasmalogens)	18.6 ± 0.9	18.9 ± 0.6	19.9 ± 0.9	22.9 ± 1.3

*The values in the present analysis, percentages of the total phospholipids, are mean ± S.D. obtained from three rats.

lipid extract from rat brain containing 1 μg of lipid phosphorus was derivatized. An aliquot was injected into the chromatograph. For the analysis of ethanolamine and serine plasmalogens another aliquot of the reaction mixture was exposed to hydrogen chloride fumes before HPLC analysis in a manner similar to that described by Jungalwala et al. [7]. This is based on previous observations that hydrogen chloride fumes quantitatively hydrolyze alk-1-enyl group from phosphoglycerides and neutral glycerides [11, 12]. The chromatogram of the original lipid extract (Fig. 5A) reveals no detectable amounts of lysophosphatidylethanolamine and lysophosphatidylserine in rat brain. The exposure to hydrogen chloride fumes converted the ethanolamine plasmalogen derivative into a lysophosphatidylethanolamine derivative, since Fig. 5B shows that the peak corresponding to PE derivative decreased, while a peak corresponding to LysoPE derivative appeared. From peak areas in the chromatogram we calculated the quantities of phosphatidylethanolamine, ethanolamine plasmalogen (converted to lysophosphatidylethanolamine derivative) and phosphatidylserine in rat brain by reference to standard curves obtained from known amounts of phospholipids. In Table I data are compared with those obtained by using other HPLC methods. The results were in good agreement. Day-to-day precision of the analysis was evaluated by measuring aliquots of same lipid extracts for four times over a period of 18 days. Three lipid extracts were analyzed. Coefficients of variation (standard deviation/mean) averaged 8%, 18%, and 11% for PE, PS, and ethanolamine plasmalogen, respectively.

DISCUSSION

N-Succinimidyl 2-naphthoxyacetate has been previously used for the detection of amino acids on paper chromatograms [13]. The present method is the first use of this reagent for labelling ethanolamine- and serine-containing

phospholipids. Using rat brains we showed that the method was applicable to the analysis of phospholipids containing these amino groups in tissue samples. It appears to offer several advantages over TLC and other HPLC methods. The most important of these is probably the saving in time and labor, since the derivatization is easy to perform and the HPLC analysis is rapid. If tissue extracts do not contain detectable amount of LysoPE or LysoPS, one of these commercially available lipids can be added to Folch lipid extracts and used as an internal standard. This will improve the speed and the accuracy of quantitation. The sensitivity of measurement is another striking feature. Trace amounts of phospholipids in the pmole range can be determined. Also noteworthy about the method is the specificity introduced by derivatization, chromatographic separation and fluorescence detection. It is free from interfering compounds that might cause error in the analysis.

ACKNOWLEDGEMENT

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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CATECHOLAMINES IN RAT BRAIN USING A LASER FLUORIMETRIC DETECTION SYSTEM

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SUMMARY

A sensitive high-performance liquid chromatographic method for the determination of catecholamines in rat brains has been developed using a fluorescence detector equipped with a continuous wave laser as an excitation light source. A new pre-purification and derivatization method was established and confirmed to be useful for the determination of catecholamines in biological samples. This pre-treatment method was simple, reproducible and specific. About 1 mg of the rat brain tissue was enough to determine catecholamines levels. The levels of dopamine and norepinephrine in rat brain were 0.40 and 0.87 ng, respectively, which agree with the findings of other workers.

INTRODUCTION

The measurements of micro amounts of catecholamines (CA) in brain are of obvious importance in neurochemistry. Various methods for the determination of CA have been reported, including high-performance liquid chromatography (HPLC) with fluorimetric [1—4] or electrochemical detection [5—9], gas chromatography [10] and gas chromatography—mass spectrometry [11, 12]. Of these, HPLC was suitable for the precise, sensitive and rapid determination of CA in biological samples. In particular, HPLC with fluorescence detection (HPLC—FD) is a sensitive and economic method for determining CA.

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In HPLC—FD analysis, both pre- and post-column derivatization methods have been widely used. Mell et al. [13] described the post-column chromatographic detection of biogenic amines, which necessitated the use of complex post-column derivatization components. Refshauge et al. [5] also reported that the trihydroxyindole fluorescence technique, which is a post-column method, has disadvantages, in that it requires substantial investment in equipment and the assay is complex and not easy to perform. In this work, the pre-column derivatization method was employed for the highly sensitive detection of CA using a laser as an excitation light source because the post-column method involves a seriously large background compared with the pre-column method.

Some papers have already been published on the pre-column method for CA using various kinds of fluorescence reagents. Davis et al. [14] derivatized various amines in biological samples with *o*-phthalaldehyde (OPA)—2-mercaptoethanol, and the fluorescent derivatives were immediately extracted with ethyl acetate prior to HPLC analysis. Mell et al. [13] examined CA in urine by means of OPA—2-mercaptoethanol derivatization, and an aliquot of reaction mixture was injected directly on to a column. Imai and Tamura [15] derivatized CA in urine with fluorescamine, and the fluorophore mixture was injected on to a TSK LS-160 column. Schwedt and Bussemas [16] found that CA derivatized with dansyl chloride could be separated and analysed by means of a Zorbax-ODS column.

In a previous paper [17], we stated that the sensitivity of a detector for HPLC could be substantially increased by use of a continuous wave argon laser as an excitation source combined with an optical fibre as a wave guide. The sensitivity obtained with this system was about 20 times higher than that with conventional fluorescence detection systems. The laser fluorimetric detection system was applied to the determination of norepinephrine (NE) and dopamine (DA) in rat brain by use of a newly developed pre-treatment method. The pre-column derivatization methods mentioned above were employed in HPLC analysis using the laser fluorimetric detection system. However, interfering peaks derived from the large excess of reagents appeared on the chromatogram. This paper describes a new pre-treatment method for the highly sensitive HPLC determination of CA, and also reports the determination of CA in rat brain tissue.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph (Model TRI-ROTOR I; Japan Spectroscopic Co., Tokyo, Japan) equipped with a laser fluorimetric detection system constructed as shown in Fig. 1 was used. The HPLC separation was carried out with a 250 × 4 mm I.D. stainless-steel column packed with LiChrosorb RP-18 (5 μm) using the balanced slurry packing method. The column temperature was kept at 50°C by circulating water. The eluate from the column was introduced into the laser fluorimetric detector, which was constructed from a Model FP-110 spectrofluorimeter (Japan Spectroscopic Co.), optical fibre (Machida Endoscope Co., Tokyo, Japan) and a Model 165-05

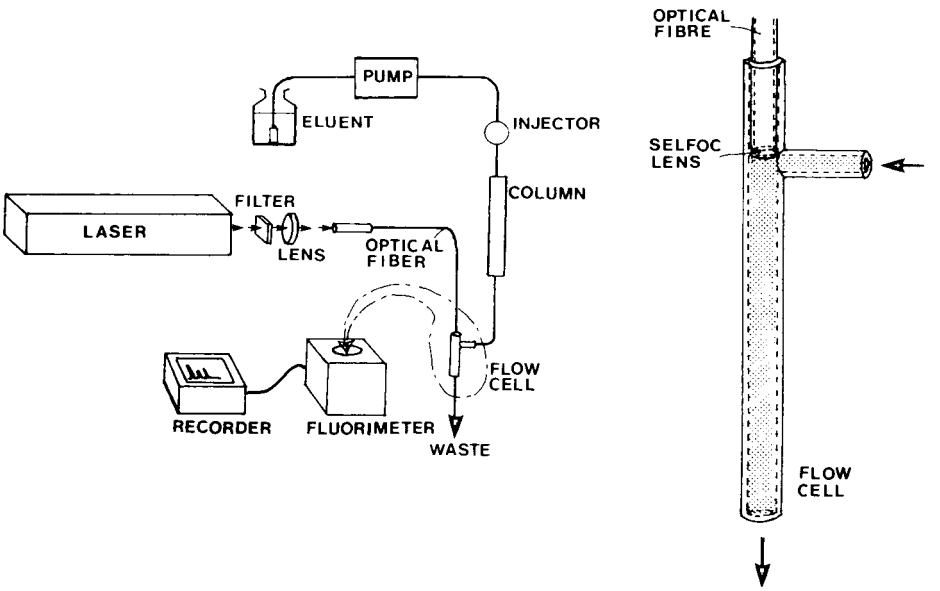

Fig. 1. Schematic diagram of the experimental system for HPLC with laser fluorimetric detection. The argon laser beam transmits along an optical fibre which is axially introduced into a flow cell system to excite the fluorophores of a flowing sample in a capillary cell, without irradiating the cell walls and/or the liquid surface.

argon ion laser (Spectra Physics, Santa Clara, CA, U.S.A.). The flow cell used in the detector was made by Kyowa Seimitsu Co. (Tokyo, Japan) according to our specification. The structure of the flow cell is also shown in Fig. 1. The chromatogram was recorded with a Model 3066 recorder (Yokogawa Electronics Works, Tokyo, Japan).

Reagents

Norepinephrine hydrochloride (NE · HCl) and dopamine hydrochloride (DA · HCl) were purchased from Nakarai Chemicals (Kyoto, Japan) and 3,4-dihydroxybenzylamine hydrobromide (DHBA · HBr) was obtained from Aldrich (Milwaukee, WI, U.S.A.). *o*-Phthalaldehyde (OPA) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and sublimed under reduced pressure (heated to 45—50°C before use). Ethanethiol was obtained from Wako (Osaka, Japan). Alumina (Woelm neutral activity grade I) was purified according to Anton and Sayre [18].

Preparation of reagent solutions

OPA solution was prepared daily by dissolving 5 mg of OPA and 100 μl of ethanethiol and 1 ml of ethanol in 10 ml of 0.1 *M* phosphate buffer (pH 8.60) solution containing sodium metabisulphate (0.05 mg/ml). A standard mixture of CA was prepared by dissolution in and dilution with 0.04 *N* perchloric acid solution so that it contained 80 and 160 ng/ml of NE and DA, respectively. The solution of DHBA which was used as an internal standard was prepared in a similar manner (25 ng/ml). The 0.4 *N* perchloric acid solution

used for the deproteinization contained 0.1 *N* EDTA disodium salt (20 μl/ml). Tris—HCl buffer (pH 8.60) solution (3.0 *M*) was prepared by dissolving 0.4 mg/ml of EDTA disodium salt.

Procedure

Rat brain tissue was homogenized in 0.4 *N* perchloric acid solution in a Potter homogenizer. After centrifugation (10,000 *g* for 10 min at 4°C), 10 μl of the supernatant (corresponding to 1 mg of rat brain tissue) were transferred into an Eppendorf micro tube with 40 μl of the internal standard solution, 15 mg of acid-washed alumina and 50 μl of 3.0 *M* Tris—HCl buffer solution (pH 8.60). The tube was rotated on the rotary rod for 5 min to allow the adsorption of CA on the alumina as shown in Fig. 2. Then, the alumina was allowed to fall to the bottom of the tube and the supernatant was aspirated off. The alumina was washed with about 10 ml of water as shown in Fig. 2.

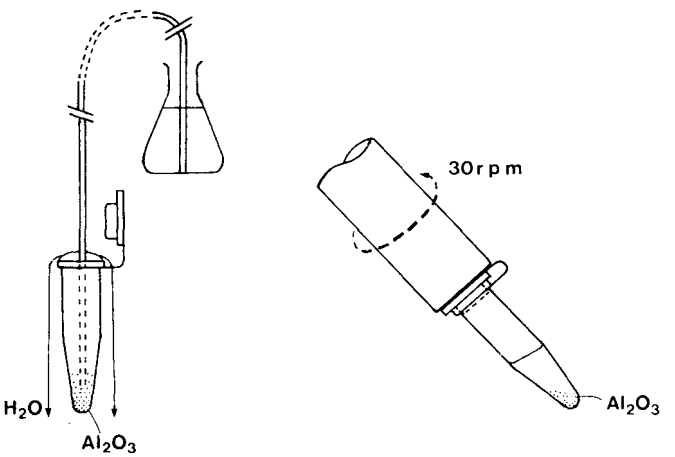

Fig. 2. Schematic diagram of the CA pre-treatment method. Right: to mix the alumina with Tris—HCl buffer or OPA solution, the Eppendorf tube attached on the rotary rod was rotated at a constant rate (30 rpm). Left: to wash the alumina with water, the water, which was continuously flowing through the PTFE tube, was circulated in the Eppendorf tube. The water, but not the alumina, overflowed from the tube and fresh water was applied constantly to the tube from the water reservoir.

After the washings had been aspirated off, 500 μl of OPA reagent solution were added to the tube for the derivatization. To complete the reaction, the mixture was rotated for 10 min in a manner similar to that of adsorption of CA on to alumina. After the removal of the excess of reagent solution by aspiration, the alumina was washed with about 10 ml of water as shown in Fig. 2. After the last washing, the derivatives of CA on alumina were eluted by gentle rotation for 30 sec with 50 μl of 0.4 *N* acetic acid in ethanol (Fig. 2). Then, 20 μl of the supernatant were injected on to the HPLC column. HPLC separation and detection were carried out under the conditions described in the legend of Fig. 4.

RESULTS AND DISCUSSION

Both pre- and post-column derivatization using fluorescent labelling reagents have been widely used. For sensitive analysis, post-column derivatization for HPLC—FD generally seems to be unsuitable for highly sensitive analyses because of the large excess of fluorescence reagent in the mobile phase, which causes a large background on the chromatogram. The pre-column derivatization method, on the other hand, may provide a low background level on the chromatogram. Therefore, the pre-column derivatization method should be applicable to highly sensitive analyses.

With the CA pre-treatment method of Mell et al. [13] and Davis et al. [14], the background derived from the large excess of reagent appeared on the chromatogram in our detection system. To overcome this problem, we investigated a new CA pre-treatment method. An outline of the method is given in Fig. 3.

The procedure for the pretreatment of CA consists of three steps.

In step (1), the adsorption of CA on to the alumina can be performed by adjusting the pH of the sample solution to be slightly alkaline. For this purpose, 50 μl of 3.0 *M* Tris- HCl buffer were added to the mixture of sample solution and alumina. Further adjustment was not necessary. Interfering substances were washed out from the alumina with water. The adsorption of CA on to the alumina and washing were performed by a simplified method shown in Fig. 2. Higa et al. [19] reported that the use of boric acid gel for the pre-purification

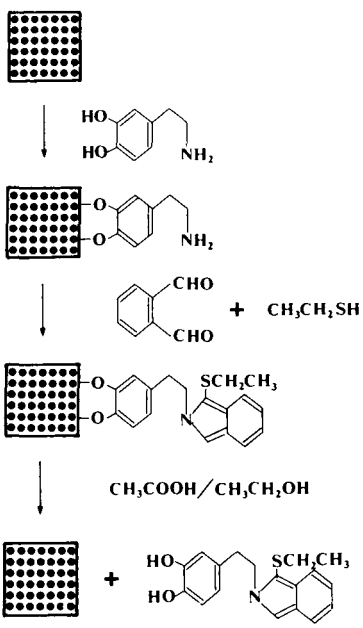

Fig. 3. Procedure for the pre-treatment of CA (for example, DA) consists of three steps. (1) The CA selectively forms a complex with alumina in a weakly alkaline solution. The alumina was washed with water. (2) The adsorbed CA on alumina was derivatized to the fluorophore with OPA—ethanethiol, and the alumina was washed with water. (3) The fluorophore was desorbed from the alumina using a small amount of acid, and the acidic solution was then injected directly on to the column.

provided better recoveries of CA than those with alumina. However, the background level derived from boric acid gel was very high, making it impossible to use boric acid gel in the pre-treatment of CA for the laser fluorimetric HPLC detection. On the other hand, virtually no increase was found in the background level derived from the alumina.

In step (2), the CA on the alumina was derivatized with OPA—ethanethiol into a fluorophore. During the derivatization, the CA should be stable. It is generally considered that the free molecule of CA is unstable and decomposes rapidly under alkaline conditions, probably because of autoxidation of catecholic groups [20]. However, a certain amount of CA adsorbed on the alumina was allowed to stand overnight in weakly alkaline solution, then derivatized with OPA—ethanethiol into a fluorophore and analysed chromatographically. The result was almost the same as with that derivatized immediately after the treatment with alumina. These results suggested that CA adsorbed on to alumina is stable at least overnight in a weakly alkaline solution. 2-Mercaptoethanol is generally used for the derivatization of compounds with a primary amino group into fluorescent products with OPA. It is well known, however, that such fluorescent products are not very stable.

Simons and Johnson [21] reported that the products produced using ethanethiol are more stable than those obtained using 2-mercaptoethanol. Therefore, ethanethiol was used with OPA for the derivatization of CA into a fluorescent compound. However, some decomposition of the fluorescent compound was found in the period necessary for the procedure. Therefore, it is important that the operation after the addition of the reagent solution is carried out in a constant time.

Chen et al. [22] reported that the reaction of CA and OPA was essentially complete within a few seconds. However, we adopted a reaction time of 10 min, because the reactivity between the adsorbed CA molecule on the alumina and the reagents might be lower than that in solution. In order to complete the reaction in a short time, the test tube was rotated on a rotary rod.

An advantage in step (2) is that the interfering excess of fluorescence reagents can be removed. After the derivatization, this was carried out by washing with water. Even after washing with water, the adsorbed CA fluorophore remained on the alumina, and the excess of fluorescence reagents was almost totally eluted. With the procedure of Davis et al. [14] and Mell et al. [13], it was difficult to separate OPA-labelled CA from the peaks of the excess of reagent. In our procedure, only small front peaks on the chromatogram were found, as shown in Fig. 4.

In step (3), the fluorophore on the alumina was desorbed with 0.4 *N* ethanolic acetic acid. When the tube was vigorously rotated, it became impossible to obtain reproducible results, probably because the fluorophore was decomposed by mixing with air. The rotation of the tube was carried out in the same manner as described earlier.

Fig. 4 shows the chromatogram of an authentic mixture of CA. Each CA was effectively separated with isocratic elution. The laser fluorimetric HPLC detection system was compared with a conventional fluorimetric HPLC detector (in this instance, the JASCO FP-110) equipped with a high-pressure mercury lamp (excited at 365 nm). Both fluorimetric measurements were per-

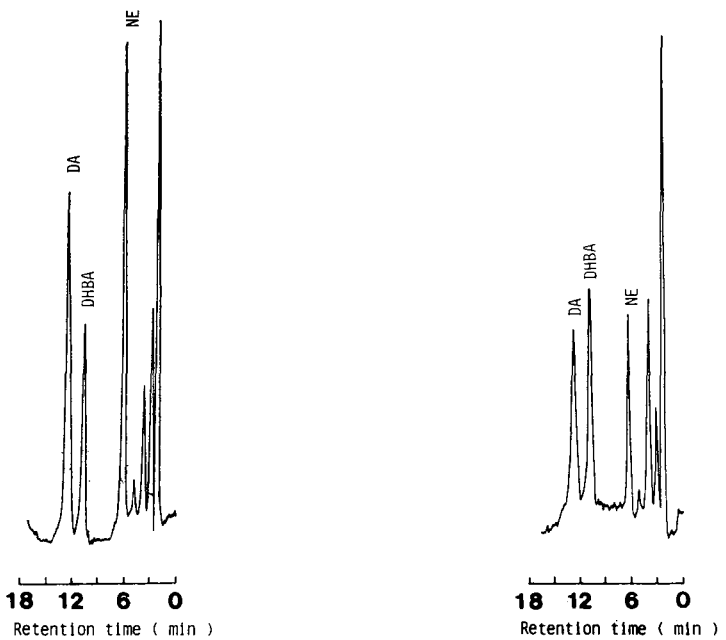

Fig. 4. High-performance liquid chromatogram using the laser fluorimetric detection system obtained from a standard mixture of catecholamines. Operating conditions: column, 250 mm × 4.0 mm I.D., LiChrosorb RP-18 (5 μm); column temperature, 50°C; mobile phase, acetonitrile—0.05 *M* monochloroacetate buffer, pH 2.50 (35:65); flow-rate, 1.0 ml/min; detector, fluorescence spectrophotometer (detection at 450 nm, excitation at 351 and 363 nm of excitation lines of argon laser, laser power ca. 15 mW).

Fig. 5. High-performance liquid chromatogram using the laser fluorimetric detection system obtained from an aliquot of whole rat brain sample (corresponding to 1 mg): DA, 0.87; NE, 0.40; DHBA, 1.0 ng per sample.

TABLE I

TEST OF RECOVERY OF CATECHOLAMINE FROM RAT BRAIN

Sample	Sample No.	NE (ng per sample)	DA (ng per sample)
Added brain	1	1.04	1.93
	2	0.95	1.91
	3	0.99	2.02
	4	1.04	2.09
	5	1.00	2.12
	6	0.92	2.03
	Average	0.99	2.02
	C.V.* (%)	4.9	4.2
Brain		0.40	0.87
Added		0.60	1.20
Recovery (%)		98.3	95.8

*C.V. = coefficient of variation.

formed with the same detector except for the light source. With a laser, the signal-to-noise ratio for 0.8 ng of NE per sample was about 300, for 1.6 ng of DA per sample it was about 200 and for 1.0 ng of DHBA per sample it was about 120. On the other hand, when the light source was a mercury lamp, the signal-to-noise ratio for 0.8 ng of NE was 15 and for 1.6 ng of DA it was about 10. These results suggest that the sensitivity of the laser fluorimetric HPLC detection system is 20 times higher than that with the conventional fluorimetric HPLC detection system.

Linear relationships between the peak height ratio of both NE and DA to DHBA and the amount present were obtained over ranges of at least 0.2–1.6 ng for NE and 0.4—3.2 ng for DA.

The results of the precision and recovery test with this method are shown in Table I; a series of determinations were made on the same rat brain sample. Good results were obtained, as shown by the precision and recovery of CA.

The rat whole brain sample prepared as described above provided the chromatogram shown in Fig. 5. The values obtained by the present method were in good agreement with those reported by other workers [23].

Some investigators [24—27] have reported that the sensitivity of fluorescence detection in HPLC could be increased by the use of a laser as the excitation light source. Diebold and Zare [24] reported a high sensitivity of a laser fluorimetric detector with a wall-less flow cell in the determination of picogram levels of aflatoxins. In a previous paper [17], we reported that the detection limit of dansylalanine using the present detection system with a laser was 2 pg (signal-to-noise ratio = 2), which was more sensitive by an order of magnitude than that obtained with the same fluorimeter with a mercury lamp.

It is interesting to compare the sensitivity of the present method with that of conventional HPLC methods for CA. An exact comparison of the practical sensitivity of the methods is not easy, however, because the limits of quantitation were reported in different terms. The detection limits of CA in HPLC methods reported so far are shown in Table II.

A comparison of these values shows that the detection limits of CA in biological samples using our method exceed those using the OPA pre-column derivatization method [14] and the native fluorescence method [30, 31]

TABLE II

COMPARISON OF DETECTION LIMITS OF CATECHOLAMINE IN BIOLOGICAL SAMPLES USING VARIOUS HPLC DETECTION SYSTEMS

Method	Detection limit (compound analysed)	Sample	Ref.
Laser fluorescence	5 pg (NE), 16 pg (DA)	Rat brain	This work
Trihydroxyindole	7 pg (NE)	Plasma	[28]
Trihydroxyindole	20 pg (NE, DA)	Plasma	[29]
OPA pre-column	50—100 pg (NE, DA)	Plasma, tissue	[14]
Native fluorescence	100—500 pg (NE, DA)	Urine	[30]
Native fluorescence	100 pg (NE), 300 pg (DA)	Rat brain	[31]
Electrochemical detection	10 pg (NE, DA)	Rat brain	[32]
Electrochemical detection	100 pg (NE, DA)	Rat brain	[33]

by more than an order of magnitude. Also, the sensitivity of our method was slightly better than with the trihydroxyindole method [28, 29] and the electrochemical detection method [32, 33].

To use this laser fluorimetric HPLC for the sensitive determination of CA in biological samples, it is very important to reduce background signals caused mainly by an excess of fluorescent reagents. Although a new pre-column derivatization method has been established in this work to reduce the background, the HPLC system could not be used with such a large range of sensitivity. Therefore, if better derivatization methods for CA could be developed, it might be possible to determine CA in biological samples with greater sensitivity using this laser fluorimetric HPLC system. Moreover, if a laser providing a stable high output power in the UV region can be developed, highly sensitive quantitation might be achieved with the fluorescence method.

CONCLUSION

A highly sensitive HPLC method for the determination of CA using new pre-purification and derivatization methods has been developed. The pretreatment method is simple and effective not only for the clean-up of CA from the sample but also for the removal of excess of reagents from the reaction mixture. As a result, a highly sensitive fluorescence detection system using a laser as the excitation light source was used for the determination of trace amounts of CA in rat brain tissue.

Although derivatization with OPA and ethanethiol as fluorescent labelling reagents was used in this work, other reagents such as dansyl chloride and fluorescamine could also be used.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF HUMAN PARATHYROID HORMONE IN REFERENCE STANDARDS, PARATHYROID TISSUE AND BIOLOGICAL FLUIDS

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been used to fractionate human parathyroid hormone (hPTH) from a variety of natural sources and to compare it with synthetic hPTH and hPTH fragments. Multiple radioimmunoassay systems for amino, mid and carboxyl regions of hPTH were used to monitor various preparations of hPTH previously prepared by conventional methods and ampouled in nanogram amounts for reference standard and reagent purposes. Results confirmed that they were free of detectable cleavage products, but showed that the intact hPTH comprised three or four closely associated components. A similar pattern of heterogeneity was obtained when hPTH was extracted from stored human parathyroid adenomata by a simple rapid HPLC bulk fractionation method. Comparison with synthetic 1—84 hPTH and modification of sample handling to minimize oxidative conditions, indicate that some of these components are probably intermediate oxidation products. A number of less hydrophobic components, with carboxyl region immunoreactivities, were obtained from the individual adenoma samples, human parathyroid cyst fluid, ampouled samples of human adenoma tissue culture medium, and secondary hyperparathyroid plasma ultrafiltrate when they were fractionated by RP-HPLC. The results strongly suggest that the biological degradation of hPTH is more complex than generally believed, and that RP-HPLC offers a new dimension in its analysis.

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INTRODUCTION

Reversed-phase high-performance liquid chromatographic methods (RP-HPLC) have now been used to separate a wide variety of polypeptide [1,2] and protein hormones [3,4]. We have recently applied these techniques, which are based on the interaction of hydrophobic residues with alkylsilane-bonded silicas of various carbon loadings, chain-lengths and pore sizes, to the characterization of bovine parathyroid hormone (bPTH) [5]. The results based on UV absorbance, endogenous tryptophan fluorescence chromatographic profiles, region specific radioimmunoassays (RIA) and in vivo bioassay showed that high yields of biologically active material could be readily separated from oxidation and other degradation products using an octadecylsilane-bonded (C-18) 8-nm pore-size packing. These results suggested that similar methods coupled with region-specific RIA might provide a high-resolution, high-recovery system for the analysis of human parathyroid hormone (hPTH) and associated materials of biological origin.

This paper reports the fractionation, by RP-HPLC, of components in a variety of preparations of hPTH, a polypeptide of 84 residues. The preparations included the World Health Organisation (WHO) International Reference Preparation which was prepared and purified by conventional lengthy extraction and chromatographic procedures, other ampouled preparations similar to those used as working standards in immuno-assay systems, as well as materials extracted from individual parathyroid adenomata and related biological fluids and processed solely by rapid HPLC methods.

Three ampouled preparations of extracted hormone and the two ampouled samples of biological fluids analysed here have been included in a recently completed international collaborative study, organised on behalf of the WHO, to characterize and calibrate by RIA and in vitro bioassay the First International Reference Preparation of Parathyroid Hormone, Human, for Immunoassay (IRP hPTH) [6].

EXPERIMENTAL

The five ampouled preparations included in this study have been described in detail in the report of the WHO international collaborative study [6]. In summary the preparations comprised two groups:

1. Preparations derived from extracts of pooled human parathyroid adenomata and hyperplastic tissues

NIBSC research reagent, ampoule code 78/551. Unpurified hPTH was extracted from pooled lyophilized tissues using the urea—HCl procedure [7] and precipitated with trichloroacetic acid (TCA). The extract prepared and donated to NIBSC by Drs. C. Arnaud and B. Brewer, was estimated to be between 2% and 5% pure. Ampoules were estimated to contain the equivalent of approximately 3.6 pmole (36 ng) of hPTH.

NIBSC Research Standard hPTH for immunoassay, ampoule code 75/549. Material was extracted from pooled fresh frozen tissues by the phenol method [8], precipitated with TCA and partially purified by gel chromatography. The

material, prepared and donated to NIBSC by Drs. J.S. Woodhead and M. Peacock, was estimated to be approximately 10% pure. Ampoules contained the equivalent of approximately 2.5 pmole (25 ng) of hPTH [9].

International Reference Preparation for Parathyroid Hormone, Human, for Immunoassay (IRP hPTH), ampoule code 79/500. Material was extracted from pooled lyophilized tissue by the urea—HCl method [7] and precipitated with TCA. The TCA hPTH was purified and re-purified using gel chromatography and ion-exchange chromatography; the final product, donated to WHO by Drs. C.D. Arnaud and B. Brewer, was stated to be 95% pure. Ampoules were estimated to contain approximately 10 pmole (100 ng) of hPTH [6].

2. Preparations derived from biological fluids

Tissue culture hPTH. The culture medium in which human parathyroid adenomata had been maintained in culture for 6—9 days was fractionated by gel chromatography; "peak 2" covering the elution region associated with bPTH was pooled as described by Dorn and Montz [10]. A portion of the fluid, donated to NIBSC by Drs. R. Montz and G. Dorn, was diluted and freeze-dried in ampoules codes 78/616. Estimates of the content of immunoreactive hPTH varied from 2.6—146 mI.U. per ampoule depending on the immunoassay system used [6].

Plasma filtrate secondary hyperparathyroid hPTH. Plasma ultrafiltrate obtained during haemodialysis of one patient with hyperparathyroidism secondary to renal failure [11], was donated to NIBSC by Professor R. Ziegler and Dr. H. Minne. One-ml aliquots of this fluid were freeze-dried in ampoules coded 78/618. Estimates of the content of immunoreactive hPTH varied from 0.5—166 mI.U. per ampoule depending on the immunoassay systems used [6].

Ampouling

Ampouling was carried out according to procedures used for WHO International Biological Standards described by the WHO Expert Committee on Biological Standardization [12]. The preparations of extracted hPTH, ampoule code numbers 78/551, 75/549 and 79/500 contained 250 μg of human albumin, free of peptidase activity [13] as carrier, and 5 mg lactose.

Materials

Other materials used in this study included pepstatin A (Sigma, Poole, Great Britain), synthetic human calcitonin (Ciba-Geigy, Basle, Switzerland); synthetic peptide fragments of hPTH, sequences 1—34, 28—48, 44—68 and 53—84 (Bachem Fine Chemicals, Torrance, CA, U.S.A., or Uniscience, Cambridge, Great Britain); highly purified bPTH preparations characterized and described previously [6], and cathepsin products 1—30 and 37—84 of bPTH, prepared and characterized by Dr. J. Morrissey (St. Louis, MO, U.S.A.) according to published procedures [14]. Synthetic 1—84 hPTH (Asp^{76}) was generously donated to us by Dr. S. Sakakibara (Protein Research Foundation, Osaka, Japan). Details of synthesis, purification and characterization are described elsewhere [15]. Individual human parathyroid adenomata were generously provided by Drs. I. Marschner and W.G. Wood (Munich, G.F.R.); fluid aspirated from a parathyroid cyst was kindly made available by Drs. R. Ardaillou and D. Raymond (Paris, France).

High-performance liquid chromatography

The systems used in this study have been described previously in detail [1,2,5]. Briefly, polypeptides were separated on 150 mm × 4.6 mm I.D. columns, slurry packed with ODS-Hypersil (8 nm pore size, 5 μm particle size, Shandon Southern, Runcorn, Great Britain), using a primary solvent of 0.155 *M* sodium chloride—0.01 *M* hydrochloric acid (pH 2.1) with acetonitrile (Rathburn Chemicals, Walkerburn, Great Britain) as secondary solvent. Peptides were dissolved in the aqueous primary solvent for injection. The initial conditions consisted of a 2.5-min loading phase with primary (aqueous) solvent and a short rapid gradient step to 10% acetonitrile over the next 2.5 min. The peptides were then routinely eluted using a linear gradient of 10—60% acetonitrile over the next 67 min (the rate of change was 0.75% acetonitrile per min) at a constant flow-rate of 1 ml/min and a temperature of 45°C, with an Altex 324-40 chromatograph. For increased resolution of materials eluting close to intact hPTH 1—84, a slow gradient of 24—44% acetonitrile over 60 min (i.e. 0.33%/min) was used. The eluate was monitored for UV absorbance at 215 nm (LDC Spectromonitor III) and for endogenous fluorescence of tryptophan-containing peptides (225/340 nm, Schoeffel FS-970).

In all cases fractions were collected at 0.2-, 0.5- or 1-min intervals into polypropylene microcentrifuge tubes (Sarstedt U.K., Leicester, Great Britain), or

TABLE I

RIA SYSTEMS USED FOR hPTH DETERMINATIONS

Antiserum	Tracer type	Assay standard	Final antiserum dilution	Incubation time	Non-specific binding (% total)	Specific binding (% total)
I + N	^{125}I bPTH (1—84)	hPTH (1—84)	1:300,000	1 day	7	38
			1:500,000	3 + 3 days	7	29
C + I	^{125}I bPTH (1—84)	hPTH (1—84)	1:300,000	1 day	10	35
			1:500,000	3 + 3 days	8	27
N	^{125}I hPTH (1—34)	hPTH (1—34)	1:300,000	1 day	3	31
		hPTH (1—84)	1:600,000	3 + 3 days	3	22
M	^{125}I bPTH (1—84)	hPTH (44—68)	1:26,000	1 day	8	36
C	^{125}I bPTH (1—84)	hPTH (53—84)	1:600,000	1 day	8	27

*Molar ratio at 50% displacement.
**ND = Not done.
***Indicates non-parallelism of displacement curves.
§No displacement at >10-fold molar excess.

into glass tubes. For collection of pmole quantities of hPTH for immunoassay and/or bioassay, tubes containing 10 μl of a solution of bovine serum albumin (1 mg/ml Fraction V RIA grade crystalline, Sigma) were used. All eluate fractions were rapidly frozen on dry ice, freeze-dried to remove acetonitrile and stored at −40°C.

For bulk fractionation of individual adenomata and parathyroid cyst fluid prior to analytical HPLC as described above, a mini-column (80 mm × 4.6 mm I.D.) tap-packed with Partisil 10 ODS (Whatman, Maidstone, Great Britain) was used. Between 0.5 and 2 g of tissue (which had been stored at −70°C) was homogenised (UltraTurrax, Janke and Kunkel, Staufen, G.F.R.) over ice in 10 ml/g of 0.155 *M* sodium chloride—0.01 *M* hydrochloric acid (pH 2.1) readjusted to pH 2.1 after homogenisation and then centrifuged at 105,000 *g* for 30 min. In some experiments 20 μg/ml pepstatin and 5 μg/ml human calcitonin (hCT) were added to the homogenate as an inhibitor of proteolysis and an internal chromatography marker, respectively. The supernatant containing acid-soluble proteins and peptides was then trace enriched onto a mini-column, the retained materials washed with 5 ml of acid saline and polypeptides eluted by stepwise additions of 5 ml each of 20% and 60% acetonitrile in 0.155 *M* sodium chloride—0.01 *M* hydrochloric acid (pH 2.1) [2].

The acetonitrile-containing eluates were immediately subjected to analytical

Detection limit ($M \cdot 10^{-9}$)	50% Displacement ($M \cdot 10^{-9}$)	Relative affinity for*:				Comments
		hPTH (1—84)	hPTH (1—34)	hPTH (44—68)	hPTH (53—84)	
0.1	0.9	1	ND**	§	ND	Predominantly Intact and N-region hPTH
0.05	0.2	1	0.12***	ND	§	
0.1	0.6	1	ND	§	ND	Predominantly C-region and Intact hPTH
0.05	0.2	1	0.07	ND	3.6	
0.3	1.6	ND	1	ND	ND	N-region: recognizes Intact hPTH on an equimolar basis
0.06	0.6	1	1	§	§	
0.04	0.2	0.37	§	1	§	Mid-region: some recognition of Intact hPTH
0.05	0.3	0.08	0.01	§	1	Predominantly C-region, poor recognition of Intact hPTH

fractionation as described above, after removal of the organic modifier by evaporation under nitrogen. The entire process from thawing and homogenisation of solid tissues to collecting and freezing of fractions from analytical HPLC took less than 2.5 hours. An identical procedure was used for parathyroid cyst fluid, except that the homogenisation step was omitted.

Immunoassay

Freeze-dried residues from HPLC and analytical fractionation were routinely dissolved in 0.25—0.5 ml of acetic acid (0.001—0.1 *M*) and aliquots diluted in assay buffer appropriate to the immunoassay system to be used for measurement of immunoreactive hPTH, or for localisation of the hCT internal marker. Aliquots were routinely assayed undiluted or at low dilution in order to screen for non-specific interference or for low concentrations of immunoreactivity, and at multiple further dilutions for measurement of high concentrations of eluted peptides. Repeat assays were carried out as required.

Five RIA systems, with different recognition characteristics for intact (I) hPTH and for the amino (N), mid and carboxyl (C) regions of the hPTH molecule were used. Four were heterologous reagent systems based on antisera raised to bPTH, radioiodinated bPTH as tracer and hPTH as standard. In addition to antiserum Burroughs Wellcome 211/32, antisera 1127/21 and 266/5 resulting from collaborative studies with Wellcome Research Laboratories (Beckenham, Great Britain) were used [16]. The fifth RIA was an homologous system in which the synthetic h1—34 PTH peptide had been used as immunogen to raise antiserum and radioiodinated as tracer. (Antiserum G-017 was generously provided by Drs. B. Moukhtar and C. Desplan, Paris, France.) Other details of reagents and methodology are as described elsewhere [17] except that concentrations of reagents were increased and separation of bound and free tracer modified as required for the more rapid one day RIA systems.

The comparison of cross-reactivities with I, N-, mid- and C-region hPTH peptides are summarized in Table I.

RIA for hCT was modified from a published method [18] and used antiserum Burroughs Wellcome 824/7.

RESULTS

With an octadecylsilane (C-18) packing the highly purified native bPTH 1—84, the synthetic hPTH 1—84 and the marker peptide, synthetic hCT, can readily be separated using a linear gradient of acetonitrile in pH 2.1 isotonic saline as shown in the chromatogram in Fig. 1. The retention time for the hCT marker was 42 min, with bPTH 1—84 eluting a minute later at 43 min, and hPTH 1—84 eluting slightly more than a minute earlier than hCT at 40.5 min. The gradient system also gives a clear separation of the synthetic hPTH peptide fragments, 1—34, 28—48, 44—68 and 53—84 with retention times of 39, 33, 19.8 and 26.5 min, respectively (the relative elution positions for these synthetic peptides are marked on Fig. 1 for comparison). The precision with which the retention times of individual synthetic peptides could be reproduced on different occasions and with different columns of the same dimensions was ±1 min. Nevertheless, because the biological preparations

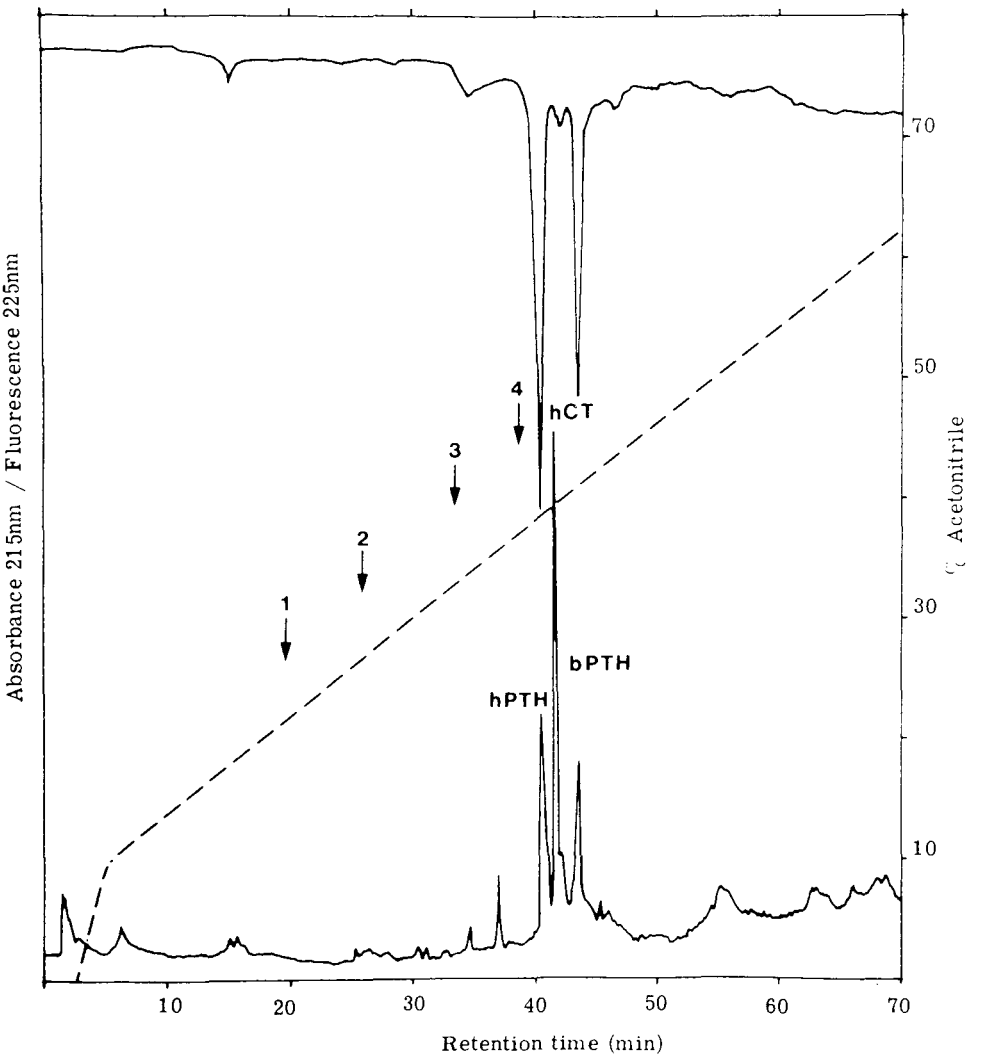

Fig. 1. UV absorbance (215 nm) (lower trace) and endogenous tryptophan fluorescence (225/340 nm) (upper trace), chromatograms of synthetic hPTH (Asp^{76}), the marker peptide synthetic hCT and highly purified native bPTH. The acetonitrile gradient as used throughout all studies in this report is shown (— — —). Arrows indicate the characteristic retention times for the synthetic peptide fragments of hPTH, in order of elution, as described in the text: (1) 44—68, (2) 53—84, (3) 28—48 and (4) 1—34.

analysed included samples with pmole quantities of hPTH which could not be detected directly by UV absorbance or fluorescence, hCT was included as an internal peptide marker in all such samples. hCT was particularly suitable for this purpose as it elutes close to the main regions associated with bPTH or hPTH and it can be localized to individual tubes by RIA as it does not cross-react in immunoassay systems for hPTH.

Ampouled preparations containing 3%, 10% and 95% pure extracted hPTH, as detailed in Experimental, and originally prepared by conventional proce-

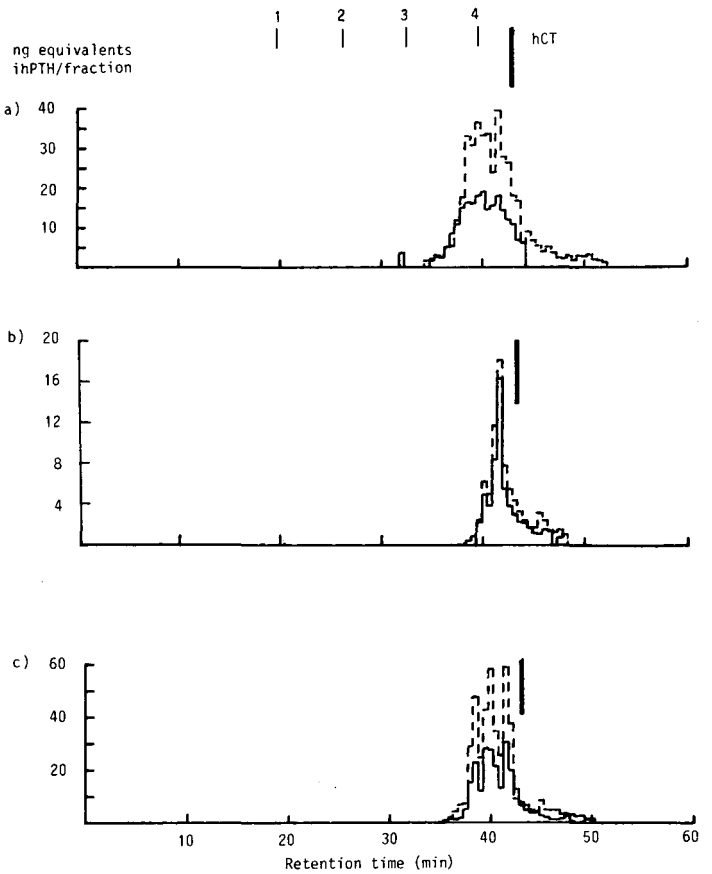

Fig. 2. Immunoreactivity profiles for the three ampouled preparations of unpurified (a, ampoule code 78/551), partially purified (b, ampoule code 75/549) and highly purified (c, ampoule code 79/500) extracts from pooled human parathyroid adenomata. RIA systems, I+N (— — —) and C+I (———) were used to assay fractions collected at 1-min intervals during gradient elution as shown in Fig. 1 and described in the text. In each instance, the contents of three ampoules (approx. 120, 75 and 300 ng respectively) were dissolved in the primary solvent for injection onto the HPLC column. The position of the hCT internal marker peptide is indicated for reference.

dures from a pool of parathyroid adenomata, were chromatographed and the eluate fractions were monitored for both N- and C-region immunoreactivity. The results illustrated in Fig. 2a, b and c show that virtually all major immunoreactivities detectable by the N- and C-region immunoassay systems are associated with a 6-min elution period, immediately preceding the hCT internal marker. There is no indication of dissociation of N- and C-immunoreactivity profiles and no dissociation of N- and C-region immunoreactivity could be demonstrated even when a shallower (0.33%/min) acetonitrile gradient was used to provide greater resolution (data not shown). However, none of these ampouled materials elute as a single sharp peak of immunoreactivity, despite the fact that the immunoreactive hCT was always localized to, at most, two consecutive eluate fractions, corresponding to the UV absorbance peak width

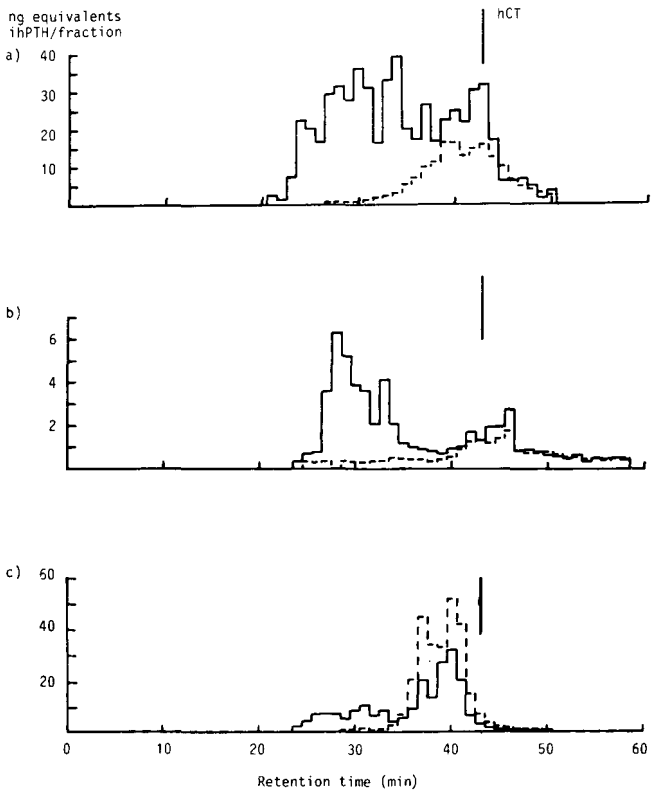

Fig. 3. Immunoreactivity profiles for three samples of biological fluids: (a) tissue culture fluid, ampoule code 78/616; (b) plasma diafiltrate, ampoule code 78/618, and (c) parathyroid cyst fluid (not ampouled). Two RIA systems, I+N (— — —) and C+I (———) were used to assay fractions collected at 1-min intervals during gradient elution shown in Fig. 1 and as described in the text. The contents of five ampoules of each of 78/616 and 78/618 were dissolved in the primary solvent for injection onto the HPLC column. The high protein content of the resulting solutions of 78/616 and 78/618 may have resulted in size exclusion phenomena not evident with other samples. Such artefacts may partly account for the apparent delay in elution of immunoreactivities in comparison with other samples. One ml of the cyst fluid had been processed through the mini-column, as described in the text, and the 20—60% acetonitrile elution step concentrated for analytical HPLC and collection of fractions. The elution position of the hCT marker is indicated.

of less than 1 min. Previous work with native bPTH, of varying degrees of purity showed that the components were readily resolved by both the chromatographic profiles and immunoreactivity profiles [5].

In contrast to these results from material prepared by conventional chemical procedures from human parathyroid tissues, there was clear evidence of the presence of hormone fragments in the biological fluids. Thus, the ampouled samples of partially-purified material from tissue culture supernatants from parathyroid adenomata (Fig. 3a), a plasma ultrafiltrate from a case of secondary hyperparathyroidism (Fig. 3b), and a sample (not ampouled) of fluid aspirated from a parathyroid cyst (Fig. 3c), gave several early-eluting peaks of C-regional immunoreactivity which were not detected with antisera with recognition of N-region immunoreactivity.

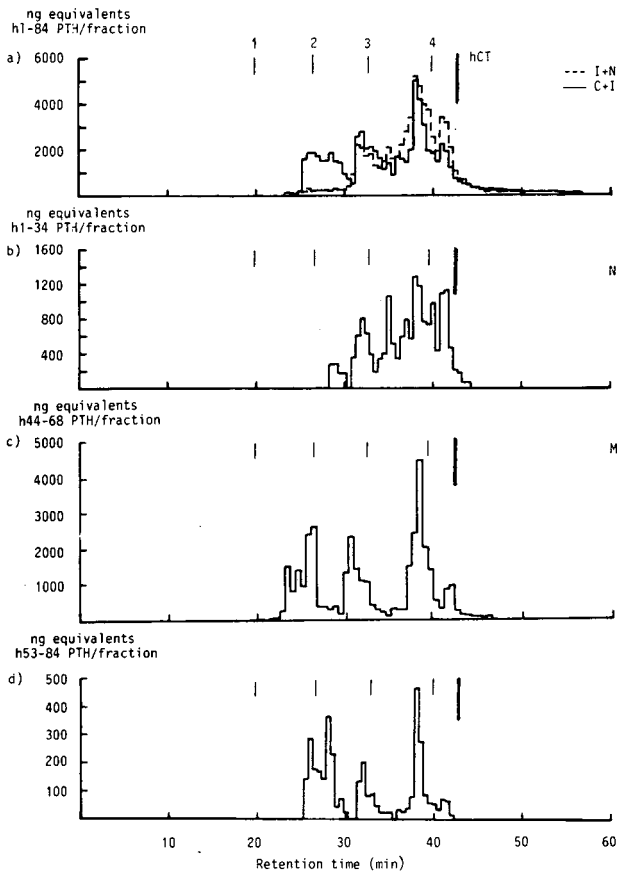

Fig. 4. I+N (— — —) and C+I (——) immunoreactivity profiles of parathyroid adenoma components on fractions collected at 0.5-min intervals (a). The gradient conditions used are shown in Fig. 1 and described in the text. Other specific region RIAs were also used to assay the same fractions, namely the homologous h1—34 PTH RIA (b), the heterologous h44—68 PTH RIA (c) and heterologous h53—84 PTH RIA (d). The elution position of the hCT marker is indicated. Small lines indicate the characteristic elution positions for the synthetic hPTH peptide fragments 44—68 (1); 53—84 (2); 28—48 (3); 1—34 (4).

It is evident from Figs. 2 and 3 that hPTH preparations processed by a variety of conventional methods show significant heterogeneity when analysed by RP-HPLC. To determine to what extent this heterogeneity was a function of type of sample and to what extent it was due to the method of purification used, a series of single human parathyroid adenomata were individually processed using only a rapid method of bulk fractionation by HPLC followed by analytical chromatography, as described in Experimental. A typical result is illustrated in Fig. 4.

Three main areas of interest can be discerned in the immunoreactivity profiles of such rapidly processed parathyroid tissue. The early-eluting materials can be seen at 25—30 min and show predominantly mid- and C-region immunoreactivity. Materials pooled from this region had no bioactivity in the canine renal membrane adenylate cyclase assay [19]. In the next period, at 30—35

min, the eluted materials are detected by N-, mid- and C-region immunoassay systems and had weak but significant bioactivity. A major area of N-, mid- and C-region immunoreactivity was evident in the later 35—40 min region, immediately preceding the hCT internal marker as in all hPTH preparations tested (cf. Figs. 2 and 3). A pooled sample from this region gave a full dose—response curve parallel to that of hPTH 1—84 in the in vitro bioassay (data not shown). Similar complex immunoreactivity profiles were obtained with separately processed tissue from two other adenomata and although the relative proportions of the different components varied (data not shown), none of these preparations gave a single, sharp peak of hPTH (N+C) immunoreactivity, when the standard bulk fractionation procedure using a mini-column, described in Experimental, was used. Other region-specific immunoassays (Fig. 4b, c and d) confirmed the heterogeneity of material prepared in this manner. Similar bioassay results were obtained on the pooled fractions from the other adenomas, confirming that the components with the longer retention times were associated with biological activity.

When, however, the evaporation of acetonitrile from the mini-column fractions was dispensed with and the samples rendered suitable for analytical chromatography with continuous gradient elution by dilution with acid saline to lower the organic modifier concentration sufficiently to allow hydrophobic interaction with the RP support, some, but not all, of the heterogeneity associated with the immunoreactivity just in front of the hCT was no longer apparent (Fig. 5). In this instance, the major immunoreactive peak eluted just 1—2 min earlier than hCT and occupied only two peak fractions corresponding to a peak width of about 1 min. This material probably, therefore, represents the intact, unmodified hPTH 1—84 and is very similar in retention time and peak width to the synthetic hPTH 1—84 (Fig. 1). The broader peaks in the hPTH from other preparations (cf. Fig. 2) must therefore be due to the presence, in this region, of other materials. We have shown previously that the oxidized forms of bPTH elute close to the unoxidized material [5] and it is a possibility, therefore, that some of the peak spreading seen in the hPTH region of some preparations is due to the presence of multiple oxidation products, as the trap fractions of 1 min were not small enough to separate all possible components as

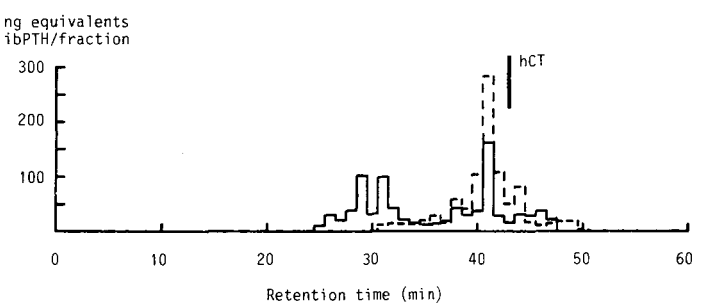

Fig. 5. I+N (— — —) and C+I (——) immunoreactivity profiles of an HPLC extraction of a single human parathyroid adenoma in which the HPLC extract was not processed (other than by dilution) prior to analytical HPLC under the gradient conditions described in the text, with collection of fractions at 1-min intervals for RIA. The position of the hCT marker is indicated.

individual peaks. It is not due to a loading effect with large amounts of biological materials as in all cases the hCT internal standard was recovered as a sharp (<1 min) peak and within two consecutive fractions by immunoassay.

DISCUSSION

To date, the characterization of products obtained from human parathyroid tissue has been restricted by the limited amounts of human tissue available and by the very low yield of peptide for chemical and biological study after the lengthy chemical extraction and purification procedures. The rapid and efficient high-yield, high-resolution RP-HPLC extraction system developed for study of endocrine tissues [2] and the HPLC fractionation systems, developed for the study of bPTH [5] have now been extended to hPTH.

Application of RP-HPLC to the analysis of the three ampouled samples of biologically active hPTH extracted from pooled human parathyroid tissue confirmed that the samples, prepared by conventional methods, precipitated with TCA and further processed by gel and ion-exchange chromatography to different degrees of purity consisted of intact immunoreactive hPTH and did not contain detectable amounts of immunoreactive cleavage products. These findings are in agreement with the results of other groups working with products extracted from human parathyroid tissue and purified by conventional methods [20—22]. The HPLC results on three ampouled preparations were thus in accord with the WHO international collaborative study which showed that homogeneity of potency estimates were obtained from a wide variety of different immunoassay systems and by in vitro bioassay systems [6].

The two ampouled samples of biological fluids, on the other hand, apparently contained little intact hormone as predicted by the marked lack of homogeneity of potency estimates obtained from immunoassay systems and low in vitro bioactivity in the WHO study. They contained multiple components with predominantly C-region immunoreactivity (Fig. 2).

However, the IRP hPTH, consisting of hPTH estimated by conventional chemical, immunochemical and biological methods to be a homogeneous product approximately 95% pure, proved to be unexpectedly heterogeneous on RP-HPLC.

Several factors might contribute to this heterogeneity of extracted intact hPTH. It could be due to the presence of a much wider spectrum of isohormones [20,21,23], and some clarification can be expected when synthesized polypeptides with alternative sequences [24,25] are available. Conformational effects do not appear to be responsible as these would affect all PTH 1—84 preparations, including the synthetic peptides.

The demonstration that rapid processing of hPTH, without evaporation, gave a sharp peak in the intact PTH region (Fig. 5) indicates that oxidation may be an important factor in generating at least some of the heterogeneity in the other preparations. The deliberate oxidation of bPTH [5] and synthetic hPTH 1—84 [26] generates a range of slightly less hydrophobic compounds.

In a previous study of hPTH by RP-HPLC, hPTH was isolated from a small pool of parathyroid adenomata [27]. Three closely associated components

were resolved. However, only one of these, the most hydrophobic, was selected for further study and cleavage and degradation products, if any, from these solid tissues were not mentioned. RP-HPLC systems with the addition of hydrophobic ion-pairing additives such as the heptafluorobutyric acid (and trifluoroacetic acid) [27] can, in some instances, resolve other components of protein mixtures [28]. Detectable heterogeneity of hPTH preparations may well, therefore, be dependent upon the mobile phase used, as well as the handling conditions and source of materials.

As noted in the Results, there appear to be three main elution areas or periods of interest when considering the immunoreactivity profiles obtained from the parathyroid tissues. The third or latest period, between 35 and 40 min, consists of immunoreactive components which are associated with heterogeneity of the intact hormone. The nature of the immunoreactive components associated with the first and second periods, i.e. eluting between 25 and 30 min and between 30 and 35 min respectively in the present studies, cannot be determined directly or indirectly. Further studies are precluded by the small amounts of human hormone available. Nevertheless, certain inferences can be drawn from the known properties of the molecule in terms of cleavage, and the predicted hydrophobic domains.

The majority of the early (25—30 min) eluting materials (see Figs. 2 and 3) could be equivalent to the larger two-thirds mid/carboxyl region fragment now generally believed to be a typical cleavage product. This interpretation is consistent with the fact that material pooled from this region showed no activation of canine renal membrane adenylate cyclase in the in vitro biological system [19].

Theoretical studies [29] indicate that the 46—55 sequence of PTH has little or no hydrophobicity and links the N- and C-terminal regions which each contain a separate hydrophobic core. Synthetic 44—68 was, in practice, the least hydrophobic of the chemically defined substances available for testing; a larger two-thirds, mid/carboxyl region of bPTH, the bovine cathepsin product, 37—84 PTH, also eluted within the 25—30 min region. These considerations would tend to support the hypothesis that the least hydrophobic components correspond to the larger mid- and C-region fragments. The possibility that parathyroid tissue may also contain or be capable of producing a variety of smaller mid- and C-region cleavage products cannot be excluded.

Human PTH products pooled from the second period, i.e. between 30 and 35 min, showed evidence of weak but significant bioactivity and were associated with N-, mid- and C-region immunoreactivities. It could be postulated that parathyroid tissue may contain or be capable of producing a variety of large N- and mid-region bioactive cleavage products although such fragments are perhaps less easy to reconcile with classical views of hPTH cleavage. There is, however, a suggestion from other workers that glandular release of fragments with mid-region but lacking C-terminal immunoreactivity may occur [30].

Definitive identification of the multiple components of hPTH which can readily be resolved by the RP-HPLC system described here, and by the system of Bennett et al. [27], is still dependent upon the availability of human parathyroid tissue, and upon micro-scale chemical analysis and biological assessment. However, it is evident that RP-HPLC systems add a major new dimension

to the future analysis of hPTH, its fragments and congeners, in tissue and biological fluids.

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CHROMATOGRAPHIC SEPARATION OF 24(*R*),25-DIHYDROXYVITAMIN D_3 AND 25-HYDROXYVITAMIN D_3-26,23-LACTONE USING A CYANO-BONDED PHASE PACKING

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SUMMARY

A high-performance liquid chromatographic system is described for the baseline resolution of 25-hydroxyvitamin D_3, 24(*R*),25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3-26,23-lactone, the three principal circulating metabolites of vitamin D_3 in the vitamin D-replete animal. The system is based upon a cyano-bonded phase packing and the solvent hexane—isopropanol—methanol (94:5:1). Of particular interest is the strong retention of carbonyl-containing vitamin D metabolites. The new system can be used for unequivocal analysis of vitamin D metabolites in plasma samples from clinical and animal studies and in the separation and identification of renal metabolites generated in vitro.

INTRODUCTION

During the early seventies, it was believed that the major metabolism of 25-hydroxyvitamin D_3 [25(OH)D_3] occurring in the kidney was into 1,25-$(OH)_2D_3$, the biologically active form of vitamin D_3, or into 24(*R*),25-$(OH)_2D_3$ [1]. Recently it has become clear that the kidney is capable of synthesising a number of other side-chain hydroxylated metabolites including: 25(*S*),26-$(OH)_2D_3$ [2, 3], 23(*S*),25-$(OH)_2D_3$ [4—6] and 25(OH)D_3-26,23-lactone [7]. The close structural similarity of the side-chain hydroxylated metabolites has placed an increased burden on the chromatographic techniques used in the routine separation of vitamin D compounds. Methods based upon silica microparticles and isopropanol—hexane mixtures, established for the separation of 25(OH)D_3, 24(*R*),25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ [8, 9] offer poor resolution of 24(*R*),25-$(OH)_2D_3$ and 25(OH)D_3-26,23-lactone. Alternatively, methods [10, 11] utilizing silica and methylene chloride in the mobile phase give good resolution of 24(*R*),25-$(OH)_2D_3$ and 25(OH)D_3-

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26,23-lactone but offer poor resolution of $25(OH)D_3$ and $25(OH)D_3$-26,23-lactone.

The recent surge of interest in the metabolism and biological activity of $24(R),25\text{-}(OH)_2D_3$ [12—14] has created a need for chromatographic systems capable of baseline resolution of $25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$ and $25(OH)D_3$-26,23-lactone. Such a system would permit detailed study of renal metabolism of $25(OH)D_3$ and also allow for convenient assay of $24(R),25\text{-}(OH)_2D_3$ in clinical and animal plasma without the interference of $25(OH)D_3$-26,23-lactone. This paper describes a high-performance liquid chromatographic (HPLC) system based upon a cyano-bonded packing that takes advantage of the strong retention of $25(OH)D_3$-26,23-lactone and other carbonyl-containing vitamin D_3 molecules making possible excellent resolution of $25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$ and $25(OH)D_3$-26,23-lactone.

EXPERIMENTAL

Vitamin D metabolites

Crystalline $25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$, $25(S),26\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ were generous gifts of Dr. Milan Uskokovic of Hoffman-LaRoche, Nutley, NJ, U.S.A. 24-Keto-$25(OH)D_3$ [15, 16] and $25(OH)D_3$-26,23-lactone [17] were kindly supplied by Dr. Sachiko Yamada, Teikyo University and Dr. Tatsuo Suda, Showa University, Japan. Compound A, identified as 24-keto,$23,25\text{-}(OH)_2D_3$ [18] was generated from $25(OH)D_3$ using the perfused rat kidney [19].

Solvents

All solvents were distilled-in-glass spectroscopic grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Chromatography

HPLC was performed on a Model LC204 fitted with a Model 6000A pumping system, WISP automatic sample injector and Model 440 UV fixed-wavelength (254 nm) detector (all from Waters Assoc., Milford, MA, U.S.A.). Integration of peaks was performed using a Sigma 10 chromatography data station in the peak area mode (Perkin-Elmer, Norwalk, CT, U.S.A.). A stainless-steel Zorbax-CN column (25 cm × 4.6 mm I.D., 6 μm microspheres) was a gift of Dr. John Larmann (DuPont Instruments, Wilmington, DE, U.S.A.). Solvent systems used in the study were originally developed for silica column and based upon mixtures of hexane—isopropanol—methanol [9].

Competitive protein binding assay

$25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$, $25(S),26\text{-}(OH)_2D_3$ and $25(OH)D_3$-26,23-lactone were measured in some experiments by a competitive-protein binding assay utilizing vitamin D binding globulin (DBP) present in rachitic rat serum. Such an assay is based upon that of Belsey et al. [20] modified to include bovine serum albumin [21]. The metabolite under investigation was tested for its ability to displace $[^3H]25(OH)D_3$ from DBP and compared to a standard curve of non-radioactive $25(OH)D_3$ ranging from 25 to 1000 pg per

tube. Since $25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$ and $25(S),26\text{-}(OH)_2D_3$ each displace $[^3H]25(OH)D_3$ equally well [22, 23] results obtained with these metabolites are directly comparable. However, $25(OH)D_3$-26,23-lactone has been reported to have a 5-fold higher affinity for $[^3H]25(OH)D_3$ than $25(OH)D_3$ in this assay [10]. Though in our hands this was only a 2.5-fold difference a correction factor must be applied to the results for this compound when using a standard curve of $25(OH)D_3$.

Animal experiments

Adult male Wistar rats (Camm Laboratories, Toronto, Canada) were fed a standard rodent diet (Masterfeed Laboratory, Toronto, Canada). Kidneys were cannulated, surgically removed and perfused with a Krebs—Henseleit buffer containing 6% bovine serum albumin [19]. $25(OH)D_3$ (500 ng/ml) mixed with 1 μCi $[26,27\text{-}^3H]25(OH)D_3$ (20 Ci/mmol, Amersham, Arlington Heights, IL, U.S.A.) was added to the perfusate (100 ml) in 200 μl ethanol. Aliquots of perfusate (2 ml) were removed at hourly intervals and lipid extracted according to the method of Bligh and Dyer [24]. After nitrogen evaporation of the organic phase each extract was redissolved in hexane—isopropanol—methanol (94:5:1), centrifuged at 1000 *g* for 5 min and the supernatant fluid (200 μl) subjected directly to chromatography.

Female rabbits (Reimans Fur Ranch, St. Agatha, Canada) were orally dosed with 250 μg vitamin D_3/kg body weight/day (in ethanol). Daily blood samples were taken from the ear vein. Lipid extraction of 250-μl samples of plasma paralleled the analysis of kidney perfusates. Chromatography of plasma extracts again employed direct injection of the total lipid material.

RESULTS

The baseline separation of $25(OH)D_3$, $24(R),25\text{-}(OH)_2D_3$, 24-keto,$23,25\text{-}(OH)_2D_3$ and $25(OH)D_3$-26,23-lactone on Zorbax-CN using hexane—isopropanol—methanol (94:5:1) is shown in Fig. 1. Retention times of these com-

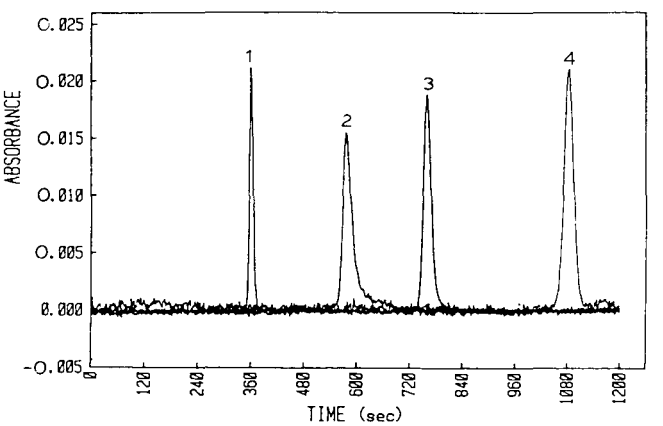

Fig. 1. Chromatographic separation of $25(OH)D_3$ (1), $24(R),25\text{-}(OH)_2D_3$ (2), 24-keto,$23,25\text{-}(OH)_2D_3$ (3) and $25(OH)D_3$-26,23-lactone (4) on Zorbax-CN using the solvent system hexane—isopropanol—methanol (94:5:1) and a flow-rate of 1.4 ml/min.

TABLE I

RETENTION TIMES OF VITAMIN D METABOLITES ON ZORBAX-CN AND ZORBAX-SIL

Metabolites	Retention time (min)	
	Zorbax-CN*	Zorbax-SIL**
Vitamin D_3	3.87	3.60
25(OH)D_2	6.66	6.28
25(OH)D_3	7.11	6.78
24-Keto,25-OH-D_3	9.78	7.54
24(*R*),25-$(OH)_2D_2$	11.30	10.39
24(*R*),25-$(OH)_2D_3$	11.43	11.08
24-Keto,23,25-$(OH)_2D_3$	14.82	11.32
25(*S*),26-$(OH)_2D_3$	14.09	14.49
1α,25-$(OH)_2D_3$	18.66	21.90
25(OH)D_3-26,23-lactone	20.71	11.71

*Chromatographic conditions: column, 25 cm × 4.6 mm; mobile phase, hexane—isopropanol—methanol (94:5:1); flow-rate, 1.3 ml/min.
**Chromatographic conditions: column, 25 cm × 4.6 mm; mobile phase, hexane—isopropanol—methanol (91:7:2); flow-rate, 1.5 ml/min.

pounds, and a series of other D compounds, on Zorbax-CN are shown in Table I. For comparison are shown the retention times of the same compounds on Zorbax-SIL using a slightly more polar solvent system hexane–isopropanol–methanol (91:7:2). It is clear that in general a more polar solvent is required to elute vitamin D metabolites from Zorbax-SIL than from Zorbax-CN. However, it is also evident that within the vitamin D group, those metabolites containing a carbonyl residue [24-keto-25(OH)D_3, 24-keto-23,25-$(OH)_2D_3$ and 25(OH)D_3-26,23-lactone] are more strongly retained on Zorbax-CN than their dihydroxy and monohydroxy analogues. Thus, 24(*R*),25-$(OH)_2D_3$ and 25(OH)D_3-26,23-lactone, which are poorly resolved on Zorbax-SIL, become widely separated on Zorbax-CN with no appreciable change in the solvent selectivity.

We applied this chromatographic procedure to the analysis of rabbit plasma in an attempt to separate the principal circulating metabolites. Application of the method to the analysis of hypervitaminotic D plasma enabled us to observe the UV (254 nm) absorption of each separated metabolite and measure it using an integrator (Fig. 2). Alternatively, collection of 0.5-min fractions of chromatographic effluent, evaporation of mobile phase and dissolution in ethanol permitted the application of a competitive protein binding assay to quantitate the separated D metabolites (Fig. 3). Both assay techniques gave similar results with measurable peaks of 25(OH)D_3, 24(*R*),25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$ and 25(OH)D_3-26,23-lactone. Note that peaks are displaced to the right in the binding assay trace because of delays involved during collection. Results for 25(OH)D_3-26,23-lactone in the binding assay are expressed in equivalents of 25(OH)D_3 and reflect its 2.5-fold higher affinity for DBP. Levels of other metabolites [e.g. 23,25-$(OH)_2D_3$] are below the

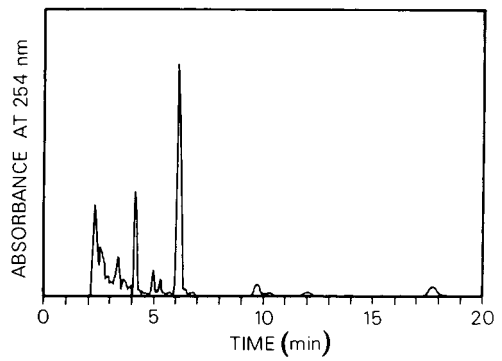

Fig. 2. UV_{254} chromatographic profile of hypervitaminotic rabbit plasma on Zorbax-CN. Conditions as in Fig. 1. Peaks were identified by comparison to standards. Absorbance scale = 0.1 absorbance units full scale. 250 µl plasma were extracted and the recovery of added tracer [^{3}H]25(OH)D_3 was 76%. Integrator gave 25(OH)D_3 = 6.15 min, 202 ng; 24,25-$(OH)_2D_3$ = 9.83 min, 21.5 ng; 25,26-$(OH)_2D_3$ = 12.20 min, 8.53 ng; 25(OH)D_3-26,23-lactone = 17.99 min, 32.2 ng.

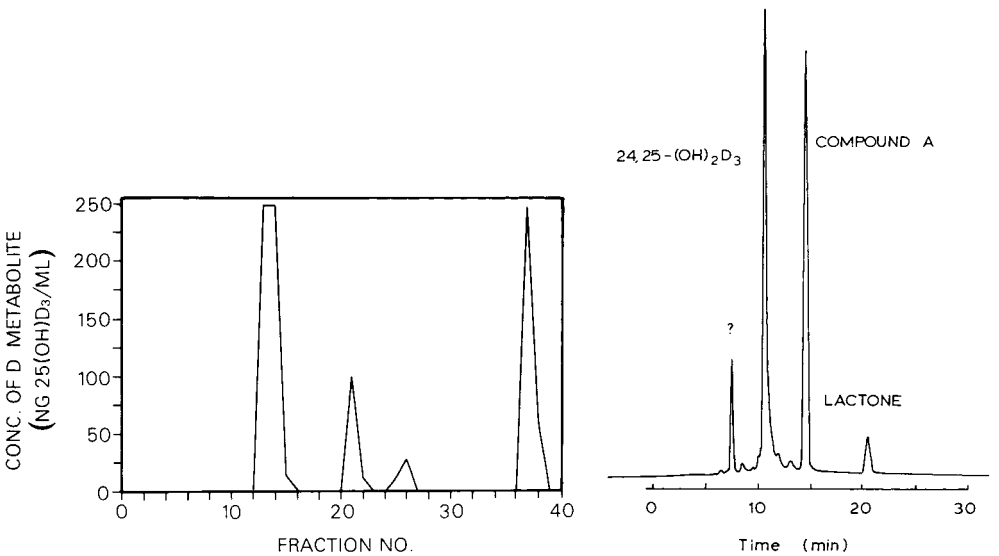

Fig. 3. Competitive protein binding assay of fractions from the chromatographic run shown in Fig. 2. Each fraction was dissolved in 1 ml of ethanol and 50 µl were assayed for ability to displace [^{3}H]25(OH)D_3 from DBP. Each data point represents the mean of triplicate determinations and results are expressed in nanogram equivalent of 25(OH)D_3 per ml plasma.

Fig. 4. Separation of the 24,25-$(OH)_2D_3$ fraction from Zorbax-SIL by Zorbax-CN chromatography. Conditions: Solvent, hexane—isopropanol—methanol (94:5:1); flow-rate, 1.4 ml/min. Compound A was subsequently identified as 24-keto,23,25-$(OH)_2D_3$.

detection limit of each assay technique or else it is possible that these minor components co-migrate with one of the major components.

Kidney perfusate extracts were also well resolved by Zorbax-CN chromatography (Fig. 4). The 24(R),25-$(OH)_2D_3$ region from Zorbax-SIL chromatography containing a number of closely migrating, partially resolved peaks was

well separated on Zorbax-CN. Again, the presence of a >C=0 group in 24-keto-23,25-$(OH)_2D_3$ increased its retention on Zorbax-CN permitting it to be obtained in pure form for identification and enabling us to measure its production rate independently of 24(*R*),25-$(OH)_2D_3$ and 25$(OH)D_3$-26,23-lactone.

DISCUSSION

We report in this paper the novel use of a cyano-bonded phase packing in the separation of vitamin D metabolites. The chromatographic system is particularly useful for the separation of >C=0 containing vitamin D compounds from their hydroxylated analogues and in particular for the resolution of 24(*R*),25-$(OH)_2D_3$ and 25$(OH)D_3$-26,23-lactone. This is a difficult separation using isopropanol—hexane solvent mixtures [8] and although feasible using methylene chloride [10] it is only possible at the expense of losing the separation of 25$(OH)D_3$ and 25$(OH)D_3$-26,23-lactone. The new system utilizing Zorbax-CN and hexane—isopropanol—methanol solvent mixtures permits resolution of all three principal circulating metabolites.

The new chromatographic system described here allows for the simultaneous analysis of 25$(OH)D_3$, 24(*R*),25-$(OH)_2D_3$ and 25$(OH)D_3$-26,23-lactone by a procedure involving extraction, one chromatographic step and measurement by either UV_{254} peak integration or competitive protein binding assay. This is of great potential in the clinical setting where unequivocal assay of these metabolites is required in order to determine the clinical usefulness of each.

The chromatographic system using UV_{254} peak integration promises to be of great potential to the clinician monitoring vitamin D-intoxication in patients given large doses of vitamin D_3. Small plasma volumes (e.g. 250 μl used here) are sufficient for this purpose. Where greater sensitivity is required the chromatographic system must be used in conjunction with a competitive protein binding assay of the type described here (B_{50} ca. 200 pg per tube or less). Such a combination permits analysis of 25$(OH)D_3$, 24(*R*),25-$(OH)_2D_3$ and 25$(OH)D_3$-26,23-lactone in as little as 250 μl if the plasma concentration of each metabolite is greater than 1 ng/ml. Plasma levels below 1 ng/ml are best measured by scaling up the conditions employed here (e.g. 500 μl or 1 ml plasma extracted and directly chromatographed on a 25 cm × 6.2 mm Zorbax-CN column) along the lines suggested by our previous work [25].

The other area where Zorbax-CN chromatography has proved to be of value is in the purification of vitamin D metabolites prior to identification. The technique has already allowed us to recognise the existence of and identify a potentially important renal product, 24-keto,23,25-$(OH)_2D_3$. In addition, it will provide a useful procedure to aid the researcher who wishes to confirm identity of a vitamin D peak during metabolic studies.

In previous work [9] we have demonstrated the importance of the chemical composition (or selectivity, α) of the solvent in the separation of vitamin D metabolites. Here we have demonstrated the importance of the chemical properties of the column packing on this separation. We assume that the increased retention of >C=0 containing vitamin D metabolites is due to their

chemical interaction with —C≡N on the surface of the chromatographic packing. This example serves to illustrate the potential value of this —C≡N packing to the more general field of steroids containing >C=0 groups.

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ANALYSIS AND KINETICS OF 2,4-DINITROPHENOL IN TISSUES BY CAPILLARY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Five groups of six ICR mice were orally dosed with 22.5 mg/kg 2,4-dinitrophenol. Groups were sacrificed at 1, 3, 6, 12, and 24 h post treatment, and serum, liver, and kidney tissues were collected for analysis of dinitrophenol content. Quantitation was performed via a capillary gas chromatography—mass spectrometry technique after liquid—liquid extraction of biological specimens spiked with a trideuterated dinitrophenol internal standard. Concentration versus time data for each tissue were subjected to pharmacokinetic analysis. Similar two-compartment open models were found to characterize most phases of the disposition of this compound. The kidney appears to maintain a more persistent low concentration of 2,4-dinitrophenol.

INTRODUCTION

The compound 2,4-dinitrophenol (2,4-DNP) [51-28-5] possesses a number of well characterized biological activities [1] and toxicological properties [2]. This compound is a member of the original group of compounds identified by the United States Environmental Protection Agency as priority pollutants. While many toxicological properties of 2,4-DNP are known, its potential for more subtle genotoxic or reproductive effects remains inadequately studied [3, 4]. There is a single report in the literature describing a teratogenic interaction between insulin and 2,4-DNP [5]. Furthermore, the specific influence of pharmacokinetic factors upon reproductive toxicity is an area of developing interest in the field of toxicology. Thus, our interest in 2,4-DNP kinetics has arisen from the proposition that this factor plays a significant role in reproductive toxicity.

As is the case with most environmental contaminants, little is known about the biological fate of 2,4-DNP in vitro [6, 7], and an early study of in vivo

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kinetics has utilized rather non-specific and insensitive photometric techniques [8]. We have developed a sensitive and specific quantitative analysis for 2,4-DNP utilizing capillary gas chromatography coupled with mass spectrometry (GC—MS). This method employs a liquid—liquid extraction procedure which is applicable to biological tissue specimens. A study of the kinetic disposition of 2,4-DNP in serum, liver, and kidney tissues in the mouse was undertaken utilizing concentration data obtained with the analytical method described in this report. The kinetic data derived from this study may be of practical use in further toxicological investigations upon 2,4-DNP.

EXPERIMENTAL

Subjects

Thirty-six ICR mice were used in this study. Six animals were used as controls and 30 were orally dosed via intubation with one half the reported oral dose LD_{50} of 2,4-DNP in this species [9]. The dose was 22.5 mg/kg and it was administered as a solution in isotonic $NaHCO_3$ (1.4%) such that 150 μl of the preparation were used per 10.0 g of mouse body weight. Groups of six mice each were killed by exsanguination at intervals of 1, 3, 6, 12, and 24 h post treatment. Blood was collected, allowed to clot, and centrifuged to obtain small volumes of serum. In addition, liver and kidney tissues were collected. Serum and all tissues were stored at —20° C until analysis.

Assay

All specimens and standards were subjected to a liquid—liquid extraction procedure to clean up the samples prior to chromatography. Specimens of serum ranging in volume from 100 to 300 μl were diluted to a final volume of 1.0 ml with saline. A 100-μl aliquot of distilled water containing 1.0 μg of 2,4-dinitrophenol-3,5,6-d_3 (d_3-2,4-DNP, Merck) was added as a stable isotope internal standard to all serum samples and to standards prepared in normal human serum. Liver and kidney samples were mechanically homogenized (1:20, w/v) in saline. Homogenate (0.5 ml) was added to 0.5 ml saline to yield 25 mg tissue per 1 ml total aqueous tissue preparation in each tube. Tissue 2,4-DNP standards were prepared by adding known amounts of analyte to homogenates of control (untreated) mouse tissues. These tissue homogenates, specimens and standards, also received 100 μl of d_3-2,4-DNP internal standard solution. All samples were acidified with 1.0 ml McIlvaine's citrate buffer (pH 3.0) [10] and extracted twice with 5 ml diethyl ether (Burdick & Jackson Labs., double distilled in house before use). The organic phase was counter-extracted with two 1-ml volumes of freshly prepared 5% aqueous $NaHCO_3$. The resulting aqueous phase was acidified with 250 μl of 6 *N* HCl and re-extracted twice into 5 ml diethyl ether. The organic phase was dried with 60 mg anhydrous sodium sulfate (Suprapur®, E.M. Reagents). Following removal to a clean conical tube, the diethyl ether was evaporated away at 60° C under a stream of Zero Grade nitrogen (Air Products). Samples were reconstituted with 10 μl benzene (Burdick & Jackson Labs.) and a 1.0-μl aliquot was taken for injection into the gas chromatograph.

Quantitation of 2,4-DNP was performed using a Finnigan 4000 GC—MS

system. Chromatographic separation of 2,4-DNP was achieved on a 30 m × 0.25 mm I.D. DB-5 (1.0 μm thick film) bonded phase fused silica capillary column (J&W Scientific) with helium carrier at a flow-rate of 29 cm/sec using splitless injection. Conditions required for this separation were: injector 220°C, oven 70°C for 30 sec then programmed to 210°C at 8°C/min. At 30 sec post injection, the injector was vented with a helium flow-rate of 32 ml/min to eliminate excess solvent vapors. The septum was swept with 13 ml/min helium. The interface chamber was maintained at 250°C and the capillary column was led from the oven, through the interface, and directly into the ion source assembly of the mass spectrometer. Mass spectrometer data acquisition was performed using electron ionization (ion source temperature, 250°C; emission current, 0.30 mA; electron multiplier voltage, −1490 V; electron energy, 70 V) and multiple ion detection (MID) for the base peak (also molecular ion) of 2,4-DNP (*m/z* 184) and d_3-2,4-DNP (*m/z* 187) with an instrument dwell time of 0.1 sec for each mass.

Peak area ratios of analyte and internal standard ion peaks (*m/z* 184/187) were determined for the standards containing known amounts of 2,4-DNP. Linear standard curves of concentration vs. peak area ratios resulted by which unknown samples were quantified via interpolation from the regression line. Standard curves were generated each day analyses were performed using the appropriate sample matrix for the samples in question. The concentration data derived from each mouse tissue were grouped within each sampling time post treatment and then subjected to pharmacokinetic data analysis. The ESTRIP

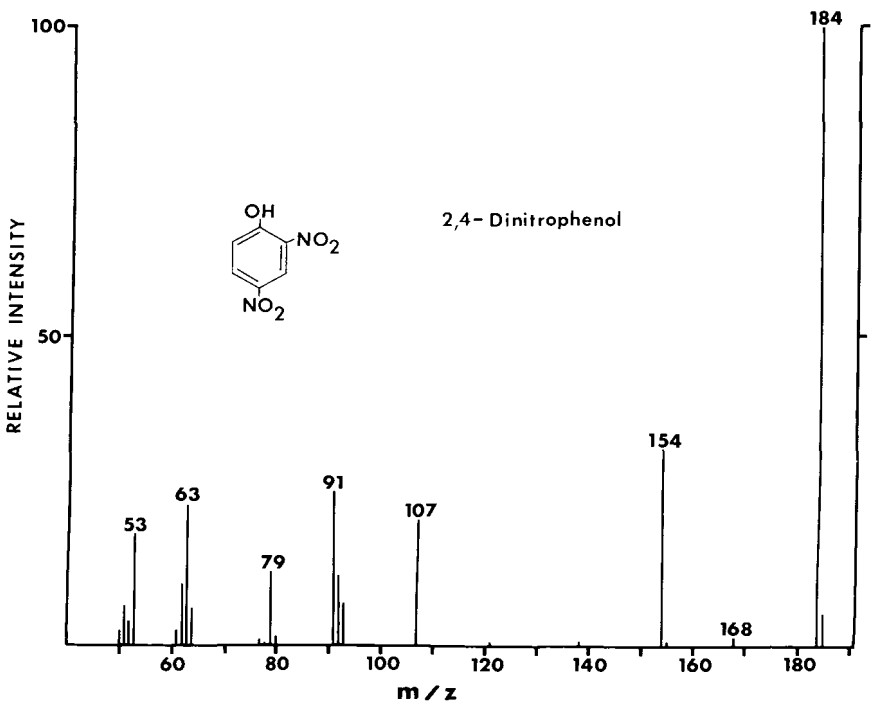

Fig. 1. Electron ionization spectrum of 2,4-dinitrophenol. The parent ion (*m/z* 184) is also the base peak of this compound. Trideuterated 2,4-DNP also yields a molecular ion (*m/z* 187) that is the base peak.

program [11], run on a Microproducts minicomputer, was used to generate the best fitting polyexponential function for the concentration vs. time data obtained in this study. These equations were used to generate the curves representing mean disposition of 2,4-DNP in all three tissues as a function of time with a Hewlett-Packard 8925A calculator and plotter.

RESULTS

Standards

A complete low-resolution mass spectrum of 2,4-DNP is shown in Fig. 1 to illustrate the suitability of molecular ion monitoring in this assay. The compound is sufficiently stable to yield a molecular ion base peak. Thus the base peak from the trideuterated internal standard is conveniently three mass units greater than that of the analyte. Sets of standards run with each batch of samples were used to quantitate that group of specimens. These daily batches of standards were intended to accommodate any variability resulting from instrumental parameter changes or column degradation. When all standard runs for each tissue are examined as a group (Fig. 2), quite good reproducibility is evidenced by small coefficients of variation at each data point. Key coefficients

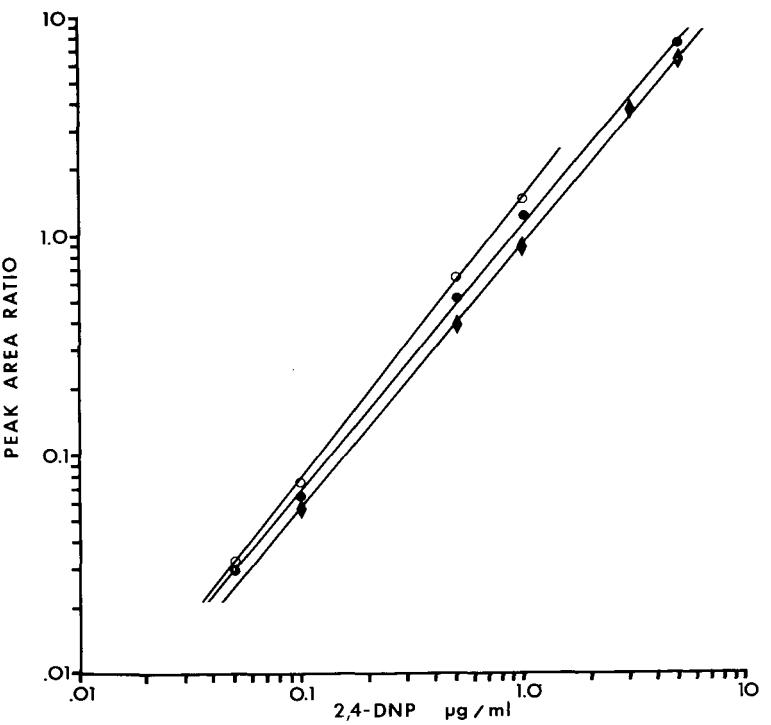

Fig. 2. Means of standard curves which were generated during each daily analysis and used to quantitate 2,4-DNP in serum, liver, and kidney tissues. Peak area ratios of 2,4-DNP and d_3-2,4-DNP base peaks were similar in the different tissue matrices. Standard errors of the mean are not shown as the errors were smaller than the symbols designating each mean value. The method exhibited satisfactory day-to-day reproducibility for all three tissues. Serum, n = 5 (♦); kidney, n = 5 (•); liver, n = 4 (○).

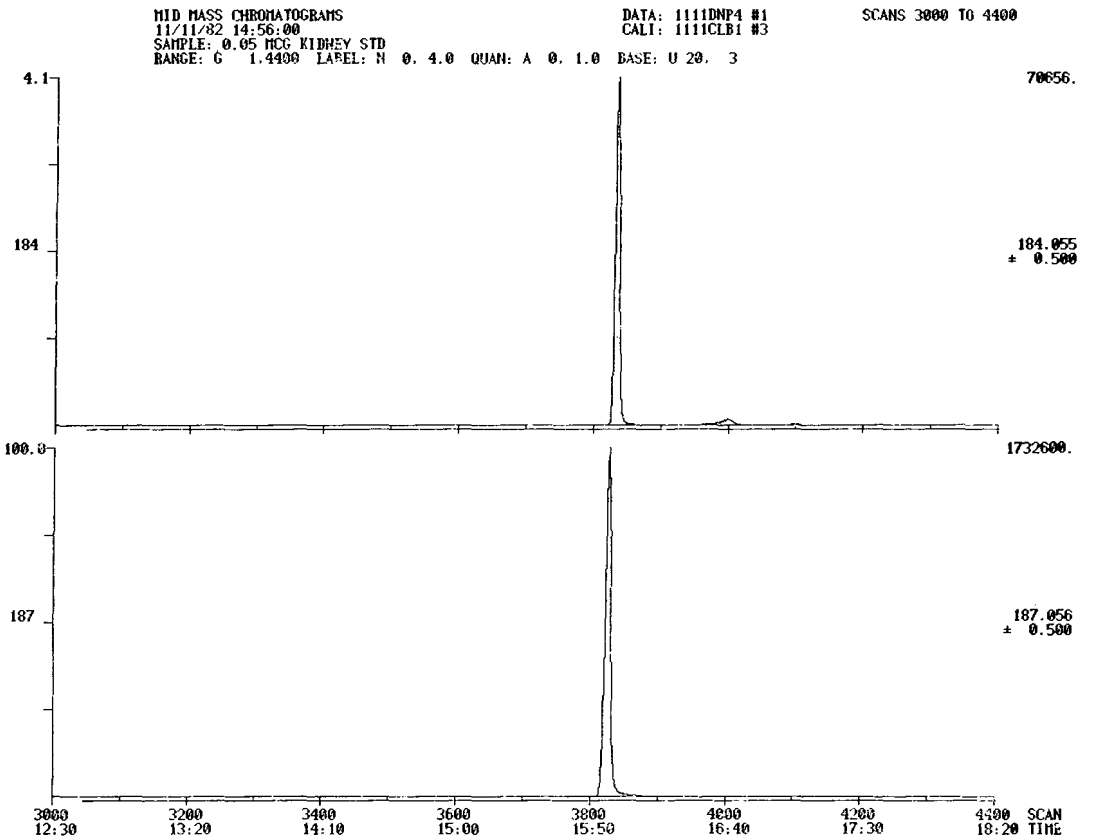

Fig. 3. Base peak mass chromatograms of 2,4-dinitrophenol and the trideuterated 2,4-dinitrophenol-3,5,6-d_3 internal standard obtained from a 50-ng (in 25 mg) kidney tissue standard. The retention time for 2,4-DNP was 16 min under the conditions employed in this assay and this standard gave a molecular ion signal of 70,656 ion counts. Note how this acidic compound exhibits little peak tailing on the DB-5 capillary column.

of variation were as follows: 1 μg/ml serum, 3.5%; 1 μg per 25 mg liver, 2.5%; 1 μg per 25 mg kidney, 10.0%. This analytical technique is also characterized by high sensitivity. Fig. 3 represents a typical ion chromatogram obtained from the 50-ng (in 25 mg tissue) kidney standard. The very high ion count (70,656) derived from this technique is suitable for other applications requiring much greater sensitivity or employing smaller samples of biological material. The method appears to be at least an order of magnitude more sensitive than required for this study. Examination of extraction efficiency of standards indicated that the double extractions used throughout this study yielded about 70% recovery of 2,4-DNP. Aqueous solutions of 2,4-DNP and d_3-2,4-DNP were found to be stable for more than a month while maintained at 4°C.

Pharmacokinetics

Results of the 2,4-DNP concentration measurements for each tissue were averaged in each sampling time period and analyzed with ESTRIP. With the minimal number of sampling intervals used in this study, a two-compartment open model was found to best represent the disposition of 2,4-DNP in all tissues. The summary of calculated kinetic parameters is presented in Table I. It is apparent from the quite similar half-times for absorption, distribution, and elimination (except in kidney), observed in these three tissues that rapid 2,4-DNP exchange occurs between these sites. Major differences are seen in the coefficients of the exponential equations indicating different relative peak concentrations of 2,4-DNP among the tissues. In Fig. 4 we have plotted the best fitting functions for the concentration vs. time data for each tissue. Here it is very clear that the primary difference among the serum, liver, and kidney kinetics is the maximum concentration attained and the uniquely slow elimination from the kidney. Curve shape is obviously similar with calculated peak concentrations occurring at the same time, approximately 1 h post treatment.

TABLE I

SUMMARY OF ESTRIP PHARMACOKINETIC PARAMETERS FOR 2,4-DINITROPHENOL IN THE MOUSE

Data are based upon the best fitting triexponential equation of the form $C_t = A_3\exp(-B_3t) + A_2\exp(-B_2t) + A_1\exp(-B_1t)$ where $B_1 = \beta$; $B_2 = \alpha$; and $B_3 = k_{abs}$ in other notation.

Tissue	Subscript	Coefficient (A)	Exponential (B)	$t_{1/2}$ (h)	F*	r^2**
Serum	1	2.821	0.09	7.70	6.81	0.997
	2	165.300	0.57	1.20		
	3	−168.100	1.39	0.50		
Liver	1	0.213	0.08	8.70	1.77	0.974
	2	44.410	0.69	1.00		
	3	−44.620	1.17	0.59		
Kidney	1	0.173	$9.1 \cdot 10^{-3}$	76.20	2.87	0.971
	2	46.410	0.61	1.14		
	3	−46.590	1.11	0.62		

*F = sum of squared variances.
**r^2 = estimate of goodness of fit between calculated function and data.

DISCUSSION

The description of 2,4-DNP kinetics reported here is apparently the first such data based upon a highly sensitive and specific GC—MS analytical technique. Many years ago a spectrophotometric technique was developed for quantitating 2,4-DNP [6]. This relatively nonspecific method was used in an elegant kinetic study of disposition in serum and ocular tissues of the duck and rabbit [8]. In that study of the relationship between kinetics and cataractogenic potential of 2,4-DNP, clearance from duck serum was found to be a biexponential process with rate constants similar to those reported here in the mouse. Various other analytical techniques have also been applied to the

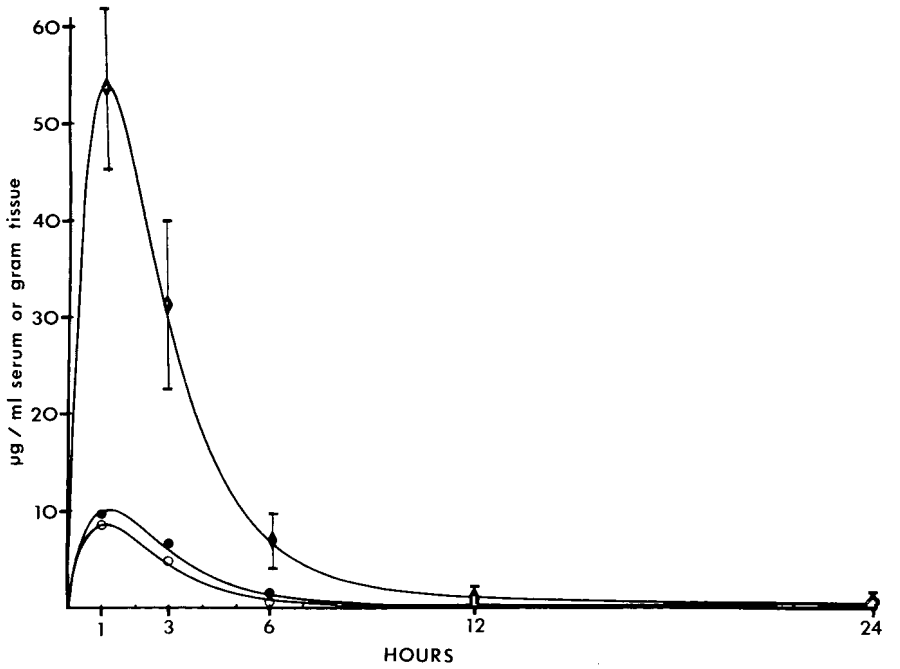

Fig. 4. Computer-plotted curves of 2,4-dinitrophenol disposition in mouse serum (♦), liver (○), and kidney (•) after oral administration of 22.5 mg/kg. Mean values are plotted for each time period and standard errors for the serum data are designated by brackets. Liver and kidney S.E.M. values were too small to be plotted. It is apparent that the major difference among these curves is their magnitude.

qualitative determination of 2,4-DNP. An extremely sensitive bioluminescent assay has been reported quite recently [12]. Also, a high-performance liquid chromatographic technique has been described which is very sensitive [13]. While these techniques are very capable of quantitating trace amounts of 2,4-DNP, their selectivity may be questionable when dealing with complex biological materials. These methods have not been applied to the assessment of 2,4-DNP kinetics in tissues.

Prior to the development of capillary columns with covalently bonded liquid phases, gas—liquid chromatography of polar compounds had been difficult. Derivatization of 2,4-DNP in industrial effluents has been attempted to improve gas—liquid chromatographic separation, but this was reported to result in little improvement [14]. Under the conditions used in this analytical technique, excellent chromatographic resolution was attained with the very acidic 2,4-DNP molecule in underivatized condition. We had originally hoped to apply a technique similar to this analysis to quantify selected metabolites of 2,4-DNP. The nitro-reduced metabolites are of particular interest as they have been identified in vitro [7], and the 4-amino-2-nitrophenol species has been identified as a potential carcinogen [15]. Technical difficulties associated with extraction of the metabolites have to date foiled attempts to analyze these amino derivatives in biological samples in our laboratory. Pure 4-amino-2-nitrophenol does elute from the DB-5 capillary column (data not shown)

under the conditions employed for 2,4-DNP analysis as do acetylated derivatives of 2,4-DNP, 4-amino-2-nitrophenol and 2-amino-4-nitrophenol. Future work with reduced metabolites may utilize such acylated derivatives.

The pharmacokinetics reported here must be viewed as preliminary data. Since a two-compartment open model seems to fit observed concentration vs. time data in all tissues examined, additional time points should be utilized to more accurately characterize each phase of disposition. Ideally, at least four data points should be employed in defining each exponential term of the kinetic function. The merit of this study and, indeed, the major point of interest lie in the fact that parallel samples were obtained and that the results indicate a quite close correspondence in concentrations among these tissues at all sampling times. The very close similarities in absorption and distribution may well result from the fact that 2,4-DNP is soluble in both lipids and water. The apparent persistence of 2,4-DNP in the kidney tissues ($t_{½}$ 76 h) may be a result of tissue binding of this compound, since protein binding is a recognized phenomenon with this material [8]. While 2,4-DNP binding sites have been assumed to exhibit uniform affinity, our data suggest that there may exist some differences among tissues.

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CHROMBIO. 1718

DETERMINATION OF NOMIFENSINE IN HUMAN SERUM

A COMPARISON OF HIGH-PERFORMANCE LIQUID AND GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid (HPLC) and gas—liquid chromatographic (GLC) methods for the measurement of the antidepressant nomifensine in human serum were developed and compared for precision, accuracy, sensitivity and convenience. No significant difference was found between these two techniques with regard to sensitivity and precision. Both methods can accurately measure serum nomifensine concentrations down to 8 nmol/l. The coefficient of variation (C.V.) for intra-assay variability of nomifensine was 4.8% (HPLC) and 5.5% (GLC) at 150 nmol/l. The HPLC method proved to be both simpler and more selective than the GLC method. The calibration graph was linear over the range 8—1000 nmol/l in the HPLC method, but only up to 150 nmol/l in the GLC method. The selectivity and simplicity of the HPLC method make it useful for both pharmacokinetic studies and therapeutic serum level monitoring of nomifensine. The HPLC method was applied to the analysis of serum samples obtained from four healthy individuals receiving therapeutic dosages of nomifensine.

INTRODUCTION

Nomifensine is a new antidepressant agent with a pharmacological profile different from the classical tricyclic antidepressants [1—3]. A number of methods have been described for the measurement of nomifensine, gas chromatographic procedures having been applied successfully to its analysis in serum [4—6]. This approach, however, requires prior derivatization to a fluorinated derivative and is relatively time consuming.

A radioimmunoassay (RIA) for nomifensine in serum has also been reported [7]. Since a correction for cross-reactivity of the nomifensine conjugate has

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to be made, the accuracy of that method may be suspect. Only one high-performance liquid chromatographic (HPLC) method for nomifensine has been published [8]. The detection limit of that method, about 84 nmol/l, is insufficient for pharmacokinetic studies.

As the currently available techniques for the analysis of nomifensine are either time consuming or lack sensitivity and accuracy, a selective and sensitive HPLC method for the determination of low levels of nomifensine in human serum has been developed. A gas—liquid (GLC) chromatographic method was also developed and then compared for accuracy, reliability and convenience with HPLC.

A study of the pharmacokinetics of nomifensine in healthy individuals after a single dose was made using the HPLC method described.

EXPERIMENTAL

Materials

Nomifensine maleate (M.W. 238.3 as nomifensine base) and *p*-chlorodisopyramide were obtained from Leiras Pharmaceutical Plant (Turku, Finland) and desmethylmaprotiline from Giba-Geigy (Basle, Switzerland). *n*-Hexane, acetonitrile and ethyl acetate were of analytical-reagent grade (E. Merck, Darmstadt, G.F.R.). Heptafluorobutyric anhydride (Pierce, Rockford, IL, U.S.A.) was used to prepare heptafluorobutyrate derivates of nomifensine.

p-Chlorodisopyramide solution in methanol (5 μg/ml) was used as the internal standard in the HPLC method and desmethylmaprotiline solution in ethanol (10 μg/ml) in the GLC method. Serum standards were prepared by spiking fresh human serum to concentrations within the range 12.5—1000 nmol/l.

High-performance liquid chromatography

Extraction procedure. A serum sample (1 ml) was made alkaline with 1 ml of 2 *M* sodium hydroxide solution and 125 μl of internal standard solution (5 μg/ml) were added. This mixture was extracted twice with *n*-hexane (5 ml) and the combined organic extracts were evaporated to dryness at 40°C under a gentle stream of pure nitrogen. The dry residue was dissolved in 100 μl of 0.05 *M* phosphoric acid and 50 μl were injected into the chromatograph.

Chromatographic analysis. Analyses were carried out on a Model SP 740 HPLC system (Spectra-Physics, Santa Clara, CA, U.S.A.) using an HP 1030B UV detector (Hewlett-Packard, Waldbronn, G.F.R.) set at 205 nm. An SP 4100 computing integrator (Spectra-Physics) was used to calculate peak heights. The mobile phase consisted of acetonitrile—0.05 *M* potassium phosphate buffer (26:74, v/v) (pH 3.5) and the flow-rate was 1.6 ml/min. The reversed-phase column was a 10-μm μBondapak C_{18}, 30 cm × 3.9 mm I.D. (Waters Assoc., Milford, MA, U.S.A.).

Gas—liquid chromatography

Extraction procedure. Internal standard (150 μl), 1 *M* sodium hydroxide solution (1 ml) and *n*-hexane (5 ml) were added to the serum sample (1 ml) and the mixture was shaken for 15 min. After centrifugation, the hexane layer

was transferred into a conical tube and 0.5 *M* hydrochloric acid (2.5 ml) was added. After shaking for 15 min the organic phase was carefully withdrawn and the acidic aqueous phase was washed with *n*-hexane (5 ml). The organic phase was removed and 2 *M* sodium hydroxide solution (1 ml) was added to the acidic aqueous phase and shaken with *n*-hexane (5 ml) for 15 min. After centrifugation the hexane layer (4.5 ml) was transferred into a clean test tube and evaporated to dryness at 40°C under a gentle stream of pure nitrogen. To the dry residue 100 μl of heptafluorobutyric anhydride (1:10 solution in ethyl acetate) were added. The tube was shaken in a mixer for about 15 sec, then incubated at 40°C for 30 min. The reaction mixture was taken to dryness under a stream of nitrogen at 40°C and finally reconstituted in 400 μl of ethyl acetate. A 2-μl volume of this solution was taken for GLC.

Chromatographic analysis. Analyses were performed on a Varian Series 2100 gas chromatograph equipped with a ^{63}Ni electron-capture detector (Varian Aerograph, Walnut Creek, CA, U.S.A.). The chromatographic column was a coiled glass tube, 2 m × 2 mm I.D., packed with 3% OV-17 on 80–100 mesh Chromosorb W HP (Applied Science Labs., State College, PA, U.S.A.). The temperatures were column 250°C, injector 270°C and detector 300°C, and the carrier gas (nitrogen) flow-rate was 30 ml/min. Chromatograms were recorded with a laboratory potentiometric recorder.

Pharmacokinetic application of the HPLC method

Four healthy volunteers in a fasting state were given 100 mg nomifensine orally in capsule form. All persons abstained from smoking and drinking alcohol at least 2 days before and during the experiments. They received a standardized breakfast 2 h and a standardized lunch 4 h after the drug administration. During 24 h (after drug administration) twelve blood collections were taken. Serum levels were determined by the HPLC method described above.

RESULTS AND DISCUSSION

High-performance liquid chromatography

Nomifensine and the internal standard, *p*-chlorodisopyramide, show good HPLC characteristics. Nomifensine produced a symmetrical, sharp peak having a retention time of 4.15 min. The internal standard eluted after nomifensine and was well separated from it (Figs. 1a, 2a and 3a).

Fig. 4 shows a typical calibration graph produced by HPLC analysis for known amounts of nomifensine in serum. The response is linear for samples containing 8–1000 nmol/l and the correlation coefficient is $\geqslant 0.998$. The precision of the assay was assessed by multiple analyses of the serum pools and standard control samples. The coefficient of variation (C.V.) for intra-assay variability of nomifensine was 4.8% at 150 nmol/l and 4.0% at 500 nmol/l ($n = 9$).

The detection limit was defined as the signal that was three times higher than the background noise. In this way the detection limit of the HPLC technique was found to be 8 nmol/l, which is sufficient for pharmacokinetic studies and therapeutic serum level monitoring. The HPLC method published earlier [8] is

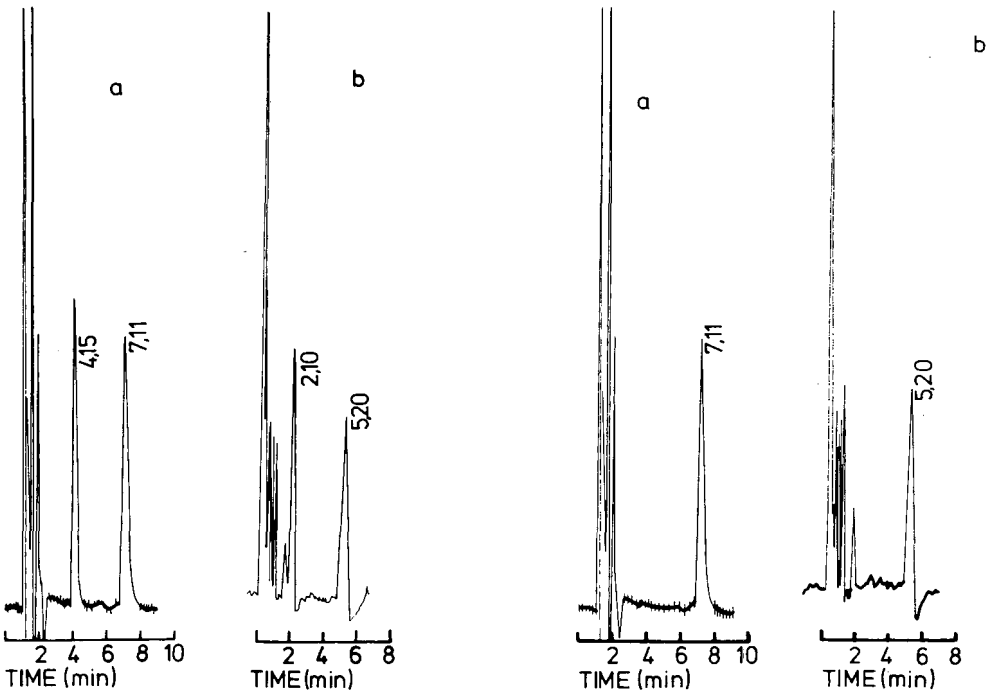

Fig. 1. (a) Liquid chromatogram of the extract from the serum sample of a volunteer 3 h after oral administration of nomifensine. The retention time of nomifensine is 4.15 min and that of the internal standard, *p*-chlorodisopyramide, is 7.11 min. The concentration of nomifensine is 354 nmol/l. (b) Gas chromatogram of the extract from blank serum spiked with 250 nmol/l of nomifensine. The retention times of nomifensine and of the internal standard, desmethylmaprotiline, are 2.10 and 5.20 min, respectively.

Fig. 2. (a) Liquid chromatogram of the extract from the blank serum. The retention time of the internal standard, *p*-chlorodisopyramide, is 7.11 min. (b) Gas chromatogram of the extract from the blank serum. The retention time of the internal standard, desmethylmaprotiline, is 5.20 min.

too insensitive for the determination of low levels of nomifensine, the detection limit being only 84 nmol/l.

The extraction efficiency was tested by determining admixtures of known amounts of nomifensine to serum in the range 25–1000 nmol/l. Comparison of the peak heights for nomifensine extracted from serum samples with those obtained after direct injection of the drug solutions into the chromatograph indicated that the extraction efficiency was 72.1% (S.D. 2.6%; $n = 8$).

Interference by other compounds was also examined. Other commonly prescribed psychotropic drugs were well separated from nomifensine and the internal standard in the column. The capacity factors for these drugs were calculated under the chromatographic conditions described (Table I).

Barbiturates sometimes encountered in the course of antidepressant therapy were found not to interfere. They are not extracted with hexane from alkalinized serum. Endogenous compounds in human serum also did not affect the HPLC analysis of nomifensine.

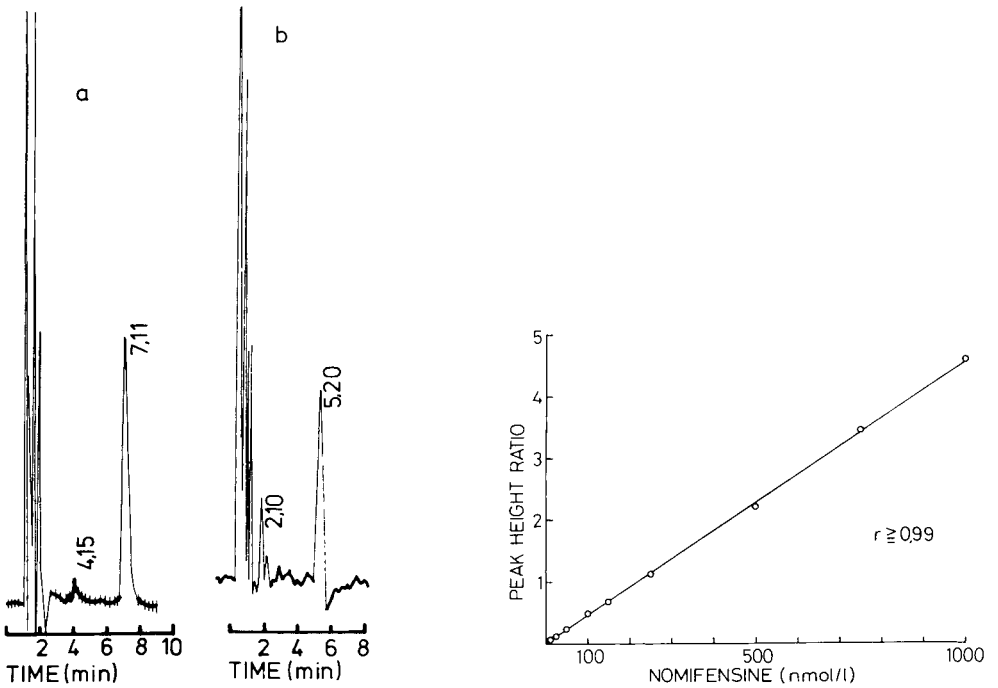

Fig. 3. (a) Liquid chromatogram of the extract from the serum sample of a volunteer 12 h after oral administration of nomifensine. The retention time of nomifensine is 4.15 min and that of the internal standard, *p*-chlorodisopyramide, is 7.11 min. The concentration of nomifensine is 17 nmol/l. (b) Gas chromatogram of the extract from blank serum spiked with 25 nmol/l of nomifensine. The retention time of nomifensine and of the internal standard, desmethylmaprotiline, are 2.10 and 5.20 min, respectively.

Fig. 4. Calibration graph for nomifensine in HPLC. Peak-height ratios of nomifensine to those of the internal standard are plotted against nomifensine concentration in serum. The correlation coefficient ($r \geqslant 0.998$) indicates linearity over the concentration range studied.

TABLE I

CAPACITY FACTORS OF SOME PSYCHOTROPIC DRUGS

For liquid chromatographic conditions, see the text.

Drug	k'	Drug	k'
Chlordiazepoxide	1.8	Oxazepam	9.2
Nomifensine	2.0	Lorazepam	11.3
Perphenazine	3.5	Desmethylimipramine	11.7
Internal standard	3.8	Desmethylmaprotiline	12.9
Desmethyldoxepin	6.4	Imipramine	13.9
Mianserin	6.7	Nortriptyline	14.8
Doxepin	7.6	Maprotiline	15.3
Haloperidol	8.2	Amitriptyline	17.3

Possible interference of the metabolites of nomifensine in serum after nomifensine administration was considered to be negligible. We noticed that the metabolites are not extractable in our extraction procedure. The assumed metabolites, hydroxynomifensine and hydroxymethoxynomifensine, are extracted at pH 8 [9].

Gas—liquid chromatography

Typical gas chromatograms of nomifensine and the internal standard, desmethylmaprotiline are shown in Figs. 1b, 2b and 3b. The retention times are

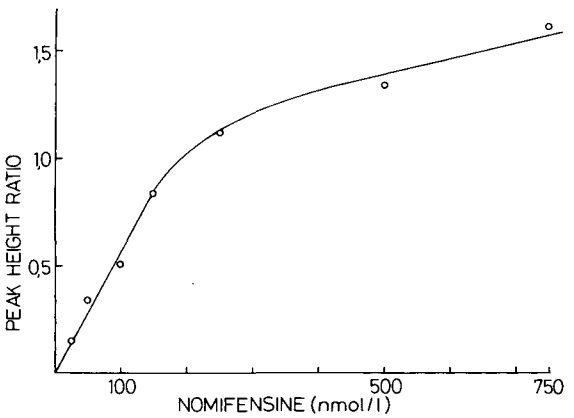

Fig. 5. Calibration graph for nomifensine in GLC. Peak-height ratios of nomifensine to those of the internal standard are plotted against nomifensine concentration in serum. The graph is linear over the concentration range 8—150 nmol/l.

TABLE II

COMPARATIVE PROPERTIES OF HPLC AND GLC FOR THE MEASUREMENT OF NOMIFENSINE IN HUMAN SERUM

Parameter		HPLC	GLC
Extraction of serum samples		One step	Three steps and derivatization
Extraction efficiency:			
at 25—1000 nmol/l (± S.D.)	(n = 8)	72.1 ± 2.6%	65.6 ± 7.7%
at 12.5	(n = 9)	68.2 ± 2.8%	—
at 25	(n = 9)	69.2 ± 5.2%	—
at 50	(n = 9)	74.8 ± 5.5%	—
at 100	(n = 9)	73.5 ± 4.9%	—
Precision (n = 9):			
at 12.5 nmol/l		4.2% (C.V.)	—
at 25		6.7%	—
at 50		5.3%	—
at 100		6.6%	—
at 150		4.8%	5.5% (C.V.)
at 500		4.0%	—
Sensitivity		8 nmol/l	8 nmol/l
Linearity		8—1000 nmol/l	8—150 nmol/l

2.10 min for nomifensine and 5.20 min for the internal standard, which indicates good resolution for these compounds. However, under the GLC conditions described here there is an interfering endogenous compound that elutes just before nomifensine. This makes the GLC assay inaccurate for the measurement of nomifensine at levels below 25 nmol/l in human serum.

The calibration graph for known amounts of nomifensine in serum obtained with the detector used here is linear only in the range 8—150 nmol/l (Fig. 5). There is no difference between HPLC and GLC with regard to precision and sensitivity. The coefficient of variation (C.V.) for intra-assay variability of nomifensine in the GLC method was 5.5% at 150 nmol/l (n = 9) and the detection limit was 8 nmol/l. The sensitivity and precision are similar to those reported by Bailey et al. [4]. The extraction efficiency in the GLC method was checked by comparing the peak heights for nomifensine extracted from serum samples with those of authentic nomifensine in methanolic solutions. The recovery was 65.6% (S.D. 7.7%; n = 7).

The comparative properties of HPLC and GLC for nomifensine analysis are shown in Table II.

Pharmacokinetic application

The serum nomifensine levels obtained after the administration of a single dose of 100 mg to four healthy individuals demonstrate that the HPLC method has sufficient sensitivity for pharmacokinetic studies (Fig. 6). Nomifensine was absorbed rapidly from the gastrointestinal tract. The peak serum concentrations of 512—1005 nmol/l were attained after 1—2 h. The elimination half-life varied between 3.6 and 3.8 h and only very low concen-

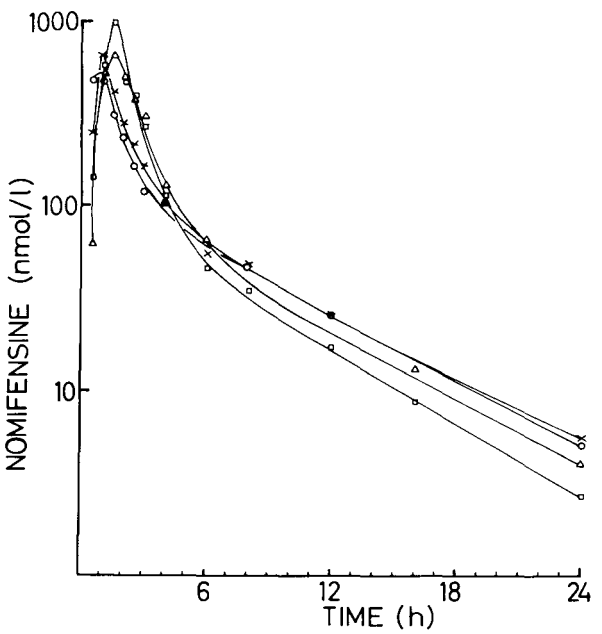

Fig. 6. Serum concentrations of nomifensine after oral administration of a single dose (100 mg) of nomifensine to four healthy volunteers.

trations were found after 24 h. These results are similar to those published earlier [10, 11].

In conclusion, the HPLC assay described here provides an efficient and accurate method for the analysis of nomifensine in human serum. We are currently using the method for pharmacokinetic studies on nomifensine after intravenous administration in dogs. The method has also been applied successfully in a study of steady-state levels in psychiatric patients. Because of the simple extraction step and the short chromatographic run time the method is useful for routine monitoring of nomifensine levels.

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ANALYSIS OF RIBOXAMIDE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED COLUMN SWITCHING

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SUMMARY

A sensitive and highly specific assay for riboxamide (TCAR) in human and canine plasma is described. The specificity of the procedure is derived from the method of sample preparation and a high-performance liquid chromatographic separation which utilizes the different selectivities of two columns. Partial separation of TCAR from plasma is achieved on a solvent-generated anion exchanger with silica gel as the solid support. The separation is completed by switching the eluent fraction containing TCAR from the first column to a second solvent-generated anion exchanger which has ODS-silica as its support. The relationship between the amount of drug injected and its peak height was linear over wide ranges of concentrations (0—10 μg/ml) and injection volumes (20—200 μl). The limit of detection for TCAR in plasma was 40 ng/ml which can be detected by injecting 200 μl of processed plasma. The recoveries from plasma were 100.2 ± 0.9% and 101.3 ± 2.3% when spiked at the 10 and 1 μg/ml levels, respectively. The applicability of the method to pharmacokinetic studies was demonstrated by following the plasma levels of TCAR after intravenous administration in the dog.

INTRODUCTION

Riboxamide (TCAR, 2-β-D-ribofuranosyl-4-thiazolcarboxamide, NSC 286193) is a C-nucleoside (Fig. 1) which was synthesized originally [1, 2] as an analogue of ribavirin. Despite exhibiting significant activity against rhino-, influenza and herpes viruses [2], the potency of TCAR was less than that of ribavirin [2]. The initial observation [2] that TCAR is a potent inhibitor of guanine synthesis led to it being investigated as an antineoplastic agent [3, 4]. It was found to have significant activity against L1210 and P388 leukemias and Lewis lung carcinoma, both in vitro [3] and in vivo [4]. As a result of these studies, TCAR is now undergoing (Phase 1) clinical trials.

High-performance liquid chromatography (HPLC) has been used to separate

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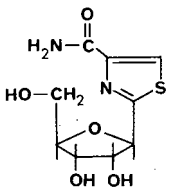

Fig. 1. The structure of riboxamide (TCAR).

the anionic metabolites of TCAR [3, 4], however, the drug itself is poorly retained on the strong anion-exchange column (Partisil 10 SAX) used. Presently, there are no analytical procedures which would be suitable for studying the pharmacokinetics of TCAR, and the development of such methodology is the subject of this investigation.

EXPERIMENTAL

Chemicals and reagents

The sample of TCAR was obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Hexadecyltrimethylammonium bromide (HTAB) was 99% pure from Aldrich (Milwaukee, WI, U.S.A.). All the other chemicals (chloroform, Na_2HPO_4, KH_2PO_4, sulfuric acid, perchloric acid and sodium hydroxide) were analytical grade from various sources. The water was distilled-in-glass, following mixed-bed deionization.

Plasma preparation

Plasma (human or canine) samples were prepared for analysis as described in Fig. 2. The plasma proteins were precipitated with perchloric acid [5] and the supernatant, obtained after centrifugation (1500 *g*), was extracted twice with chloroform. The aqueous phase was then made alkaline (pH ca. 11.5) with sodium hydroxide (10 *N*) and re-extracted twice with chloroform. HTAB was added to the recovered aqueous phase to give a final concentration of 1 m*M* HTAB. The precipitate which was formed was then removed by centrifugation (7000 *g*), and the final aqueous supernatant was analyzed by HPLC. It should be noted that the precipitate formed upon the addition of HTAB will start to resuspend if the supernatant is not removed immediately.

Chromatography

The liquid chromatography (Fig. 3) was built from various modules and designed so that selected fractions eluting from column 1 could be transferred to column 2. The system was fully automated, controlled by SLIC 1400 microprocessor (Systec, New Brighton, MN, U.S.A.) and consisted of two Altex 152 detectors (Beckman Instruments, Berkeley, CA, U.S.A.) operated at 254 nm; a WISP 710 automatic injector (Waters Assoc., Milford, MA, U.S.A.); two Altex 110A pumps; a four-port, low-pressure, switching valve (valve 1) and a Rheodyne 730 valve (valve 2, Cotati, CA, U.S.A.).

The μBondapak CN (particle size 10 μm, 300 × 3.9 mm I.D.) and Partisil 10 SAX (particle size 10 μm, 250 × 4.6 mm I.D.) columns were obtained from Waters Assoc. and Whatman (Clifton, NJ, U.S.A.), respectively. The

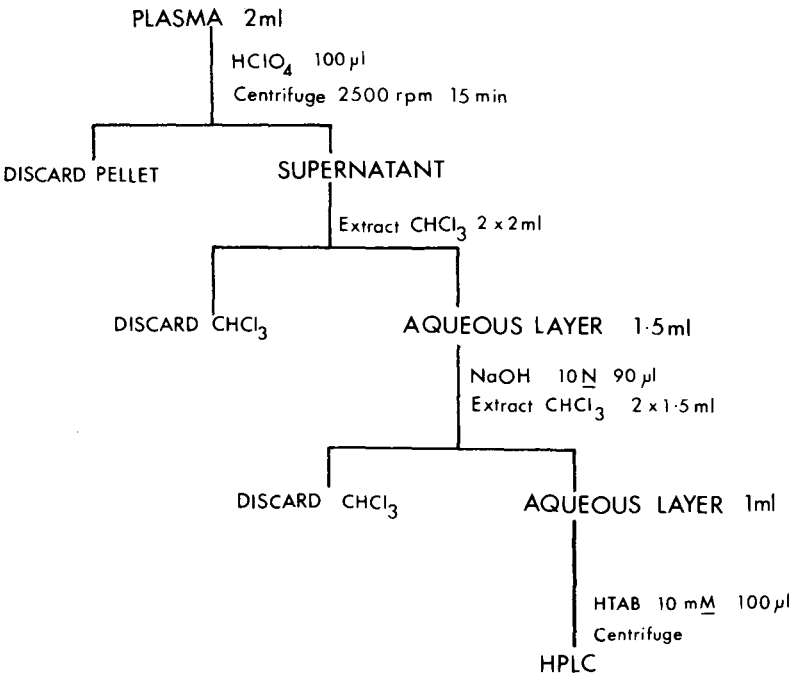

Fig. 2. Summary of the preparation of plasma samples for analysis by HPLC.

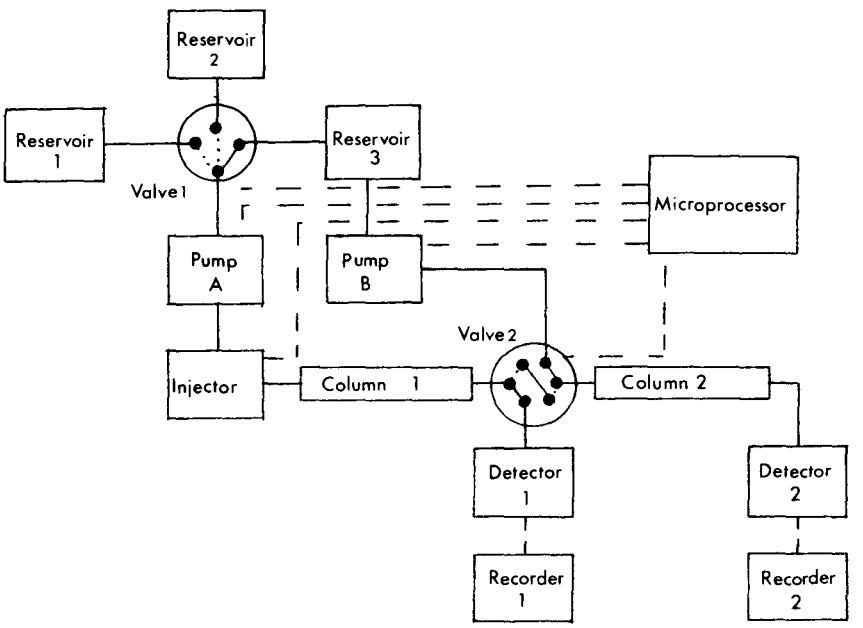

Fig. 3. Schematic representation of the chromatographic system assembled for the analysis of TCAR in plasma. See text and Fig. 4 for further explanation.

Hypersil (particle size 5 μm, 150 × 4.6 mm I.D.), ODS Hypersil (particle size 5 μm, 150 × 4.6 mm I.D.) and μBondapak C_{18} (particle size 10 μm, 250 × 4.6 mm I.D.) columns were slurry packed, as described previously [6, 7]. The Hypersil and μBondapak bulk packings were obtained from HETP (Macclesfield, Great Britain) and Waters Assoc., respectively.

For the analysis of TCAR in plasma (Figs. 3 and 4), columns 1 and 2 were packed with Hypersil and ODS Hypersil (or μBondapak C_{18}), respectively.

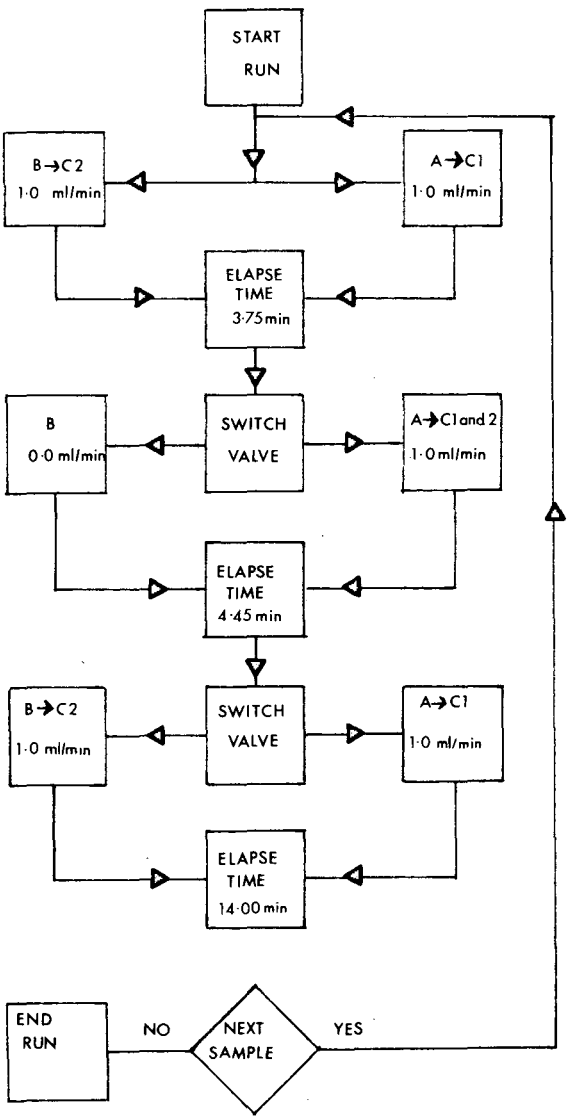

Fig. 4. Summary of the switching events for the analysis of TCAR in plasma. A, B, C1 and C2 refer to pumps A and B, column 1 (Hypersil) and column 2 (ODS Hypersil or μBondapak C_{18}), respectively. The arrows (→) within the boxes indicate the flow from the respective pumps through the columns. The elapse times are from the point of injection. See Fig. 3 for further details.

Both columns were eluted with a 10 m*M* phosphate buffer (pH 6.0) containing 1 m*M* HTAB. After injection of the sample, the fraction containing TCAR (800 μl) which eluted from column 1 was transferred to column 2, where the separation was completed. The specific details of the switching events are summarized in Fig. 4.

To remove components of the plasma preparation which failed to elute from column 1, this column was purged after each working day with water (50 ml), followed by aqueous solutions of increasing methanol concentration added in 20% increments (50 ml of each) up to 40% methanol. This procedure was facilitated by the low-pressure switching valve positioned between pump A and the three reservoirs (Fig. 3). It was not necessary to routinely wash column 2 since its chromatographic properties remained constant.

The pharmacokinetics of TCAR in the dog

TCAR (25 mg/kg) was administered to a female Beagle dog (10 kg) by injection (1 ml) into the saphenous vein. Prior to administration of the drug, the dog was fasted overnight and a "baseline" blood sample taken from the jugular vein. Blood samples (2 ml) were taken 2, 5, 15, 60, 120, 180, 270 and 360 min after administration and stored in glass vials containing EDTA. The erythrocytes were removed by centrifugation (1200 *g*, 15 min) and the plasma analyzed for TCAR, as described above.

RESULTS AND DISCUSSION

Selectivity

Analytical selectivity was achieved by the combination of sample preparation and chromatographic separation. Initially, single column systems using reversed-phase or anion-exchange stationary phases were investigated for the separation of TCAR from plasma (Table I). The drug was only adequately retained on two of the four reversed-phase columns (Table I), even with purely

TABLE I

RETENTION OF RIBOXAMIDE IN VARIOUS CHROMATOGRAPHIC SYSTEMS

Column	Mobile phase*	k'
Reversed-phase		
ODS Hypersil	A	6.20
	B	6.18
μBondapak C_{18}	B	4.16
μBondapak CN	B	0.10
Hypersil	B	0.00
Anion exchange		
Partisil SAX	B	0.10
μBondapak C_{18} + HTAB	C	1.93
ODS Hypersil + HTAB	C	1.99
Hypersil + HTAB	C	1.10

*Mobile phases: A = 0.1% sulfuric acid (pH 2.1); B = 10 m*M* phosphate buffer (pH 7.0); C = 10 m*M* phosphate buffer (pH 6.0) and 1 m*M* HTAB.

aqueous mobile phases. The highest retention was observed on ODS Hypersil ($k' = (t_R - t_0)/t_0 = 6.2$). The retention of TCAR on all the columns studied was independent of mobile phase pH (2—7), suggesting that its ionization state did not change over this range.

The lack of anionic groups on TCAR accounts for its poor retention on Partisil 10 SAX [3, 4], however, significant retention on solvent-generated anion exchangers was observed. Such columns were prepared by adsorbing a monolayer of HTAB onto the surface of silica (Hypersil) and ODS-silica (ODS Hypersil and μBondapak C_{18}) columns [8—10]. Solutions containing 10 m*M* HTAB were used to coat the columns with surfactant and the stability of the modified stationary phases was maintained, subsequently, by the presence of 1 m*M* HTAB in the mobile phase.

The retention of neutral drugs (platinum complexes) on solvent-generated anion exchangers has been observed previously [8—10], and attributed to ion—dipole interactions between the solutes and the cationic surfactant (HTAB) adsorbed onto the stationary phase. In the case of TCAR, however, these ion—dipole interactions are probably less important since the drug is retained less on ODS Hypersil and μBondapak C_{18} in the presence of HTAB than in its

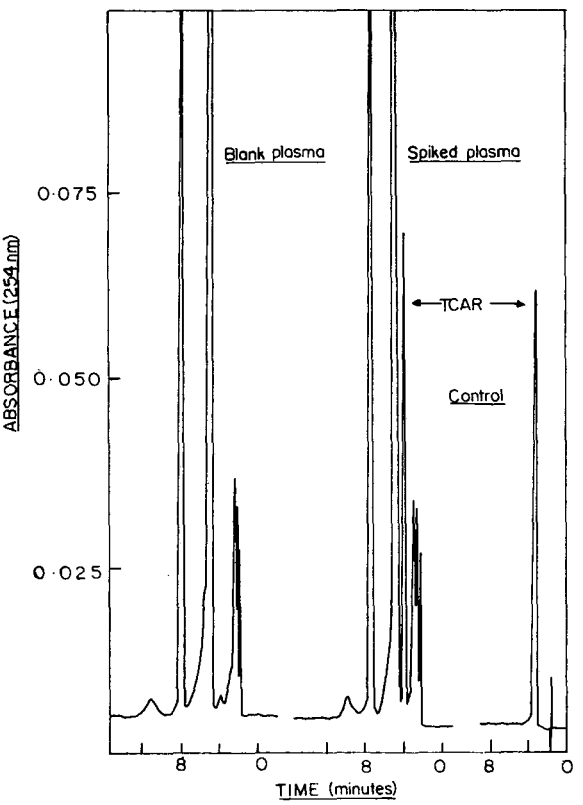

Fig. 5. Chromatogram showing the partial resolution from human plasma on ODS Hypersil, modified by the adsorption of HTAB onto its surface. Mobile phase: 10 m*M* phosphate buffer (pH 6.0) containing 1 m*M* HTAB; flow-rate: 1.0 ml/min; temperature: ambient; TCAR concentration: ca. 10 μg/ml.

absence (Table I). Similar decreased retention of neutral molecules on reversed-phase columns has been reported [11] and attributed to a reduction in the hydrocarbonaceous surface area of the stationary phase, produced by the adsorption of charged ions from the mobile phase. In contrast with ODS-silica columns, adsorption of HTAB onto the surface of silica (Hypersil) apparently enhances the hydrophobic character of the stationary phase, causing the observed increased retention of TCAR compared with that measured on bare silica (Table I).

Both reversed-phase and anion-exchange columns were investigated for the separation of TCAR from plasma, using a variety of aqueous mobile phases (pH 2—7). The weak eluents required for adequate retention of TCAR also resulted in significant retention of plasma components which interfered with TCAR. Although some of the interfering plasma components could be removed by extraction into chloroform (see Experimental), the use of two columns with different selectivities was required to achieve adequate resolution. In all cases using a single column, the TCAR peak was heterogeneous, as established subsequently by column switching techniques.

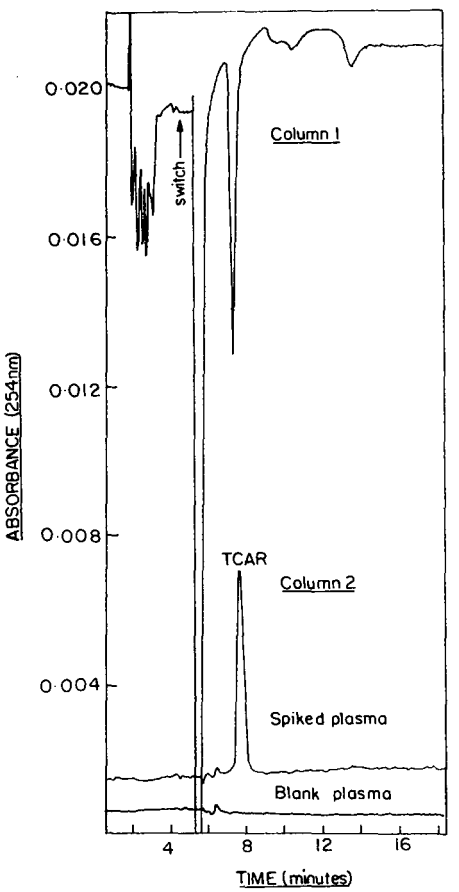

Fig. 6. Chromatogram showing the complete separation of TCAR from human plasma using column switching. See text and Figs. 3—5 for chromatographic conditions.

Optimal resolution of TCAR from plasma (Figs. 5 and 6) was achieved by partial separation on silica (Hypersil) coated with HTAB (Fig. 5); followed by transfer of the fraction containing the analyte to an ODS-silica column (ODS Hypersil or μBondapak C_{18}) coated with HTAB. It was necessary to use the same mobile phase for the elution of both columns, otherwise displacement peaks interfered with TCAR. The optimum mobile phase was 10 m*M* phosphate buffer (pH 6.0) containing 1 m*M* HTAB.

Precision and linearity

The peak height of TCAR was linearly related to the amount of solute (q in ng) injected, according to eqn. 1:

$$P = a \cdot q + b \quad (1)$$

where a and b are the slope and intercept of the linear regression (see Table II). The peak heights (P in mm) were corrected for changes in detector attenuation, using 0.02 a.u.f.s. as the reference. The response factor (a) was independent of detector attenuation (0.005—0.160), but decreased slightly with increasing injection volume (20—200 μl). This was attributed to a slight increase in band broadening with increasing volume of injection.

TABLE II

VARIATION OF RESPONSE FACTOR (a) WITH INJECTION VOLUME

Chromatographic conditions			Regression analysis*		
Injection volume (μl)	Concentration** range (μg/ml)	Detector*** setting (a.u.f.s.)	a	b	r
20	0—10	0.020	0.70	—0.26	0.9996
	0—2	0.005	0.69	—0.30	0.9993
50	0—10	0.040	0.67	—0.07	0.9998
	0—2	0.010	0.67	0.03	0.9999
100	0—10	0.080	0.65	—3.02	0.9995
	0—2	0.020	0.66	—0.21	0.9998
	0—0.2	0.005	0.64	—0.19	0.9969
200	0—10	0.160	0.62	—0.88	0.9996
	0—2	0.040	0.63	1.67	0.9998
	0—0.2	0.005	0.59	—0.02	0.9997

*Eqn. 1.

**n = 6 in all cases (samples prepared contained 0, 20, 40, 60, 80 and 100% of the concentration in each range studied).

***These represent the most appropriate detector attenuations for the analysis of TCAR in the stated ranges of concentration and injection volumes.

The day-to-day reproducibility (expressed as coefficient of variation, $n \geqslant 4$) of the response factor was 5.8%. Consequently, the response factor was checked by injection of an external standard, after every fourth plasma sample, using the same injection volume that was used for the samples. Within a single day, the coefficient of variation of the peak heights of the external standards was less than 0.7% (n = 6) for injections of 20—200 μl. The larger day-to-day

variation in response factor was probably due to fluctuations in room temperature and/or slight changes in the chromatographic properties of column 1.

Although ODS Hypersil and μBondapak C_{18}, coated with HTAB, gave adequate resolution when used as column 2, the shorter ODS Hypersil column was preferred since it offered shorter analysis times. With ODS Hypersil as column 2, the analysis time (14 min) was limited by the time required to elute the remaining plasma components from column 1. Sensitivity was not compromised by replacing μBondapak C_{18} with ODS Hypersil, since they gave very similar response factors (a = 0.73 ± 0.02 S.E.M. for ODS Hypersil and 0.70 ± 0.002 S.E.M. for μBondapak C_{18}). The limit of detection for TCAR in plasma was 40 ng/ml which gave a signal-to-noise ratio of 4:1 with an injection of 200 μl.

Recovery and compatibility of the plasma extract

After correction for dilution, the recovery of TCAR from plasma (Fig. 2) was 100.2 ± 0.9% S.E.M. and 101.3 ± 2.3% S.E.M. when spiked at the 10 and 1 μg/ml levels, respectively. This indicates that TCAR does not partition to a significant extent from water into chloroform, which was confirmed by extracting an aqueous solution containing TCAR (100 μg/ml) with an equal volume (25 ml) of chloroform. The chloroform layer was evaporated to dryness and the residue dissolved in mobile phase (200 μl). No TCAR was detected (less than 40 ng/ml) in the residue by HPLC analysis.

Initial studies indicated that the processed plasma was incompatible with the chromatographic system, because complete blockage of the column resulted after injection of a single sample. This problem was traced to the

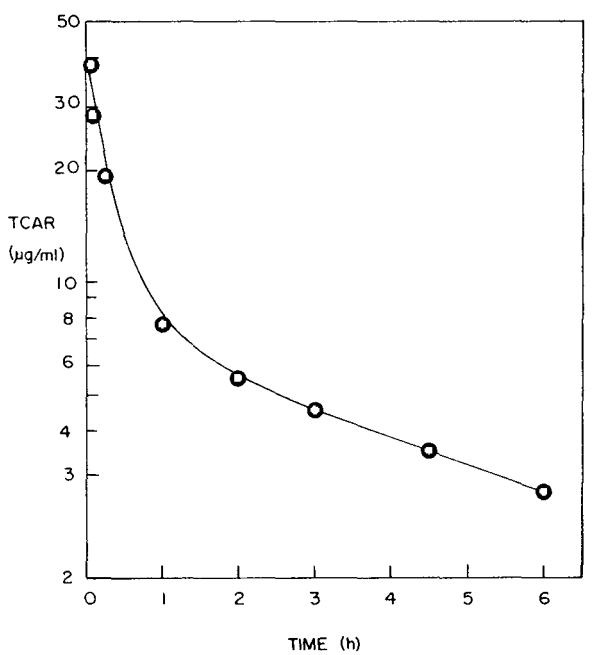

Fig. 7. Plasma concentrations of TCAR after intravenous administration (25 mg/kg) in the dog.

on-column precipitation of hexadecyltrimethylammonium perchlorate, and was circumvented by adjusting the HTAB concentration of the plasma extract to 1 m*M* and removing the resulting precipitate prior to injection into the chromatograph.

Application

The methodology was found to be suitable for the analysis of TCAR in both human and canine plasma and its applicability was demonstrated by an abbreviated pharmacokinetic study in the dog. The plasma levels of TCAR were followed for 6 h after its administration (25 mg/kg, intravenously) to a female Beagle dog, and the results are shown in Fig. 7. Over this time period, the drug exhibited biexponential decay with half-lives of 11 min (α-phase) and 4.0 h (β-phase). The extrapolated plasma concentration, at time zero, was 44.7 μg/ml which corresponds to a volume of distribution of 5.6 l.

ACKNOWLEDGEMENTS

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CHROMBIO. 1728

MEASUREMENT OF PROGABIDE AND ITS DEAMINATED METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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SUMMARY

Progabide (4-{[(4-chlorophenyl-5-fluoro-2-hydroxyphenol)-methylene]amino}butanamide) and its deaminated metabolite were measured simultaneously in plasma by high-performance liquid chromatography. Both compounds were extracted from plasma and the molecules were stabilized at the methylene—amino double bond with sodium borohydride reduction. Oxidative electrochemical detection was used for final quantitation. The method was used to measure progabide and progabide acid in plasma from a healthy volunteer who received a single 1200-mg dose of progabide orally. Lower limits of detection for progabide and progabide acid were 30 and 15 ng/ml, respectively. Coefficient of variation was less than 5% for both compounds.

INTRODUCTION

Progabide (4-{[(4-chlorophenyl-5-fluoro-2-hydroxyphenol)-methylene]-amino}butanamide) is a recently introduced compound with possible γ-aminobutyric acid (GABA) mimetic and anticonvulsant properties [1—9]. The structures of progabide (SL-76002), the deaminated acid metabolite (SL-75102), and a dichloro analogue of progabide (SL-78050) used as the internal standard (I.S.) are shown in Fig. 1.

Methods currently available for the quantitation of progabide either lack sensitivity or do not allow concurrent measurement of the active acid metabolite [2, 10, 11]. Also, an inherent problem is the instability of both the parent drug and the acid metabolite at the methylene—amino double bond [12]. The use of standard plasma extraction procedures tends to hydrolyze this bond, yielding the benzophenone and GABA amide in the case of progabide or the benzophenone and GABA in the case of progabide acid.

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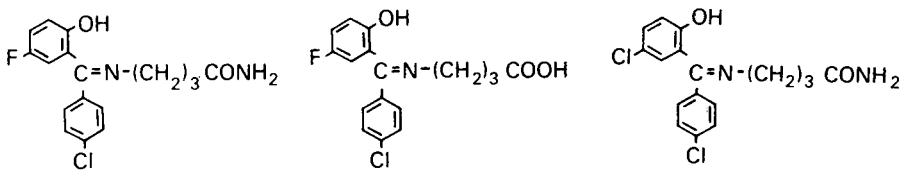

PROGABIDE (SL 76002) | PROGABIDE ACID (SL 75102) | I.S. (SL 78050)

Fig. 1. Chemical structures of progabide, progabide acid, and internal standard (I.S.).

This paper describes a quantitative high-performance liquid chromatographic (HPLC) procedure with electrochemical detection (LCEC) for the measurement of nanogram quantities of both progabide and progabide acid in plasma. By employing a sodium borohydride reduction of the double bond to stabilize the molecule, routine plasma extraction procedures can be utilized.

MATERIALS AND METHODS

Apparatus

The instruments used in this study were an Altex Model 110A pump (Altex, Berkeley, CA, U.S.A.) and a BAS LC-4A amperometric detector equipped with a TL-5 glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.) for oxidative electrochemical detection. The injector was a Model 7125 Rheodyne equipped with a 10-μl loop (Rheodyne, Cotati, CA, U.S.A.).

A RAC® Partisil 5 ODS-3 column (5-μm particle size), 10 cm $\times$ 9.4 mm I.D., with >90,000 theoretical plates per m (Whatman, Clifton, NJ, U.S.A.) was found to give good separation and ideal retention times for all compounds chromatographed, and back pressure was minimal. A Spheri-5® (5-μm particle size) RP-18 cartridge, 3 cm $\times$ 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.) served as guard column.

All chromatograms were recorded on a Beckman 10-in. strip-chart recorder (Beckman, Fullerton, CA, U.S.A.).

Reagents and chemicals

Hexane (UV), methanol, propanol-2, and methylene chloride distilled-in-glass were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and were used without further purification. HPLC grade water (J.T. Baker, Phillipsburg, NJ, U.S.A.) was used to prepare all eluent buffers. Buffers for extraction required deionized water only.

Sodium citrate, monopotassium phosphate (HPLC grade), dipotassium phosphate, sodium hydroxide, and sodium acetate (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Sodium borohydride, >99% purity, was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. Some of the glassware required pretreatment with triethylamine, Sequanal grade® (Pierce, Rockford, IL, U.S.A.) to eliminate adsorptive losses [13].

Stock standard solutions with a concentration of 100 ng/μl were prepared by using acetonitrile as solvent for progabide and I.S. and methanol for progab-

ide acid. From this stock solution, working standard solutions of 10 ng/μl (progabide), 5 ng/μl (progabide acid), and 20 ng/μl (I.S.) were prepared. These were kept at −4°C when not in use and were replaced every three weeks.

Extraction

Working standard solutions were added to 16 × 125 mm glass culture tubes in amounts ranging from 30 to 1500 ng for progabide and 15 to 750 ng for progabide acid. After 400 ng of I.S. were added, all traces of solvent were removed under nitrogen and 0.5 ml of blank plasma was added. These standards were then extracted concurrently with the patient samples by the following procedure.

A 0.5-ml volume of plasma, 0.5 ml of 2 *M* sodium acetate (adjusted to pH 4.9 with hydrochloric acid), and 9 ml of hexane—propanol-2 (96:4) were added to tubes containing 400 ng of I.S. previously dried under nitrogen. The tubes were capped with PTFE-lined screw caps, shaken for 5 min, and centrifuged. The organic layer was transferred to clean 16 × 125 mm tubes pretreated with 20% triethylamine in methanol [13].

The reduction step consisted of adding 0.5 ml of 0.4% sodium borohydride in ethanol (w/v) to each tube. After a brief vortex mixing, the tubes were allowed to set at room temperature. After exactly 10 min, 2 ml of 0.25 *M* sodium citrate (adjusted to pH 2 with hydrochloric acid) were added to each tube. The tubes were shaken for 5 min, centrifuged, and the hexane—propanol-2 was removed completely by aspiration. Another 9 ml of hexane—propanol-2 were added, the tubes were shaken for 5 min, centrifuged, and the organic layer was discarded.

To the sodium citrate remaining in the tube, 200 μl of 5 *N* sodium hydroxide, 0.5 ml of 1 *M* sodium citrate (adjusted to pH 4.8 with hydrochloric acid), and 9 ml of methylene chloride were then added. The tubes were shaken for 5 min, centrifuged, and the organic layer was transferred to clean tubes. The methylene chloride was then dried under nitrogen. The dried residue was reconstituted in 400 μl of methanol, and 10 μl were used for HPLC analysis.

Chromatographic conditions

The mobile phase for HPLC was a methanol—buffer (70:30) solution degassed with an ultrasonic bath after mixing. The buffer was a 33.3 m*M* monopotassium phosphate solution adjusted to pH 5.06 with 33.3 m*M* dipotassium phosphate. The buffer was replaced weekly. The eluent flow-rate was adjusted to 2 ml/min.

The potential of 1 V was selected on the amperometric detector electrode because it was the lowest voltage that gave acceptable sensitivity. The glassy carbon electrode was polished daily to preclude the possibility of passivation due to the high voltage required for maximum sensitivity.

RESULTS AND DISCUSSION

The ultraviolet (UV) spectra of the unreduced progabide and its reduced form are shown in Fig. 2. With use of the 0.4% sodium borohydride solution under the conditions described, the reaction was completed in less than 10

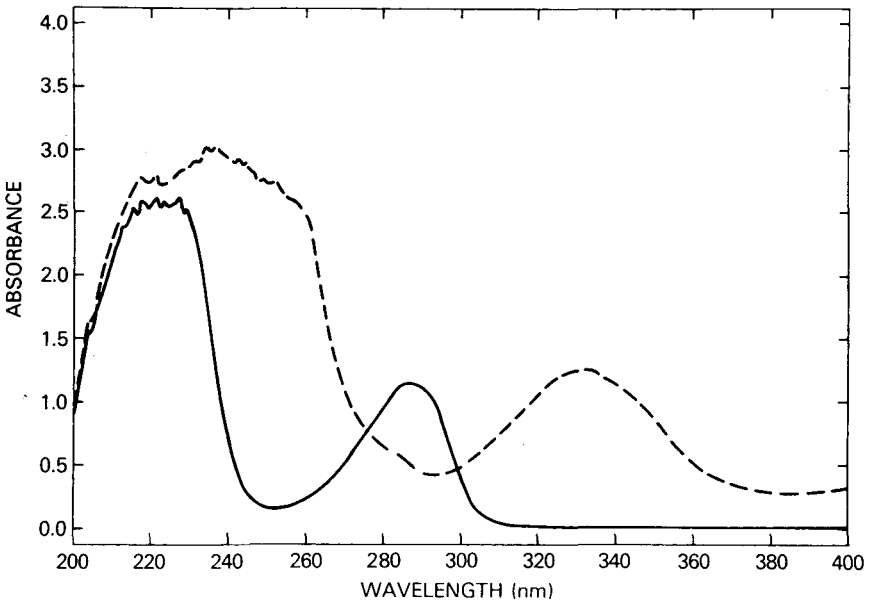

Fig. 2. UV spectra of 0.3 m*M* solution of progabide (— — —) and progabide after sodium borohydride reduction (——).

min, i.e., the maxima disappeared at ca. 332 nm, indicating the reduction of the methylene—amino double bond. The spectrum remained unchanged for at least 30 min, the maximum time the reaction was followed. The appearance of the maxima at 288 nm is thought to reflect additional ring resonance arising from the partial dissociation of the phenolic hydrogen. The electronegativity of fluorine may contribute to this dissociation. The UV spectra of progabide acid and the I.S. were similar but not identical to those of progabide. The relatively high UV absorption of these compounds indicated that UV quantitation might be a reliable method of analysis. The plasma extracts, however, manifested numerous interfering peaks throughout the UV range investigated, especially when the reduction and purification steps were not employed. The introduction of purification procedures yielded samples clean enough to allow detection at 280—290 nm, but the sensitivity was greatly attenuated. For these reasons, LCEC was chosen as the method of analysis.

The addition of 0, 100, 250, 500, or 1000 μl of ethanol in the hexane—propanol-2 (96:4) extraction solvent was compared with the percent drug reduced and showed that the reaction was facilitated by the addition of 250—1000 μl of ethanol. Therefore, 500 μl of sodium borohydride in ethanol was arbitrarily chosen as the amount exceeding the minimal effective alcohol concentration for the reaction. The effective range of the sodium borohydride concentration was also evaluated. All concentrations from 0.2% to 1% were equally effective for a complete reaction within 10 min. The 0.4% concentration was selected because it exceeded the minimal effective concentration yet was low enough to be easily neutralized in subsequent steps. It was also thought that the lower the sodium borohydride concentration, the less the possibility that the compounds might break down.

Progabide and progabide acid calibration curves obtained through linear regression analysis are described below. Each calibration curve is derived from the data of eight individual standard curves obtained over a 1-month period. These individual curves each consisted of at least six points covering the assay range.

Progabide: $Y = 0.0294 + 0.0028X$, $r = 0.9967$
95% confidence intervals: slope = 0.0028±0.0001, intercept = 0.0294±0.0469

Progabide acid: $Y = 0.0336 + 0.0035\ X$, $r = 0.0079$
95% confidence intervals: slope = 0.0035±0.0001, intercept = 0.0336±0.0432

The range of 30—1500 ng for progabide may have approached the maximal linear range of the electrochemical detector cell; occasionally a negative deviation from linearity was encountered at the high end. By diluting the final extract residue in a relatively large volume of 400 μl of methanol and injecting a constant 10 μl of this solution, several conditions that could possibly lead to this nonlinearity were minimized. Cell overloading, for example, was decreased by limiting the injection concentration to less than 40 ng. Under these dilution conditions, however, very low levels of progabide (<60 ng/ml) were difficult to quantitate unless the detector was kept meticulously clean. Also, so-called uncompensated resistance or IR drop effects were minimized by limiting the amounts of compound injected. Finally, any possible problems caused by injection volume differences were eliminated by keeping volumes constant.

The coefficient of variation was found to be <1% in a comparison of the peak height ratio between progabide or progabide acid and I.S. after repeated injections of the same sample. The coefficients of variation for a low and high determination of progabide and progabide acid extracted five times on three separate days and quantitated using three separate standard curves are shown in Table I.

A representative HPLC trace of a plasma extract from a healthy volunteer given a single oral dose of 1200 mg of progabide is shown in Fig. 3. The peaks correspond to plasma levels of 400 ng/ml for progabide and 829 ng/ml for progabide acid. The quantitation of plasma progabide levels versus time in nine healthy volunteers after single 1200-mg oral doses indicated that progabide

TABLE I

COEFFICIENTS OF VARIATION (%) OBTAINED FROM REPEATED EXTRACTIONS OF POOLED PLASMA SAMPLES (n = 15)

Coefficient of variation (%) = S.D./$\overline{X}$ × 100.

	Low	High
Progabide*	4.7	2.3
Progabide acid**	3.1	2.8

*Progabide concentration was <300 ng/ml for the low value and >2000 ng/ml for the high value.

**Progabide acid was <400 ng/ml for the low value and >1000 ng/ml for the high value.

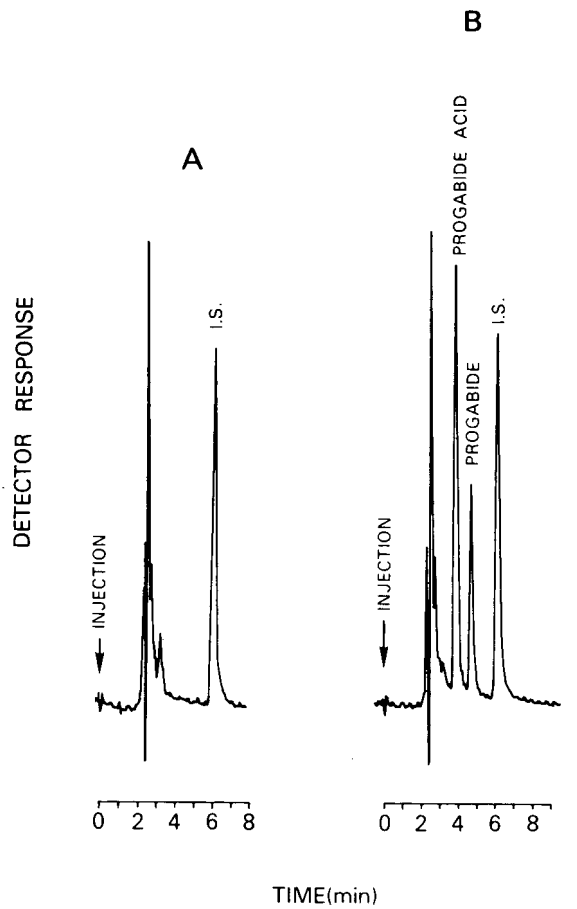

Fig. 3. Typical high-performance liquid chromatograms of plasma extracts from a volunteer before receiving progabide (A) and 12 h after orally receiving 1200 mg of progabide (B). Plasma progabide level at this time is approximately one-tenth of the peak plasma progabide level found at 3 h. The internal standard (I.S.) response is equal to 2.0 nA.

levels could reach as high as 6700 ng/ml within 2 h after administration. However, plasma progabide levels were not detectable after 48 h. The study also indicated that progabide acid levels could reach highs of 1400 ng/ml in 8 h, with detectable levels sometimes remaining after 48 h.

Fig. 4 shows a typical plasma decay curve in one of these volunteers. Progabide was rapidly absorbed, with peak plasma levels occurring 90 min after administration. Progabide also disappeared from plasma rapidly, with approximately a 2-h half-life. Progabide acid reached peak plasma levels at approximately 8 h, and plasma levels could be detected for a longer period of time.

It is obviously impossible to quantitate progabide in 0.5 ml of plasma when plasma levels of the drug are $\geqslant$3000 ng/ml; these levels exceed the highest standards, and linearity at this level is uncertain. In these cases, 200 μl of plasma were used for analysis, since our studies indicated that there is no difference in recovery when either 0.2 ml or 0.5 ml of plasma is used. However,

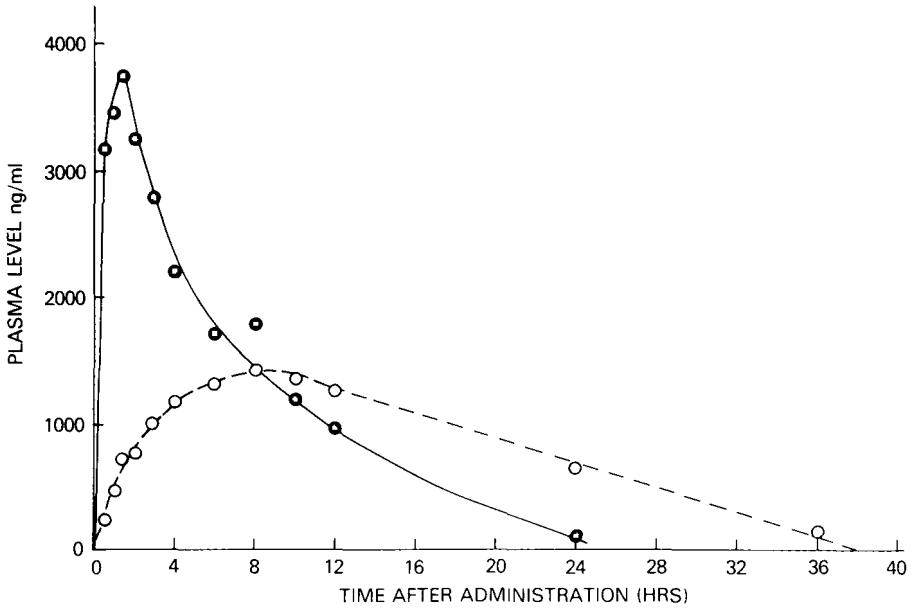

Fig. 4. Plasma progabide (•) and progabide acid (○) levels versus time after administration of a single 1200-mg oral dose of progabide to a healthy volunteer.

blank plasma was added to these samples to keep all volumes constant. When plasma progabide and progabide acid levels were low, 1 ml of plasma was used for analysis of both standards and unknowns. Recovery will decrease when more than 0.5 ml of plasma is used, but levels as low as 30 ng/ml of progabide and 15 ng/ml of progabide acid can be detected.

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CHROMBIO. 1710

IDENTIFICATION AND QUANTITATION OF A METABOLITE OF ANETHOL DITHIOLTHIONE IN RAT AND MOUSE URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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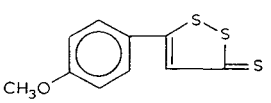
SUMMARY

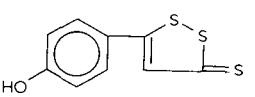
Urine samples from rats and mice fed anethol dithiolthione (ADT) [3-(*p*-methoxyphenyl)-1,2-dithiol-3-thione] were analyzed using reversed-phase high-performance liquid chromatography. Urine was introduced directly on the liquid chromatograph which was modified by replacing the sample loop with a guard column. Highly polar urine components were washed off the guard column prior to chromatography. A major metabolite and the parent compound (ADT) were separated and detected using the chromatographic conditions described in this study. The metabolite was identified as desmethyl ADT. The identification was based on co-chromatography on two columns using two mobile phases and peak height ratios of the metabolite and the reference standard. Data pertaining to the pattern of excretion of ADT and desmethyl ADT in the animals studied are reported.

INTRODUCTION

Anethol dithiolthione (ADT) [3-(*p*-methoxyphenyl)-1,2-dithiol-3-thione] has been reported to stimulate salivary secretion and to be an antidote for the dryness of the mouth produced by antidepressant drugs [1]. More recently it has been found that this and other dithiolthiones have chemo- and radioprotective properties [2]. As a first step towards the study of the metabolism and pharmacodynamics of ADT, we have identified one of its metabolites and developed a method for the determination of both the parent compound and the metabolite in the urine of rats and mice using reversed-phase high-performance liquid chromatography (HPLC).



ADT



desmethyl ADT

0378-4347/83/$03.00

EXPERIMENTAL

Instrumentation

An isocratic HPLC system assembled in our laboratory consisted of a Milton Roy reciprocating minipump, Model 396; a stainless-steel tube, 1 m × 6.4 mm O.D. as a pulsation damper, a 34.5 MPa pressure gauge; a Rheodyne injector Model 7125 and a variable-wavelength ultraviolet (UV) detector, Model Spectro-Monitor III. All parts were obtained from Laboratory Data Control (Riviera Beach, FL, U.S.A.). A Kratos variable-wavelength UV detector Model Spectroflow 769 (Westwood, NJ, U.S.A.) was connected in tandem with the Spectro-Monitor III for the simultaneous detection and identification of the metabolites, a Brownlee (Santa Clara, CA, U.S.A.) RP-18 LiChrosorb 3-cm guard column was connected in place of the sample loop of the injector. Two analytical columns were used in this study; a Whatman (Clifton, NJ, U.S.A.) Partisil PXS 10/25, ODS-2, microparticulate, 10—12 μm, reversed-phase column, and a Waters Assoc. (Milford, MA, U.S.A.) μBondapak Phenyl column. A Brownlee guard column identical to the one mentioned above was connected between the injector and the Whatman reversed-phase column to further protect the column from urine components, since most of the analyses were carried out on that column. No guard column was connected between the injector and the Waters column since very few runs were carried out on that column. A Hewlett-Packard (Avondale, PA, U.S.A.) computing integrator Model 3390A was connected to each detector. The analytical columns were kept inside a Bioanalytical Systems (West Lafayette, IN, U.S.A.) column heater Model LC-23A and column temperature was maintained at 35°C throughout this study.

Chemicals

Anethol dithiolthione (ADT) and desmethyl ADT were obtained from Dr. Baronnet of Laboratoires Therapeutique Moderne (Suresnes, France) and were used without further purification.

Methanol (glass distilled) was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was distilled, deionized and demineralized.

Reference standards were dissolved in methanol and all dilutions and mixtures were prepared also in methanol. Solutions were stored in vials provided with PTFE-lined caps and the vials were wrapped with aluminum foil to protect the solutions from light. Standard solutions were gassed with nitrogen and kept refrigerated when not in use.

Animals and treatments

Male Sprague-Dawley rats weighing approximately 200 g and CD-1 female mice weighing 20—25 g were used. The rats were placed in individual metabolism cages and urine was collected in Erlenmeyer flasks and analyzed individually. Six mice were placed in one metabolic cage and urine was collected and analyzed as one sample. A solution containing 25% glycerol and 1% Cremophor EL (BASF, Wyandott, Parsippany, NJ, U.S.A.) was used to suspend ADT prior to feeding. To 1 g of ADT, 10 ml of the above vehicle was added, the mixture was then homogenized and given to the animals by

gastric intubation to provide a dose of 1 g/kg body weight for both rats and mice.

Mobile phases

Two mobile phases containing 70% and 65% methanol in water were used. The mobile phases were degassed under vacuum immediately prior to use and kept at approximately 40°C during chromatography to prevent the introduction of air bubbles into the system. The flow-rate was kept at 1.1 ml/min throughout this study.

Detector settings

The absorption spectra of ADT and desmethyl ADT were studied using a Varian scanning spectrophotometer Model Cary 219. ADT was found to have maxima at 225, 342 and 425 nm and the extinction coefficients (α) were 61.5, 96.0 and 57.0, respectively. Desmethyl ADT was found to have maxima at 228, 350 and 425 nm and the extinction coefficients (α) were 31.0, 50.5 and 35.0, respectively. Based on these data, the variable UV detector Model Spectro-Monitor III was set at 350 nm for the routine determination of ADT and desmethyl ADT since both absorb most at or close to that wavelength. Peak height ratios for desmethyl ADT and the metabolite suspected to be desmethyl ADT were determined simultaneously using the two detectors connected in tandem. The Spectroflow 769 was set at 228 nm while the Spectro-Monitor III was set at 350 nm.

Integrator settings

Each UV detector provided a constant signal to the computing integrator of 1 absorbance unit (AU)/V. The sizes of the peaks on the chromatograms were thus determined by the attenuation of the integrator. During this study two attenuation settings were used, attenuation 3 which provides a full scale of 8 mV or attenuation 4 which provides a full scale of 16 mV. The sensitivity of the system would be 0.008 absorbance units full scale (AUFS) or 0.016 AUFS, respectively.

Sample preparation

Urine was collected in Erlenmeyer flasks which were kept on ice. The samples were collected every morning and centrifuged for 20 min at 12,100 *g* using a refrigerated Sorvall RCB-2 centrifuge set at 5°C to remove suspended particles. One ml of the centrifuged urine was filtered using Bioanalytical Systems centrifugal filters containing membranes of pore size 0.2 μm. The filtrates were directly introduced onto the chromatograph. Prior to the introduction of the sample on the guard column loop, 0.5 ml of 30% methanol was introduced, followed by the sample (5 μl) then 1 ml of 30% methanol was used to flush the highly polar urine components out of the guard column. Then the injector was turned to the inject position for the chromatography of the compounds remaining on the guard column.

The concentration of methanol used to flush the guard column was determined by preliminary experiments using Baker (Phillipsburg, NJ, U.S.A.) C_{18} disposable extraction columns. ADT and desmethyl ADT were added to

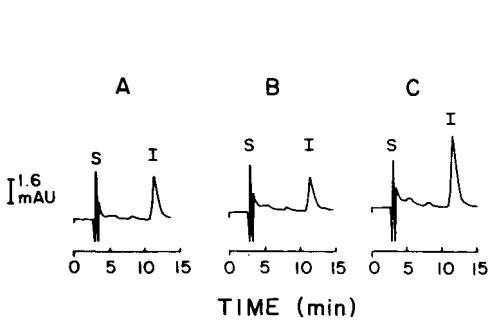
the urines and washed with various concentrations of methanol and the eluates were monitored by HPLC. The use of 30% methanol removed most of the polar urine components and did not elute the compounds of interest.

RESULTS AND DISCUSSION

Identification of the metabolite

Desmethyl ADT was added to rat urine samples and peak shape and peak heights were compared with those without added desmethyl ADT and those of desmethyl ADT standard. This was carried out using the two columns and the two mobile phases described earlier. Figs. 1 and 2 show the chromatograms on the reversed-phase column and on the phenyl column respectively using 65% methanol. As demonstrated from the peak shape and from the peak heights, it is clear that the urine metabolite is co-chromatographing with desmethyl ADT, this results in an increase in peak height without any change in peak shape in both systems. Similar results were obtained using 70% methanol on the reversed-phase column.

To further establish the identity of these compounds, chromatography was carried out at the two UV absorption maxima of desmethyl ADT, 228 nm and 350 nm. The peak height ratio of the signals produced at 228 nm to that produced at 350 nm were calculated for the reference standard and the metabolite using column A and 70% methanol. Ten samples of each were determined over a period of four days to reflect day-to-day variations of peak height ratios. The mean ($\bar{x}$) standard deviation (σ_x) and standard error of the mean ($\sigma_{\bar{x}}$) for the ten samples were determined and are presented in Table I.



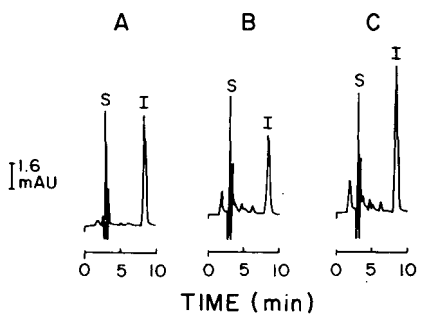


Fig. 1. Chromatograms of (A) 5 μl of 10 μg/ml desmethyl ADT; (B) rat urine of animal fed 1 g/kg of ADT, diluted with an equal volume of methanol, 5 μl injected; (C) same rat urine as in (B) spiked with an equal volume of 20 μg/ml desmethyl ADT in methanol, 5 μl injected. Chromatographic conditions: column, Whatman, Partisil PXS 10/25, ODS-2; mobile phase, 65% methanol; detector, UV 350 nm; sensitivity, 0.016 absorbance units full scale (AUFS); flow-rate, 1.1 ml/min; column temperature, 35°C; chart speed, 0.2 cm/min. Peaks: S = solvent front; I = metabolite identified as desmethyl ADT.

Fig. 2. Chromatograms of (A) 5 μl of 10 μg/ml desmethyl ADT; (B) rat urine of animal fed 1 g/kg of ADT, diluted with an equal volume of methanol, 3 μl injected; (C) same rat urine as in (B) spiked with an equal volume of 20 μg/ml desmethyl ADT in methanol, 3 μl injected. Chromatographic conditions: same as Fig. 1 except that the column used is a Waters μBondapak Phenyl. Peaks as in Fig. 1.

TABLE I

PEAK HEIGHT RATIOS OF DESMETHYL ADT AND METABOLITE

Number of samples = 10 in each case; data collected over a period of four days.

Compound	$\bar{x}^*$	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	0.617	0.0067	0.0021
Metabolite	0.628	0.0265	0.0083

*Peak height ratios of detection responses at 228 nm to those at 350 nm, using column A and 70% methanol.

Accuracy and specificity

To evaluate the accuracy and specificity of the method, retention times (t_R) for desmethyl ADT and ADT were determined several times during the course of the day to determine within-run variations, and over a period of four days to reflect day-to-day variations. The mean ($\bar{x}$), standard deviation (σ_x) and standard error of the mean ($\sigma_{\bar{x}}$) were calculated and are presented in Tables II and III. As expected the σ_x and $\sigma_{\bar{x}}$ values were smaller for the within-run determinations than the day-to-day determinations. The statistical variations were also smaller for desmethyl ADT than for ADT since the former had a shorter t_R value.

Since these compounds are well separated and no other peaks are close enough to cause interference, these variations confirmed the accuracy and specificity of the analytical method.

TABLE II

WITHIN-RUN VARIATIONS OF RETENTION TIMES (min)

Number of samples = 8 in each case; data collected the same day; column A and 70% methanol were used.

Compound	$\bar{x}$	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	8.11	0.072	0.025
ADT	17.58	0.135	0.047

TABLE III

DAY-TO-DAY VARIATIONS OF RETENTION TIMES (min)

Number of samples = 8 in each case; data collected over a period of four days; column A and 70% methanol were used.

Compound	$\bar{x}$	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	8.58	0.102	0.113
ADT	18.77	0.814	0.288

Reproducibility and precision

Desmethyl ADT and ADT were determined in one mouse urine sample containing these compounds several times during the course of one day and over a period of four days. Reference standards were chromatographed each day and detection responses for the reference standards were used to determine the amount present in the urine samples assayed on the same day. To ascertain reproducibility reference standards were chromatographed twice and the mean values were used. The statistical computation of the amounts of these compounds reflecting within-run variations and day-to-day variations are presented in Tables IV and V. The values reflecting the day-to-day variations were very similar to those reflecting the within-run variations. This is expected due to the fact that actual variations due to day-to-day variability in chromatographic conditions is eliminated by the use of detection responses of reference standards produced the same day for the computation of sample contents.

TABLE IV

WITHIN-RUN VARIATIONS OF QUANTITATIVE DETERMINATION OF COMPOUNDS STUDIED IN MOUSE URINE

Number of samples = 4 in each case; data collected the same day.

Compound	Day*	$\bar{x}$**	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	A	26.2	1.979	0.989
	B	25.3	1.319	0.659
ADT	A	8.1	0.639	0.319
	B	8.3	0.547	0.273

*Same data collected on Day A and day B.
**Amount calculated in ng on column, injection volume was 5 μl; column A, 70% methanol and 350 nm were used.

TABLE V

DAY-TO-DAY VARIATIONS OF QUANTITATIVE DETERMINATION OF COMPOUNDS STUDIED IN MOUSE URINE

Number of samples = 10 in each case; data collected over a period of four days.

Compound	$\bar{x}$*	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	25.5	1.823	0.576
ADT	8.4	0.783	0.247

*Amount calculated in ng on column, injection volume was 5 μl; column A, 70% methanol and 350 nm were used.

Detection responses and sensitivity

The detection responses for the two compounds studied were determined using Column A, 70% methanol and 350 nm. Eight determinations were carried out over a period of four days. Desmethyl ADT produced a mean

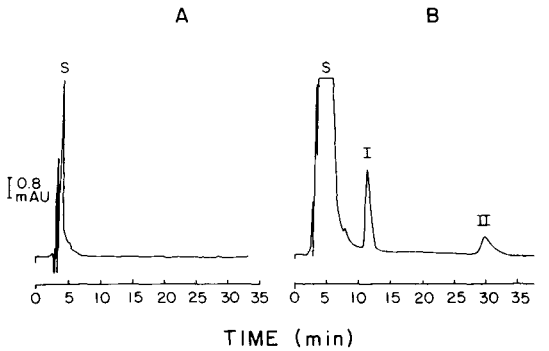


Fig. 3. Chromatograms of (A) 5 μl of control mouse urine collected prior to feeding of ADT; (B) 5 μl of same pool of mouse urine 48 h after feeding with 1 g/kg of ADT. Chromatographic conditions as in Fig. 1 except sensitivity was 0.008 AUFS. Peaks: S = solvent front; I = metabolite identified as desmethyl ADT; II = ADT.

signal size of 94.5 ± 12.9 mAU per μg on column, while ADT produced a mean signal size of 120.4 ± 9.4 mAU per μg on column. When a sensitivity setting of 8 mAUFS was used as in Fig. 3, no detectable noise was observed. Under these conditions a signal size of 5% of the full scale or 0.4 mAU will be easily detected. Such a signal represents less than 5 ng on column of both compounds studied. The sensitivity can be further increased by increasing the sensitivity setting of the detector.

Since the procedure provides both extraction and trace enrichment, as described under sample preparation, sample volumes larger than 5 μl can be introduced onto the system without affecting the baseline or the column performance.

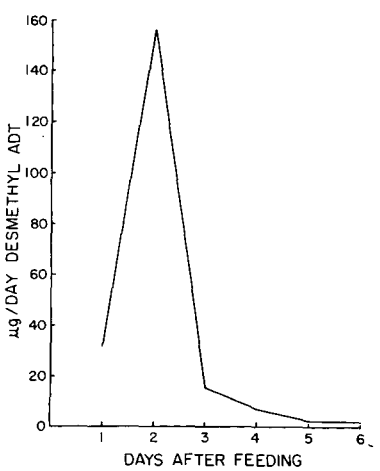


Fig. 4. Graphic representation of amounts of desmethyl ADT present in rat urine fed 1 g/kg of ADT.

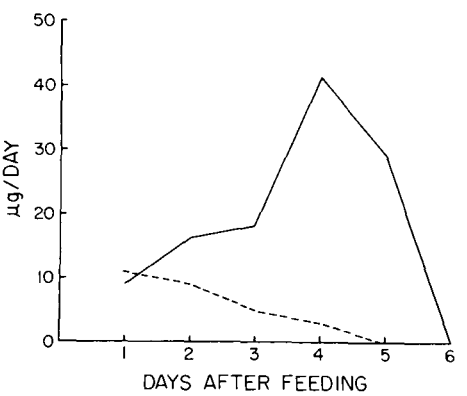


Fig. 5. Graphic representation of amounts of ADT (— — —) and desmethyl ADT (——) present in mouse urine fed 1 g/kg of ADT.

Quantitation of ADT and desmethyl ADT

ADT was not found in detectable quantities in rat urine. It was, however, detected and its quantity determined in mouse urine. Identification of ADT was based on retention characteristics using the three chromatographic conditions described above. The quantities of ADT and desmethyl ADT were determined as described above. Fig. 3 reproduces the chromatograms of urines of mice fed 1 g/kg of ADT and collected 48 h thereafter, on the reversed-phase column. The solvent front representing the polar compounds unretained under these conditions is much larger in the urine of animals fed ADT than in control animals. This suggests that this large unretained peak is due to other polar metabolites of ADT. Fig. 4 represents the amount of desmethyl ADT excreted per day in rat urine. Fig. 5 represents the amounts of ADT and desmethyl ADT excreted per day in mouse urine. ADT is absent from rat urine, while it requires five days to be cleared from the mouse. The metabolite excretion pattern is also different in the rat where the peak is reached after two days while in the mouse the peak is reached after four days.

Chromatography

The use of the RP-18 guard column in place of the injection loop and the removal of undesirable urine constituents by washing with 30% methanol after the introduction of the sample allowed the direct injection of urine on the liquid chromatograph and eliminated tedious and time consuming sample preparation. Also, the use of another guard column between the injector and the analytical column assured the complete protection of the column. The two mobile phases consisting of 65% and 70% methanol provided reasonable retention of the compounds studied. The lower methanol concentration was preferred because some urine samples produced large peaks at the solvent front which were not completely separated from desmethyl ADT when 70% methanol was used. Also, 70% methanol did not produce adequate retention on the phenyl column.

The use of two columns such as the reversed-phase column and the phenyl column for identification of unknown compounds provides stronger evidence than a single column because the physico-chemical basis for separation is different on each column. Previous work in our laboratory, in which sixteen dithiolthiones were studied indicated that the order of elution of these compounds varies on these two columns [3].

Peak height ratios at the two UV maxima of desmethyl ADT served as an additional parameter for the identification of the metabolite as desmethyl ADT.

CONCLUSION

A procedure for the separation, identification and determination of ADT and of desmethyl ADT using HPLC is described. A major metabolite of ADT in rat and mouse urine has been identified as desmethyl ADT using co-chromatography and peak height ratios. Data pertaining to the excretion of desmethyl ADT in the rat and mouse urine are reported. ADT was determined in the mouse urine and was found to decrease steadily and to disappear after five days of ingestion. Unmetabolized ADT was not detected in rat urine.

ACKNOWLEDGEMENTS

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ANALYSIS OF IODOCHLORHYDROXYQUIN IN BIOLOGICAL MATERIALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic system using a mobile phase of 0.05 *M* phosphoric acid—methanol (30:70) was developed for determination of iodochlorhydroxyquin (clioquinol, I) in biological material. I was extracted from samples with diethyl ether. Conjugates of I were hydrolyzed to free I and extracted by the same method. The ether phases were evaporated to dryness, reconstituted in the mobile phase and chromatographed using a microparticulate C18 column, a pre-column and a UV detector set at 256 nm. Quantitation of I in the range of 0.20—2.0 μg/ml of urine, 0.50—2.0 μg/g of liver, and 0.25—2.0 μg/g of feces was obtained with coefficients of variation of 0.02, 0.05, and 0.06, respectively. The detection limit of I was 0.2 μg. Extensive absorption of I upon topical application to dogs was also demonstrated.

INTRODUCTION

Iodochlorhydroxyquin (clioquinol, I) has been widely used in many countries as an antidiarrheal, antimycotic and antibacterial agent. Subacute myelo-opticoneuropathy (SMON) is the major toxicological manifestation associated with high doses of I [1—4], and an iron chelate of I has been isolated from the urine and feces of patients with SMON.

Several analytical methods have been developed to study the distribution and excretion of I. Electron-capture gas—liquid chromatography (GLC), which requires expensive instrumentation and time-consuming derivatization, has been used [5—10]. Chen et al. [10] developed a solvent extraction method for separating I and its glucuronide and sulfate conjugates in biological fluid prior to GLC. A spectrophotometric method was reported for quantitating I and its conjugates in the urine [11, 12]. This procedure is not sufficiently sensitive for the determination of the drug in other biological materials. Chen et al.

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[13] reported a method for the determination of I conjugates by high-performance liquid chromatography (HPLC). This method could not be applied to tissue or plasma because of the presence of constituents absorbing at 254 nm and free I could not be measured. Tsuji et al. [14] reported a method whereby I was chelated with aluminum and measured fluorometrically. In our laboratory a procedure for analysis of I in plasma was developed [15]. In addition, methods for analysis of I and hydrocortisone in creams and ointments [16, 17] have been developed using simple isocratic HPLC, based upon our initial procedure for the analysis of I in plasma [15]. Hayakawa et al. [18] have recently published an HPLC procedure for the analysis of I and its conjugates in biological materials.

This report describes a new HPLC method for the measurement of I in urine, feces, and liver. The procedure combines sensitivity and simplicity not attainable by previously described methods.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a reciprocating pump (Milton Roy Model 396-31), a stainless-steel tube (1 m × 6.35 mm O.D. and 4.76 mm I.D.) as a pulsation damper, a 34.5 MPa (5000 p.s.i.) pressure gauge (Laboratory Data Control), a fixed volume sample injector (Rheodyne) with a 20-μl loop, and a variable-wavelength UV detector (Spectro-Monitor III, Laboratory Data Control). A multivoltage 25.40-cm stripchart recorder (Beckman Instruments) was connected to the UV detector. A microparticulate reversed-phase chromatographic column (250 × 2.6 mm) packed with ODS-HC-SIL-X-I (particle size 10 μm, serial No. 1303, Perkin-Elmer) and a 40 X 5 mm guard column containing RP-18-MPLC (Rheodyne) were connected to the HPLC system.

Materials

Iodochlorhydroxyquin (clioquinol, I) was obtained from CIBA Pharmaceuticals (Summit, NJ, U.S.A.). Diethyl ether anhydrous analytical reagent, anhydrous sodium sulfate, benzene, sodium hydroxide, hydrochloric acid, sodium fluoride, and theophylline were purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Methanol distilled in glass, residue free, and acetone were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). β-Glucuronidase (1,000,000 units per 2.25 g) and alumina were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium acetate, acetic acid and diphenylcarbazone were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Pyridine was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). 4,7-Dichloroquinoline, 5,7-dichloro-8-hydroxyquinoline, 10-chloro-9-anthracene—methanol, 9-hydroxy-4-methoxy-acridine and α-naphthoflavone were purchased from Aldrich (Milwaukee, WI, U.S.A.). 4-Chloro-3-methylphenol, 5-nitroso-8-quinolinol, α-bromo-*p*-nitrotoluene, 4,4-dimethylbenzophenone, benzanilide, 1-naphthylamine, 2,7-naphthalenediol, and dibenzofurane were all purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Xanthin-9-ol was obtained from Matheson Coleman and Bell (Norwood, OH, U.S.A.). Trihexyphenidyl was a gift from Lederle Laboratories (Pearl River, NY, U.S.A.).

HPLC conditions

The UV detector was set at 256 nm with recorder chart speed at 0.254 mm/min. Methanol—0.05 *M* phosphoric acid (70:30) was used as mobile phase at a flow-rate of 1 ml/min. The mobile phase was filtered using a 0.2-μm filter, degassed under vacuum and maintained at 40°C during chromatography. The column was flushed at the end of each day with 100% methanol. Not more than 30 min was required for column equilibration prior to use each day.

Sample preparation

Urine. Aliquots of 1—5 ml of urine were transferred to 15-ml screw-capped and PTFE-lined centrifuge tubes. Known amounts of I working standard solution were used to spike urine samples which were then extracted three times with 1—5 ml of diethyl ether by vortexing for 10 sec, and the phases were separated by centrifuging at 3000 *g* for 10 min at 15°C. The ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness at 40°C under a stream of nitrogen. The residues were redissolved in the mobile phase and 20 μl of each was injected onto the column. When a determination of the total I in urine was made, conjugates (glucuronides and sulfates) were hydrolyzed with β-glucuronidase (final concentration 200 units/ml) at pH 5 using 1.0 *M* acetate buffer, incubating at 37°C for 2 h. Complete hydrolysis of sulfate conjugates was ensured by adjusting the solution to 1 *N* using 6 *N* hydrochloric acid and incubating at 40°C for an additional 2 h [10]. The hydrolysates were neutralized with 3 *N* sodium hydroxide prior to extraction three times with 5 ml diethyl ether as described above. After evaporation of the ether phases the residues were dissolved in 0.5 ml benzene, adsorbed on to an alumina column and washed successively with 2 ml each of benzene—pyridine (7:1), acetone, 0.1 *N* acetic acid in methanol and methanol. The alumina was transferred to another 15-ml screw-capped glass tube with 1 ml of a saturated aqueous solution of sodium fluoride and extracted twice with 5 ml diethyl ether. The ether phases were evaporated to dryness, the residues were dissolved in the moble phase, and 20-μl aliquots were injected onto the column.

Tissue. To 1-g samples of liver in 25-ml glass Potter-Elvehjem homogenizer tubes 2 ml of the mobile phase and a known quantity of the standard solution of I in the mobile phase were added and homogenized. The homogenates were transferred to 15-ml screw-capped and PTFE-lined centrifuge tubes, the homogenizer tubes were each washed with 1 ml of mobile phase and the washings were transferred to the centrifuge tubes. The homogenates were extracted three times with 5 ml diethyl ether by vortexing for 1 min and centrifuging at 3000 *g* for 10 min. The ether phases were collected and evaporated to dryness. The residues were dissolved in 0.50 ml benzene, adsorbed onto an alumina column and processed as described above for the urine.

Feces. To 1-g samples of feces in 25-ml glass Potter-Elvehjem homogenizer tubes 5 ml of mobile phase were added. Each was spiked with working standard solution of I in methanol and homogenized. The homogenized mixtures were transferred to 15-ml screw-capped and PTFE-lined centrifuge tubes and subjected to hydrolysis and the washing procedures described above for the urine extraction.

Standards. Stock solutions of I were prepared in methanol and contained 1 mg/ml and 100 μg/ml. These solutions could be maintained in the refrigerator for at least two weeks without deterioration. Working solutions were prepared fresh daily. Dilutions were made using methanol—0.05 *M* phosphoric acid (70:30) to prepare the working standards, which were used to spike samples and to calculate recovery from samples after extraction.

RESULTS AND DISCUSSIONS

The UV absorbance spectrum of a standard solution of iodochlorhydroxyquin (I) in methanol—0.05 *M* phosphoric acid (70:30) was determined, and exhibited maxima at 256 nm (absorptivity, $\delta = 0.15$) and 204 nm ($\delta = 0.11$). For optimum sensitivity the UV detector was set at 256 nm for subsequent HPLC analysis [15, 16].

Representative chromatograms of canine urine samples in the presence and absence of I using a column packed with ODS-HC-SIL-X-1 (10 μm) are given in Fig. 1. Fig. 1A represents an analysis of an extract of a blank (control) urine. No peaks which might interfere with I are present. Fig. 1B is a chromatogram of a urine sample to which I had been added. I is represented by peak 1 and has a retention time of approximately 7.5 min, while peak 4 is the solvent front with a retention time of 2.0 min. Peaks 2 and 3 are unknown substances extracted from urine with retention times of approximately 5.0 and 2.5 min, respectively. The capacity factor k' for I was 2.75.

Fig. 1C is a chromatogram of an organic extract of urine from a dog which had been treated topically with 5 g of a cream containing 3% I. The cream had been applied to the shaved back over a 200 cm^2 area. Since I is readily conjugated with glucuronic acid and sulfate [3], urine samples were hydrolyzed and washed as described under Experimental and the extracts chromatographed. The total I in the urine sample was 1.8 μg/ml. The washing procedure during sample preparation was necessary to remove interfering substances.

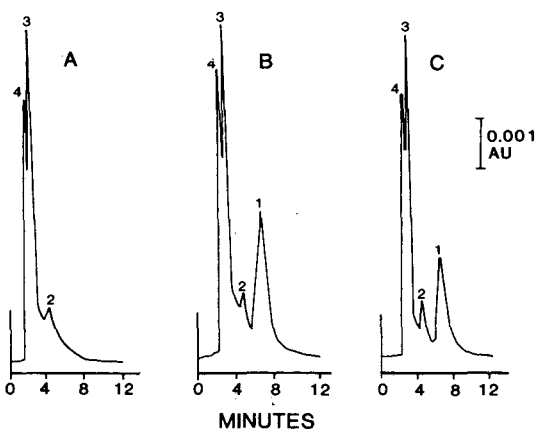


Fig. 1. Chromatograms of (A) blank canine urine; (B) urine containing 2.5 μg/ml iodochlorhydroxyquin (I); (C) urine from a dog treated topically with I following hydrolysis and alumina column clean-up procedure. The detector was set at 0.02 aufs and conditions are as described under Experimental. Peaks: 1 = I; 2, 3 = unknowns; 4 = solvent front.

The chromatograms of canine liver extracts in the absence and presence of I are shown in Fig. 2A and B, respectively. In Fig. 2B the tissue was spiked with 1.5 μg I per g. I is represented by peak 1, and peak 2 is the solvent front. As can be seen, no substances which interfered with the elution of I were extracted from the liver. Fig. 2C is a chromatogram of an organic extract of liver from a dog which had been treated with I topically. The liver contained 0.9 μg I per g of tissue.

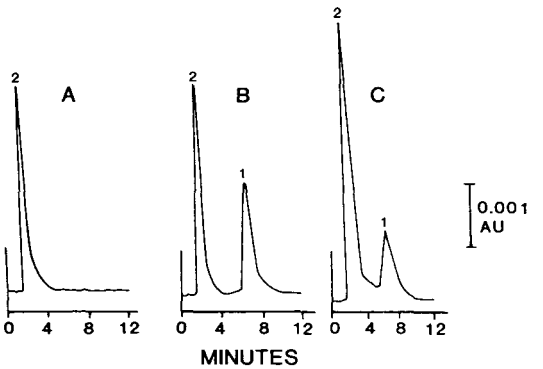
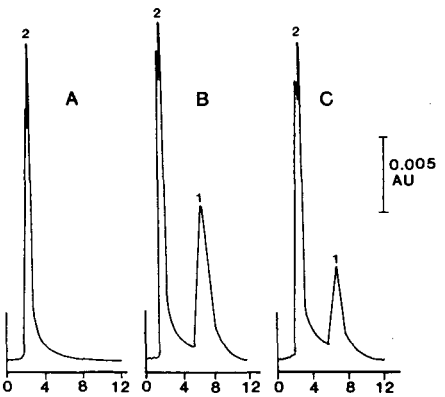


Fig. 2. Chromatograms of (A) control dog liver; (B) 1.0 g canine liver to which had been added 1.50 μg I; (C) liver from a dog treated topically with I. The detector was set at 0.02 aufs and chromatographic conditions used are described under Exprimental. Peaks: 1 = I; 2 = solvent front.

Hayakawa et al. [18] recently reported a HPLC system for the measurement of I and its conjugates in biological fluids as bile, plasma and urine. We previously reported a sensitive method for determining I in plasma [15]. Hayakawa et al. [18] also presented a chromatogram of an extract of 0.2 g of kidney containing 2 μmole (610 μg) of I. We were able to measure less than 1 μg I per g liver (Fig. 2), suggesting that our extraction procedure is more efficient. The limits of detection for I reported by Hayakawa et al. [18] and the procedure described herein were 0.3 and 0.2 μg, respectively.

Phoon and Stubley [17] developed a HPLC method for the determination of I in ointments. The system they reported is a modification of the procedure which we developed for plasma [15]. We have also modified this method for measuring I in ointments and creams [16].

Representative chromatograms of extracted feces samples from dogs in the absence and presence of I are presented in Fig. 3A and B, respectively. The feces samples employed in Fig. 3B was spiked with 20.0 μg I per g. Peak 1 represents I with a retention time of approximately 7.5 min, and peak 2 is the solvent front. As with the urine and liver samples, no peaks interfered with I. Most of the I excreted in the feces has been reported to exist as the glucuronide and sulfate conjugates [3]. Therefore, feces samples were hydrolyzed, extracted and washed as described in Experimental; a representative chromatogram is presented in Fig. 3C. The sample was obtained from a dog which had been treated topically with I, and contained approximately 11.2 μg per g. The primary route of excretion of I appears to be the feces following formation of conjugates which are concentrated in the bile [3].



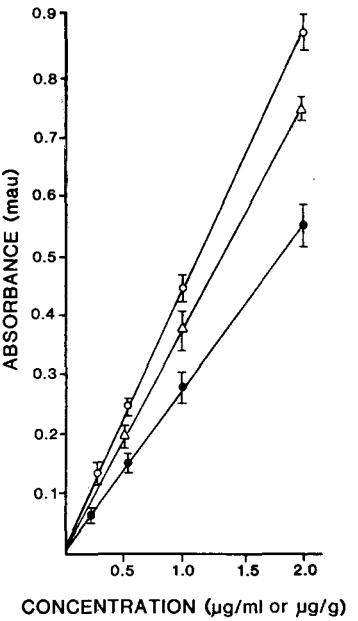


Fig. 3. Chromatograms of (A) control dog feces; (B) feces containing 20.0 μg I per g; (C) feces from a dog treated topically with I. The detector was set at 0.05 aufs, and chromatographic conditions are described under Experimental. Peaks: 1 = I; 2 = solvent front.

Fig. 4. Standard curves of iodochlorhydroxyquin (I) from urine (•——•), liver (△——△), and feces (○——○) spiked with I.

The amounts of I extracted from urine samples spiked with 0.20, 0.50, 1.0, and 2.0 μg/ml, feces samples spiked with 0.25, 0.50, 1.0, and 2.0 μg/g, and liver samples spiked with 0.50, 1.0, 2.0, and 4.0 μg/g are presented in Fig. 4. The correlation coefficient r for the standard curves for I from extracted urine, feces, and liver was 0.99 in each case. Concentrations as high as 60 μg/g of I in feces and 40 μg/ml in urine were also shown to give linear results.

The percent recovery of I following extraction of urine, feces, and liver was determined and is presented in Table I. Each value represents the mean of three determinations with the standard deviation. An average recovery of 96.85% of I was obtained by direct extraction of urine spiked with I without subjecting the samples to the hydrolysis and washing procedure which were ncessary to release conjugated I and remove interfering substances occurring as a result of the hydrolysis [1]. Approximately 28% of the I was recovered from urine following hydrolysis and the sample clean-up procedure on alumina columns.

Liver samples were spiked with I. The samples were not hydrolyzed but were subjected to the washing procedure to remove interfering substances. As such, the recovery of I was approximately 36% (Table I). The recovery of I from feces samples which had been spiked with I, hydrolyzed and washed was approximately 41% (Table I). The results indicate that the greatest loss of I occurs during the alumina column clean-up procedure which was necessary to remove substances that intefered with HPLC of I. The washing procedure was

TABLE I

RECOVERIES OF IODOCHLORHYDROXYQUIN FROM BIOLOGICAL MATERIALS

Biological sample	Added I (μg/ml or μg/g)	Percent recovery	Average percent recovery
Urine*	0.20	97.3 ± 2.1	96.8
	0.50	96.4 ± 2.3	
Urine	0.50	30.5 ± 2.7	27.8
	1.0	25.2 ± 0.4	
Liver	0.50	45.4 ± 2.9	36.0
	2.0	26.7 ± 2.0	
Feces	0.50	52.0 ± 3.1	41.0
	2.0	30.0 ± 1.5	

*Direct extraction of sample without subjection to hydrolysis and washing process.

previously employed by Tamura et al. [1] for sample preparation for the gas chromatography of I. Although the hydrolysis and washing procedures that were used reduced the percent recovery of I, extremely good reproducibility was obtained in each case.

An attempt was made to find an internal standard for the assay of I in biological tissues and fluids, and the chromatographic characteristics of the compounds presented in Table II were determined. Only α-naphthoflavone had a suitable retention time (13.5 min). However, α-naphthoflavone was sufficient as an internal standard only when samples were extracted and chromato-

TABLE II

HPLC RETENTION TIMES OF VARIOUS COMPOUNDS EXAMINED AS POSSIBLE INTERNAL STANDARDS

Substance	Retention time (min)
Iodochlorhydroxyquin	7.5
4,7-Dichloroquinoline	6.0
5,7-Dichloro-8-hydroxyquinoline	6.2
10-Chloro-9-anthracene—methanol	7.5
9-Hydroxy-4-methoxyacridine	3.0
Trihexyphenidyl hydrochloride	5.4
Diphenylcarbazone	2.6
4-Chloro-3-methylphenol	3.0
α-Bromo-*p*-nitrotoluene	3.5
1-Naphthylamine	3.0
5-Nitroso-8-quinolinol	2.5
Tribenzylamine	5.7
Xanthin-9-ol	5.5
Dibenzofurane	8.0
4,4-Dimethylbenzophenone	6.0
Theophylline	2.3
Benzanilide	3.0
2,7-Naphthalenediol	2.5
α-Naphthoflavone	13.5

graphed directly without subjection to the alumina column washing and clean-up procedure. Due to the solubility of the α-naphthoflavone in the washing solvents, the α-naphthoflavone did not adhere to the alumina and was lost. Therefore, this compound can only be used as a standard when added before injection of an extract onto the column. Hayakawa et al. [18] have used 5,7-dichloro-8-hydroxyquinoline as an internal standard without an alumina column washing and clean-up procedure.

The present method provides a rapid, sensitive, and reproducible procedure for the determination of I in different biological materials and will be useful for toxicity and metabolism studies. I has been previously shown to be well absorbed upon oral administration and is associated with neuromuscular toxicity [14]. The present study indicates that I is extensively absorbed upon topical application to dogs.

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CHROMBIO. 1699

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CEFOPERAZONE IN HUMAN SERUM AND URINE

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SUMMARY

A gradient high-performance liquid chromatographic (HPLC) procedure has been developed for the determination of microgram amounts of cefoperazone in human serum and urine. The method employes a μBondapak C_{18} column and gradient elution with two mobile phases. Excellent separation of the drug from potential degradation products as well as from representative penicillins (sodium ampicillin, sodium methicillin, potassium penicillin G) and aminoglycosides (tobramycin, gentamicin, kanamycin) has been demonstrated. Coefficients of variation of 7.3% or less were obtained for 25—100 μg/ml cefoperazone in both serum and urine. Average recoveries of the drug from spiked serum and urine samples corresponded to 97.6% and 98.6%, respectively. Amounts as low as 1 μg cefoperazone per ml of sample can be estimated using sample volumes corresponding to 0.1 ml serum or 1 ml urine.

Good correlation between the HPLC assay and a microbiological cylinder—plate assay employing *Micrococcus luteus* ATCC 9341 has been demonstrated for human serum and urine of patients treated with cefoperazone. While the microbiological method is less time-consuming, it lacks specificity in the presence of other antibiotics. The HPLC method can be used to analyze cefoperazone in the presence of penicillins and aminoglycosides which can potentially be co-administered with cefoperazone.

INTRODUCTION

Cefoperazone (I) is a recently developed cephalosporin which has been demonstrated to be bactericidal, not only against gram-positive bacteria but also against gram-negative bacteria, especially *Pseudomonas* and *Enterobacter* species [1].

A selective assay was required to determine bioavailability of this drug and to monitor its levels in serum and urine of subjects to whom cefoperazone had been administered. A recent paper published by Dupont and DeJager [2] describes an isocratic high-performance liquid chromatographic (HPLC) procedure for cefoperazone quantitation in serum. After clean-up of samples

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with Sep-Pak cartridges containing μBondapak C_{18}/Porasil (R/B), cefoperazone is injected onto the reversed-phase μBondapak C_{18} (10 μm particle size, 250 mm × 4.6 mm I.D.) HPLC column. A mobile phase consisting of methanol—water (1:1) is used. The HPLC portion of the procedure is very rapid, the retention time of cefoperazone being 2.3 min. However, the selectivity was not discussed.

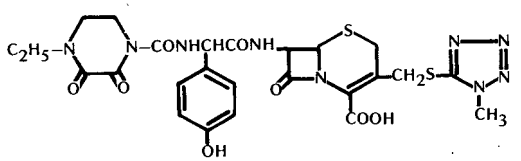
Using slower gradient HPLC described in this paper, the preliminary sample clean-up is not needed. In addition, cefoperazone can be separated from known degradation products as well as from representative penicillins and aminoglycosides.

The HPLC method has been compared to a cylinder—plate microbiological assay procedure employing *Micrococcus luteus* ATCC 9341 as the test organism.

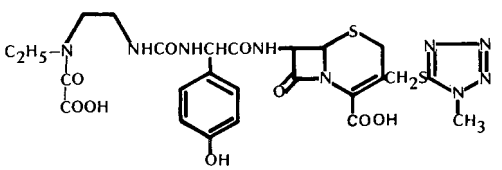
HPLC METHOD

Materials

Cefoperazone (I), cefoperazone degradation products A, B, D, E, F, (II—VI), sodium ampicillin and potassium penicillin G were supplied by Central Research of Pfizer (Groton, CT, U.S.A.). Tobramycin was obtained from Eli Lilly (Indianapolis, IN, U.S.A.). Sodium methacillin and kanamycin were



(I)



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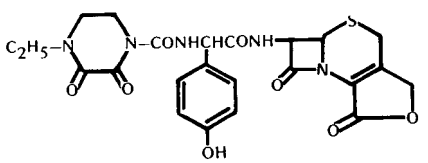

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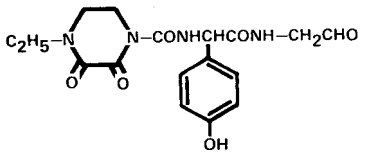

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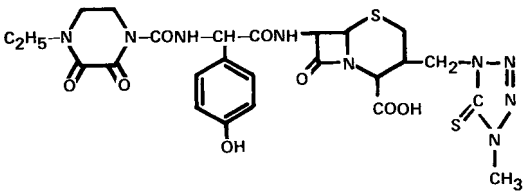
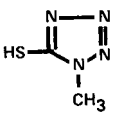


E(V)



F(VI)

acquired from Bristol Laboratories (Syracuse, NY, U.S.A.). Gentamicin was supplied by Schering (Kenilworth, NJ, U.S.A.). Glass distilled acetonitrile was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Triethylamine and acetic acid were reagent-grade quality and were used without further purification.

Apparatus and chromatographic conditions

Analyses were carried out using a μBondapak C_{18} column (10 μm particle size, 250 × 4.6 mm I.D.) attached to a UV detector at 254 nm. A dual-channel recorder, individual channels set at two different sensitivities (e.g. 0.01 a.u.f.s., 0.2 a.u.f.s.), was used in order to simultaneously record minor and major sample components. A sample injection system (e.g. Waters Assoc. U6K injector) was used to apply 20 μl of the sample or standard solution onto the column. A gradient programmer, Waters Model 660, was set using curve select-9. Two mobile phases were used. Mobile phase A was composed of 0.0012 *M* triethylamine and 0.042 *M* acetic acid in distilled water. Mobile phase B was composed of 0.0012 *M* triethylamine and 0.042 *M* acetic acid in acetonitrile—distilled water (24:76). The flow-rate of 1.5 ml/min mobile phase A and 0.5 ml/min mobile phase B was adjusted during 15 min to 1.2 ml/min mobile phase A and 0.8 ml/min mobile phase B. The assay was completed isocratically under the final conditions.

Preparation of samples and calibration curves

Serum analysis. Transfer a 1-ml aliquot of the serum sample to a 15-ml centrifuge tube and add 1 ml of methanol. Vortex the sample for 30 sec and allow it to stand at room temperature for 10 min; if necessary, dilute the sample with distilled water to obtain a cefoperazone concentration within the linearity range of 1—100 μg/ml serum and centrifuge the sample at 1022 *g* for 10 min. The procedure for a microsample is analogous. However, only 0.1 ml of sample and 0.1 ml of methanol are used.

Urine analysis. Centrifuge urine specimens at 1022 *g* for 10 min. If necessary, dilute the sample with distilled water to obtain a cefoperazone concentration within the range of 1—100 μg cefoperazone per ml urine.

Calibration for serum analysis. Prepare a series of cefoperazone standard solutions containing 25, 50, and 100 μg/ml in 1.0 ml of distilled water. Add 1.0 ml of methanol and vortex each sample for 30 sec. Inject 20 μl of each standard solution into the HPLC system. Measure peak heights of cefoperazone. Calculate the linear regression equation which characterizes the calibration curve. Prepare this curve daily.

Calibration for urine analysis. Prepare a series of cefoperazone standard solutions containing 25, 50, 100 μg in 1.0 ml of distilled water. Inject 20 μl of each standard solution into the HPLC system. Measure peak heights of cefoperazone. Calculate the regression equation which characterizes the calibration curve. Prepare this curve daily.

HPLC assay of serum and urine samples

Inject 20 μl of prepared sample into HPLC system. Measure peak heights of cefoperazone. Calculate μg cefoperazone per ml serum or urine, using regression lines obtained by the calibration of aqueous standards:

$$\mu\text{g cefoperazone per ml} = \frac{\text{peak height of sample}}{\text{slope}} \times \text{dilution factor.}$$

MICROBIOLOGICAL ASSAY

Materials and equipment

The test organism used (*Micrococcus luteus* ATCC 9341) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The plastic Petri dishes (100 × 20 mm; Falcon Plastics, Cockeysville, MD, U.S.A.) contained approximately 12 ml of Antibiotic Medium A (BBL, Cockeysville, MD, U.S.A.). Sterile normal human serum (Bio-Bee, Boston, MA, U.S.A.) and 1% potassium phosphate buffer, pH 6.0, were used to dilute standard curves and samples for the serum and urine assays, respectively. The zones of inhibition were read by an automated zone reader (AZOR) manufactured by Digital Information Science (Silver Spring, MD, U.S.A.). The calculations were performed by a PDP8E (DEC, Maynard, MA, U.S.A.) computer hard wired to the reader.

Preparation of standard and sample solutions

Serum analysis. The serum samples were analyzed against a compensatory standard curve diluted in human control serum. The standard curve ranged in

concentration from 1.5 to 6.0 μg/ml of cefoperazone. When necessary, the serum samples were diluted to contain approximately 3 μg/ml of cefoperazone using pooled human control serum.

Urine analysis. The standard curve was prepared in 1% potassium phosphate buffer, pH 6.0. The standard curve ranged in concentration from 0.3 μg to 1.2 μg/ml of cefoperazone. Samples that contained more than 1.2 μg/ml of cefoperazone were diluted with phosphate buffer to approximately 0.6 μg/ml.

Sample assay

The microbiological agar diffusion assay for cefoperazone employed a cylinder–plate technique essentially described in the United States Pharmacopeia [3]. The basic assay employed *Micrococcus luteus* ATCC 9341 as the test organism and antibiotic Medium A as the test medium. A single seeded 12-ml agar layer was used to enhance the sensitivity. After application of the standards and samples, the plates were incubated at 32—35°C for approximately 18 h. The resulting zones of inhibition were read by an automated zone reader (AZOR). The automated plate reader and computerized data handling system have been described [4]. A linear regression analysis was performed on the logarithm of the standard concentrations (μg/ml) versus their zone diameter (mm). The concentration of cefoperazone in the samples was calculated according to the following equation:

$$\text{Log } C_s = \log C_r - \Delta(m)$$

where C_s = sample concentration, C_r = reference concentration, Δ = average sample zone diameter — average reference zone diameter, and m = slope of standard curve. Potency is then calculated by the following equation:

$$\text{Potency } (\mu\text{g/ml}) = \frac{C_s \times \text{dilution}}{\text{ml}}.$$

RESULTS AND DISCUSSION

The first step in the development of the HPLC procedure was to establish selectivity. It was challenged with known cefoperazone (I) degradation products: A(II), B(III), D(IV), E(V) and F(VI).

While in the published procedure [2] cefoperazone co-eluted with cefoperazone degradation products A, E, and F, it was separated from these substances using gradient HPLC. A chromatogram depicted in Fig. 1 illustrates the good separation of all substances tested. In addition, potential interference of penicillins (e.g. sodium ampicillin, sodium methicillin, potassium penicillin G) and aminoglycosides (e.g. tobramycin, gentamicin, kanamycin) was investigated. Relative retention times summarized in Table I demonstrate that none of these substances interfere with the quantification of cefoperazone.

The HPLC response was found to be linear in the range of 1—100 μg/ml of sample. Since the calibration curves obtained by direct injection of cefoperazone standard solutions were identical with those obtained using spiked blank serum or urine, the more simple calibration using standard cefoperazone solutions directly is recommended for testing of clinical samples.

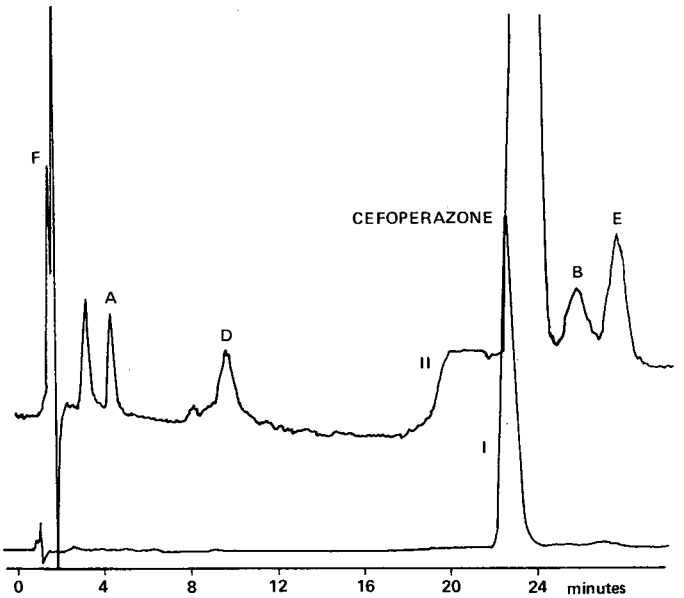


Fig. 1. Separation of cefoperazone (100 μg/ml) and cefoperazone degradation products (2 μg/ml of degradation products A, B, D and E and 0.5 μg/ml of degradation product F). I, 0.2 a.u.f.s.; II, 0.01 a.u.f.s.

TABLE I

RELATIVE RETENTION TIMES OF KNOWN CEFOPERAZONE DEGRADATION PRODUCTS, REPRESENTATIVE PENICILLINS AND AMINOGLYCOSIDES

Substance	Relative retention time
Gentamicin	0.00
Kanamycin	0.00
Tobramycin	0.00
Cefoperazone F	0.07
Cefoperazone A	0.19
Ampicillin sodium	0.23
Cefoperazone D	0.43
Methacillin sodium	0.95
Penicillin G potassium	0.95
Cefoperazone	1.00
Cefoperazone B	1.15
Cefoperazone E	1.23

The accuracy and precision were evaluated by adding known amounts of cefoperazone to blank serum and urine in both the presence and absence of penicillins and aminoglycosides. The results of the HPLC analyses are summarized in Tables II–IV. Coefficients of variation of 7.3% or less were obtained for 25—100 μg/ml cefoperazone in both serum and urine. Average recoveries of the drug from spiked serum and urine samples corresponded to 97.6% and 98.6%, respectively.

TABLE II

ACCURACY AND PRECISION OF THE HPLC ASSAY FOR CEFOPERAZONE IN URINE AND SERUM

Concentration range	*n*	Concentration of cefoperazone added	Average concentration found	Coefficient of variation (%)	Average recovery (%)
Urine 25—100 μg/ml	5	25.0	24.3	5.41	97.2
	5	50.0	50.0	0.66	100.0
	5	75.0	74.6	1.48	99.5
	5	100.0	99.8	0.42	99.8
Serum 25—100 μg/ml	5	25.0	24.9	2.04	99.6
	5	50.0	49.6	0.72	99.2
	5	100.0	98.3	1.03	98.3

TABLE III

ACCURACY AND PRECISION OF THE HPLC ASSAY FOR CEFOPERAZONE IN URINE AND SERUM IN THE PRESENCE OF SODIUM AMPICILLIN, SODIUM METHICILLIN AND POTASSIUM PENICILLIN G

Amounts of 100 μg sodium ampicillin, 100 μg sodium methicillin and 100 μg potassium penicillin G were added to 1 ml of each sample.

Concentration range	*n*	Concentration of cefoperazone added	Average concentration found	Coefficient of variation (%)	Average recovery (%)
Urine 25—100 μg/ml	5	25.0	23.6	0.93	94.4
	5	50.0	48.8	2.73	97.6
	5	100.0	98.6	4.09	98.6
Serum 25—100 μg/ml	6	25.0	24.4	3.86	97.6
	6	50.0	48.1	3.26	96.2
	6	100.0	94.6	3.67	94.6

TABLE IV

ACCURACY AND PRECISION OF THE HPLC ASSAY FOR CEFOPERAZONE IN URINE AND SERUM IN THE PRESENCE OF TOBRAMYCIN, GENTAMICIN AND KANAMYCIN

Amounts of 100 μg tobramycin, 100 μg gentamicin and 100 μg kanamycin were added to 1 ml of each sample.

Concentration range	*n*	Concentration of cefoperazone added	Average concentration found	Coefficient of variation (%)	Average recovery (%)
Urine 25—100 μg/ml	2	25.0	25.1	7.06	100.4
	2	50.0	49.5	7.29	99.0
	2	100.0	101.2	3.91	101.2
Serum 25—100 μg/ml	2	25.0	25.5	5.83	102.0
	2	50.0	48.5	7.29	97.0
	2	100.0	96.5	5.20	96.5

The HPLC method was compared to the microbiological assay by analyzing urine and serum samples of patients who had been administered only cefoperazone. A typical chromatogram of a patient serum is presented in Fig. 2. Results of parallel determinations by both methods are listed in Table V. The

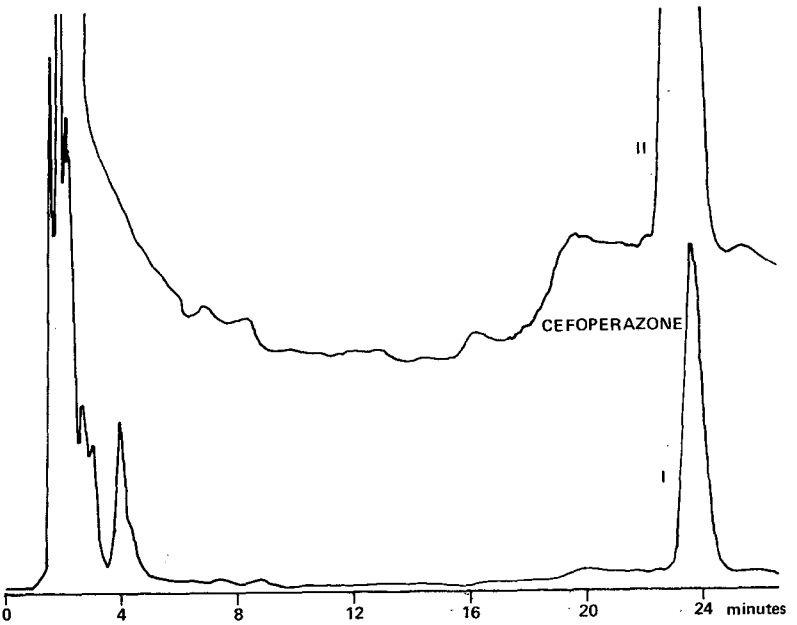


Fig. 2. Typical chromatogram of a serum of a patient who had been administered cefoperazone. I, 0.2 a.u.f.s.; II, 0.01 a.u.f.s.

TABLE V

EQUIVALENCY OF MICROBIOLOGICAL AND HPLC ANALYSES OF SERUM AND URINE OF PATIENTS ADMINISTERED CEFOPERAZONE

	Sample No.	Cefoperazone determined (μg/ml)				Sample No.	Cefoperazone determined (μg/ml)	
		HPLC assay		Bioassay			HPLC assay	Bioassay
Serum	1	166	167	156	157	8	288	277
	2	77	75	74	70	9	197	187
	3	46	43	42	40	10	126	123
	4	153	163	160	144	11	70	76
	5	72	71	67	65	12	33	37
	6	49	40	38	37	13	9	10
	7	243		235		14	2	3
Urine	1	317		333		4	67	57
	2	109		132		5	16	14
	3	101		103				

average percent agreement of the HPLC assay compared to the microbiological assay was +3.8% for the serum samples and —4.5% for the urine samples, differences which are acceptable for analysis of clinical specimens. The correlation between the serum and urine cefoperazone concentrations determined by the HPLC and microbiological methods was evaluated further using linear regression analysis. The correlation coefficients were 0.999 and 0.996 for the serum and urine determinations, respectively. The results support the conclusion regarding the excellent correlation between the two methods.

The microbiological method is less time-consuming than the HPLC method and is well suited for processing large numbers of clinical samples where sufficient sample exists and cefoperazone is the only antibiotic administered. The good agreement of the microbiological method with the HPLC technique, which specifically measures the intact drug, indicates that the microbiological method is relatively specific for the active parent compound. It further suggests that the degradation products and potential metabolites of cefoperazone do not substantially interfere with the microbiological assay.

For samples which potentially contain other penicillins or aminoglycosides, the HPLC method is clearly the method of choice due to its specificity. The HPLC method is also particularly useful in those instances where very low volumes of samples (e.g. 0.1 ml) are available as, for example, in monitoring cefoperazone levels in the serum of new born infants.

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CHROMBIO. 1714

QUANTITATIVE THIN-LAYER CHROMATOGRAPHY FOR ROUTINE DETERMINATION OF NOMIFENSINE AND ITS METABOLITES IN HUMAN URINE

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(First received August 4th, 1982; revised manuscript received March 16th, 1983)

SUMMARY

For pharmacokinetic studies with nomifensine, a thin-layer chromatographic (TLC) assay for human urine was introduced. Following acid cleavage of the N-glucuronides, nomifensine and its three main metabolites (M1, M2 and M3) were extracted at pH 10. An aliquot was transferred on to a silica gel plate. After chromatography, irradiation led to intense fluorescent yellow products, which were evaluated using a chromatogram spectrophotometer. Calibration graphs were defined by single parameters of non-linearity. The method is practicable, selective and accurate with detection limits of 0.2 μg/ml in urine for the four compounds of interest and can be used for assaying samples up to 24 h following dosage. Total nomifensine urine levels correlated well with those determined by a previous radioimmunoassay method. From cumulative excretions of nomifensine, complete relative bioavailability of a capsule formulation vs. oral solution was shown. Further, sex independence of urine excretion was demonstrated.

Pharmacokinetic data were computed using a two-compartment open model for nomifensine and its potent metabolite M1 or a one-compartment open model for M2 and M3.

INTRODUCTION

Nomifensine maleate (Alival®, Merital®) is a recently introduced antidepressive drug (Fig. 1). For pharmacokinetic purposes a practicable and selective analytical method is required for the determination of the parent drug and its metabolites M1, M2 and M3 in urine.

Previously published methods employed ^{14}C-labelled drug [1, 2], radioimmunoassay (RIA) [3], gas—liquid chromatography (GLC) [4—7] and high-performance liquid chromatography (HPLC) [8]. Using specific RIA, only the parent compound is determined in serum and urine. GLC for the

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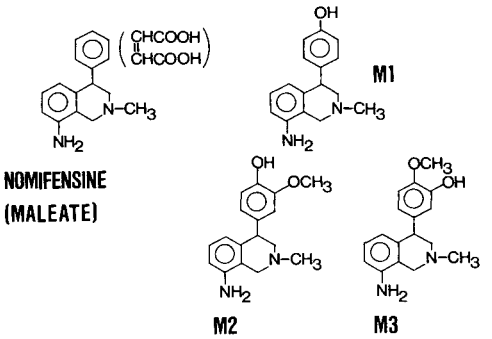

Fig. 1. Structures of nomifensine and metabolites.

determination of nomifensine in plasma requires derivatization steps. Using HPLC, nomifensine, the potent [9] metabolite M1 and the sum of M2 and M3 were determined only in serum. Total nomifensine plasma half-lives of approximately 2 h [3, 5, 7, 8] or approximately 4 h [4, 6] have been published. Total metabolite half-lives of approximately 1—2 h have been reported by Uihlein and Hajdu [8].

In urine, the glucuronides [2] of nomifensine and its metabolites are cleaved prior to analysis. On a TLC plate, fluorescent and intense yellow products of the separated compounds are formed on irradiation, which enhances the intensity of the spots and the sensitivity as well as the selectivity of the TLC method.

Analytical data have been calculated for the free base and denoted as total compound.

EXPERIMENTAL

Materials

Reagents. Analytical-reagent grade chemicals were used unless indicated otherwise. The reagents used were 0.1 mol/l hydrochloric acid, 0.1 mol/l carbonate buffer (pH 10), ethyl acetate, ethanol and concentrated ammonia solution (25%). Reference substances were supplied by Dr. K. Schmitt of Hoechst AG (nomifensine and M1 as maleates, M2 and M3 as free bases). The solvent system was ethyl acetate—ethanol—concentrated ammonia (8:2:0.2).

Equipment. A Zeiss KM 3 chromatogram spectrophotometer with micro-optics and a Servogor (Metrawatt) recorder were used. Separation was performed on silica gel HPTLC plates (No. 5641; E. Merck, Darmstadt, G.F.R.) in a Camag twin-trough HPTLC chamber, 20 × 10 cm (No. 25254). For sample clean-up and spotting, a Vortex mixer, a centrifuge, glass-stoppered tubes (ca. 8 ml) and a Desaga Autospotter* were used.

Sample preparation

Hydrolysis of N-glucuronides. Nomifensine-N-glucuronide and N-glucuronides of metabolites M1, M2 and M3 were hydrolysed by incubation in

*Modified version: Tygon tubes of larger diameter [Technicon, flow-rated, code: 116-0549-09 (white)] and 60 cm long Hostaflon tubes were used.

TABLE I

AMOUNTS OF MATERIALS USED IN HYDROLYSIS REACTION

Dose of nomifensine maleate (mg)	Equivalent to nomifensine (mg)	Urine used (μl)	0.1 mol/l HCl added (μl)
200	134.5	100	10
100	67.2	200	20
50	33.6	500	50
25	16.8	1000	100

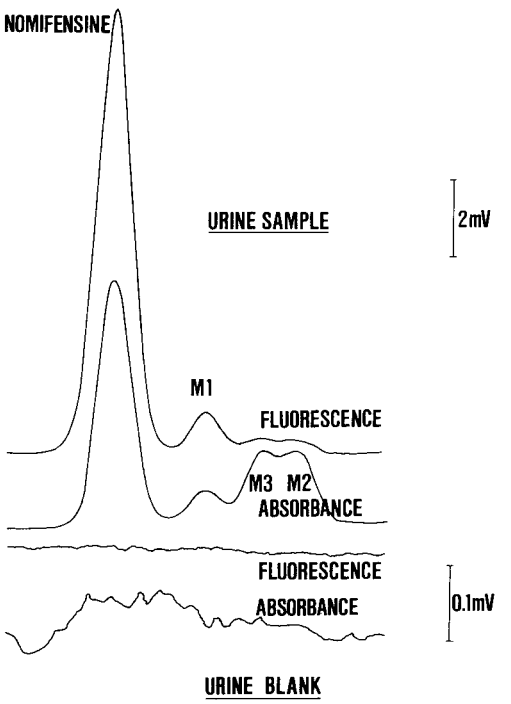

Fig. 2. Chromatography of urine extracts from a subject who had received 75 mg of nomifensine maleate orally. Urine sampled 0—5 h after application, containing 80 μl/ml of total nomifensine and 12 μg/ml of total M1, M2 and M3. A blank is shown for comparison.

0.1 mol/l hydrochloric acid. To cover the optimal range of the calibration graph, different aliquots of urine were used according to the scheme shown in Table I. The solution was incubated for 2 h in the dark at room temperature, then the pH was readjusted to pH 10 (Fig. 2) by adding 1 ml of 1 mol/l carbonate buffer (pH 10).

Extraction. The mixture was extracted with 5 ml of ethyl acetate for 30 sec on a Vortex mixer and centrifuged for 5 min. An aliquot of 100 μl (ca. 2%) of the organic phase was transferred for spotting.

Sample spotting. Using the Desaga Autospotter, 75 μl were applied on to the

HPTLC plate as a series of consecutive droplets of approximately 100 nl each. As each droplet was evaporated before the next one fell, a narrow spot was obtained suitable for HPTLC. Simultaneously, up to twelve extracts of samples, five standards and three controls were positioned on the plate (parameters: spotting speed, "2"; heating, "2"; ventilation,"2").

Chromatography

The twin-trough HPTLC developing chamber contained 10 ml of the solvent in one compartment. Chromatography was carried out at room temperature in the dark and without solvent saturation of the chamber. Within the developing time of 20 min, the solvent front moved 5 cm. Under UV light (254 nm), the HPTLC plate was heated on a heating plate for 2 h at 70°C. In this way, the parent compound and its metabolites were transformed into intense fluorescent yellow products [1].

The R_F values were as follows: nomifensine, 0.55; M1, 0.50; M2, 0.40; and M3, 0.45.

Evaluation

Measurement of nomifensine and M1 was based on their fluorescence; an excitation wavelength of 313 nm (Hg) and a secondary filter cut-off wavelength of 460 nm (FL 46) were used. In contrast, M2 and M3 were quantitated by their absorbance in the visible range (410 nm) (Fig. 2).

Calibration

Calibration functions [10] were determined for each compound from the peak heights of the standards:

$$C = \frac{C_{max} \cdot E_{rel} \cdot K_m}{1 - E_{rel} + K_m}$$

where C_{max} = maximum calibration standard, E_{rel} = peak height/maximum peak height and K_m = parameter of non-linearity. K_m was obtained from a Hofstee plot following normalization of peak heights and concentrations (Fig. 3).

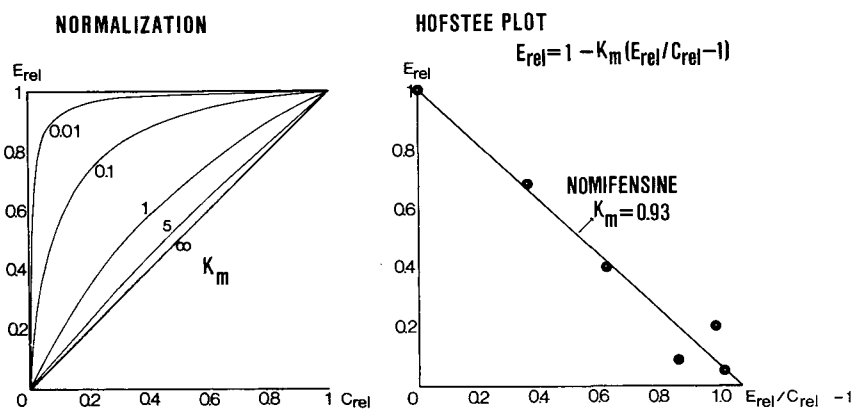

Fig. 3. Determination of K_m non-linearity parameter from a Hofstee plot.

RESULTS AND DISCUSSION

Physico-chemical properties of nomifensine and its metabolites

A knowledge of the physico-chemical properties of any compound is essential for obtaining optimal conditions for extraction from body fluids and for choosing the most suitable conditions of measurement.

Partition in octanol—water was studied in the pH range 3—12 (Fig. 4). At pH 8—10, maximal partition coefficients of 79 ± 9 were found for nomifensine (75 in diethyl ether—buffer [11]), 50 for M1 and 26 for M2.

In all instances pK_1 = 2.1 ± 0.1 (aminophenyl) was calculated from pH-dependent UV absorbances at 281 nm. For nomifensine, pK_2 = 7.0 ± 0.1 (amine) from aqueous titrations, equal to pK_2 = 7.1 ± 0.2 from the partition study. For M1, pK_2 = 7.4 ± 0.1 and pK_3 = 10.1 ± 0.1 (phenol) from titrations, equal to pK_2 = 7.5 and pK_3 = 10.1 from partition data. For M2, pK_2 = 7.5 and pK_3 = 10.2 were calculated from partition data.

Assay performance characteristics [12—14]

The compounds were mixed with blank urine at seven concentrations over the analytical ranges indicated in Table II. Each mixture was split into six portions of 0.5 ml, so that six equal series were formed. Each series was then analysed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria of an analytical method are selectivity, precision, sensitivity

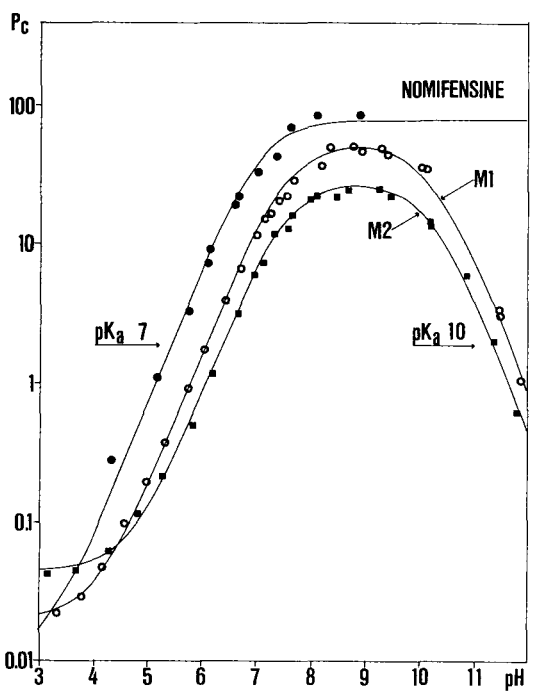

Fig. 4. Partition coefficients of nomifensine and metabolites in octanol—water.

TABLE II

NOMIFENSINE, M1, M2 AND M3 DETERMINATION BY TLC, RECOVERY AND INTRA-ASSAY PRECISION

n = 6; concentrations in μg/ml (mean ± S.D.).

Nomifensine		M1		M2		M3	
Added	Found	Added	Found	Added	Found	Added	Found
134.5	134.5 ± 1.6	34.3	34.2 ± 0.08	50.0	50.0 ± 0.57	50.0	50.0 ± 0.12
67.2	67.2 ± 1.4	13.7	14.5 ± 0.33	20.0	20.0 ± 0.39	20.0	20.6 ± 0.48
33.6	33.4 ± 0.64	6.9	6.7 ± 0.35	10.0	10.1 ± 0.20	10.0	9.9 ± 0.22
13.4	14.0 ± 0.31	3.4	3.4 ± 0.05	5.0	4.9 ± 0.10	5.0	4.7 ± 0.18
6.7	6.3 ± 0.18	1.4	1.3 ± 0.12	2.0	1.6 ± 0.21	2.0	1.6 ± 0.12
3.4	3.4 ± 0.19	0.7	0.7 ± 0.08	1.0	0.9 ± 0.15	1.0	0.9 ± 0.17
1.3	1.4 ± 0.10	0.3	0.3 ± 0.05	0.5	0.4 ± 0.12	0.5	0.5 ± 0.14
0	0	0	0	0	0	0	0
Accuracy (μg/ml):							
0.01 ± 0.31		0.06 ± 0.34		−0.09 ± 0.16		−0.04 ± 0.32	

TABLE III

NOMIFENSINE, M1, M2 AND M3 DETERMINATION BY TLC, INTER-ASSAY PRECISION

Control urines between August and November 1980; concentration in μg/ml (mean ± S.D.); C.V. = coefficient of variation.

Compound	Control 1	Control 2	Control 3
Nomifensine	65.9 ± 2.9 (C.V. 4%)	12.9 ± 1.3 (C.V. 10%)	3.4 ± 0.3 (C.V. 9%)
M1	6.8 ± 0.5 (C.V. 7%)	1.4 ± 0.2 (C.V. 14%)	0.34 ± 0.10 (C.V. 29%)
M2	9.5 ± 0.7 (C.V. 7%)	2.0 ± 0.3 (C.V. 15%)	0.49 ± 0.07 (C.V. 14%)
M3	9.7 ± 0.9 (C.V. 9%)	2.0 ± 0.3 (C.V. 15%)	0.47 ± 0.07 (C.V. 15%)

and accuracy. These parameters were derived from the analytical results given in Table II. As regards selectivity, the assay is free from interferences for all substances (Fig. 2).

Mean concentrations measured were linearly correlated with the standard deviations (S.D.s) and in this way the intra-assay precision was defined. Sensitivity was expressed as the detection limit (D.L.) and was taken as the intercept ×2. For nomifensine, precision = 1.9% of the result + 0.07 μg/ml, D.L. = 0.2 μg/ml; for M1, precision = 2.1% of the results + 0.07 μg/ml, D.L. = 0.2 μg/ml; for M2, precision = 1.2% of the result + 0.12 μg/ml, D.L. = 0.2 μg/ml; and for M3, precision = 1.6% of the result + 0.11 μg/ml, D.L. = 0.2 μg/ml.

The inter-assay precision was tested on three spiked control urines over a 4-month period. The results are presented in Table III.

Accuracy was considered to be the deviation (bias) at the mean value of the concentrations found from the concentrations mixed with blank urine. In all instances the average accuracy was < 0.1 μg/ml. Regression coefficients were greater than 0.9996.

Accuracy was further tested by comparison with radioimmunoassay. Urine samples collected after oral administration of 50 mg of nomifensine maleate

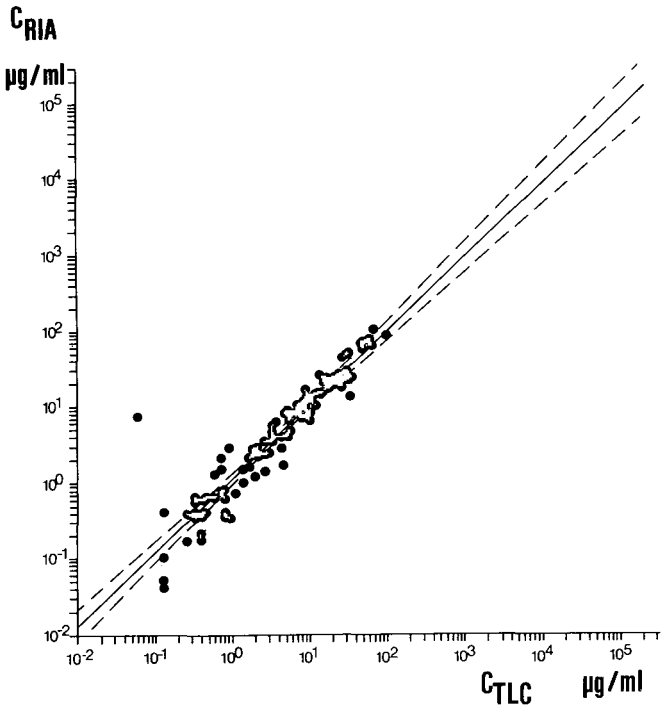

Fig. 5. Parallel determination of total nomifensine in urine by TLC and RIA (n = 131).

(solution) to healthy subjects [15] were measured by both TLC and RIA [3]. Results ranging over four decades were correlated by unweighted log–log linear regression (Fig. 5). Identity requires an intercept of zero and a slope of one. From 131 determinations, an intercept of 0.05 with a 95% probability range of −0.01 to 0.10 and a slope of 0.97 with a 95% probability range of 0.91 to 1.03 were calculated, thus including zero and one, respectively. Therefore, identity of the results of the two methods was verified.

The capacity is such that 100 samples can be processed per person per week.

Pharmacokinetics

In a cross-over study with different oral dosage forms, i.e., oral solution vs. capsule, 50 mg of nomifensine maleate were administered to 23 male volunteers. Details of the human experiment and sample collection were described previously [15].

By TLC, urine levels of the compounds were found in ranges 0.2—150 µg/ml of total nomifensine and 0.2—20 µg/ml of total metabolite(s).

By analogy with plasma levels, urinary excretion rates were used in order to obtain pharmacokinetic data (Fig. 6). For nomifensine and its potent metabolite M1, a bi-exponential decrease was found with identical terminal half-lives of 4 h. In contrast, M2 and M3 were excreted mono-exponentially with identical half-lives of 2 h.

On average, 58 ± 13% of an oral solution was found in urine, 41.0 ± 9.4%

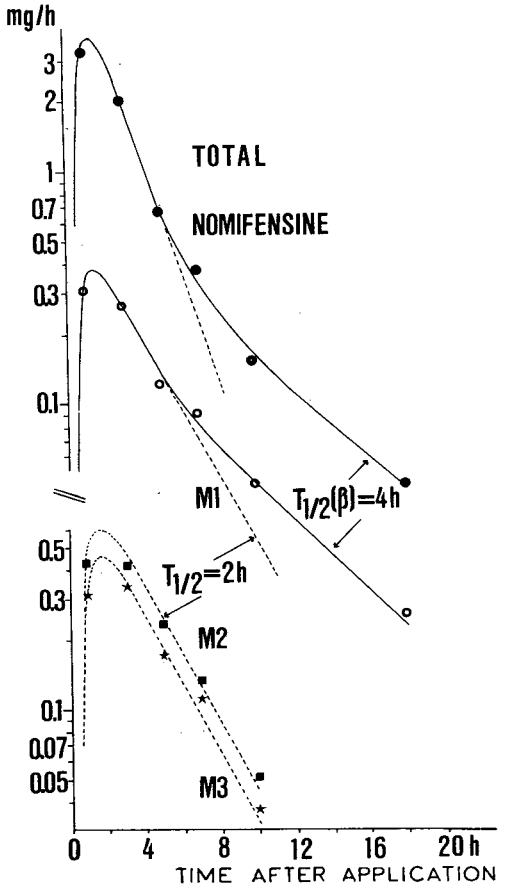

Fig. 6. Half-lives of total nomifensine and total metabolites from mean renal excretion rates (23 volunteers). ——, Two-compartment model; — — —, one-compartment model.

was excreted as total nomifensine, 5.4 ± 1.4% as total M1, 6.2 ± 2.0% as total M2 and 5.0 ± 1.5% as total M3 (Fig. 7) [1].

In capsule form, the same amount was administered to the same subjects. With a delay of just 20 min (Fig. 8), the following amounts were excreted renally: 60 ± 10% the dose was found in urine, 41.6 ± 8.0% was excreted as total nomifensine, 5.7 ± 1.4% as total M1, 7.0 ± 1.7% as total M2 and 5.7 ± 1.5% as total M3.

Excretion ratios were identical following both oral dosage forms. Therefore, nomifensine in capsules is 100% bioavailable compared with that in aqueous solution.

In a further study*, the drug was given to male and female volunteers. The details of the human experiment were similar to those of the bioavailability study (see above). Following an oral dose of 1.3 mg of nomifensine maleate

*This study was performed by Drs. W. Rupp and M.J. Badian of Hoechst AG.

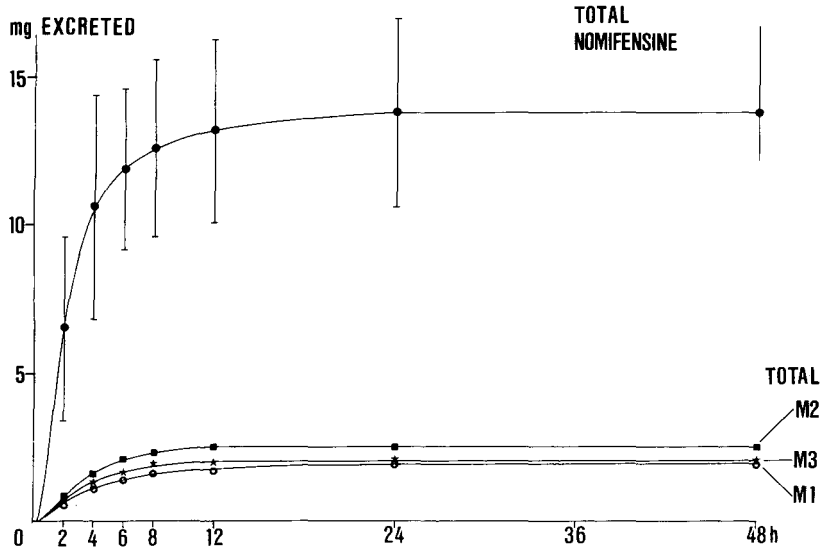

Fig. 7. Mean cumulative renal excretion of total nomifensine, M1, M2 and M3 by 23 male volunteers after administration of an oral solution of 50 mg of nomifensine maleate (= 33.6 mg of nomifensine).

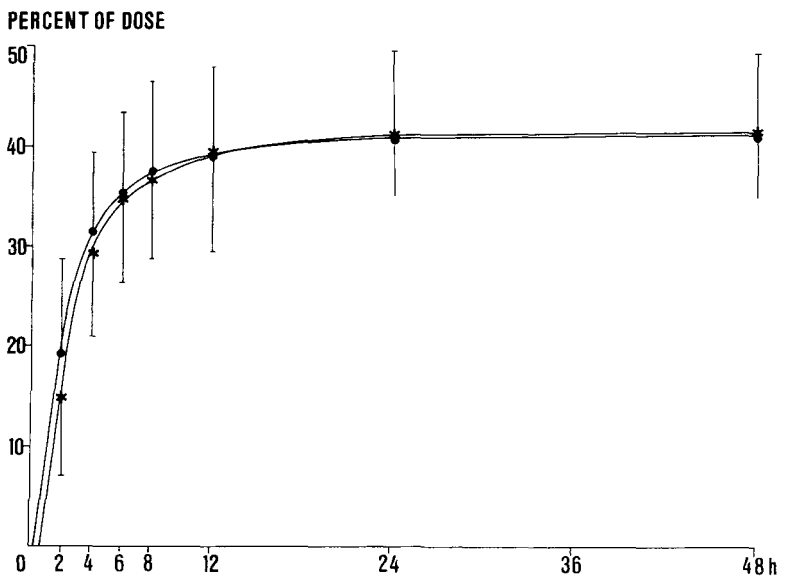

Fig. 8. Relative bioavailability: cross-over study of 23 male volunteers, capsule (⋆) vs. oral solution (•) of 50 mg of nomifensine maleate. Total nomifensine excretion as percentage of dose.

per kilogram body weight, approximately 60% of the dose was accounted for in urine (Table IV).

Sex-independent renal excretion is therefore verified.

TABLE IV

CUMULATED URINE EXCRETIONS AS PERCENTAGE OF ORAL DOSE

Compound	8 males, 69 ± 5 kg: mean ± S.D. (%)	8 females, 56 ± 4 kg: mean ± S.D. (%)
Total nomifensine	36.6 ± 6.6	35.4 ± 6.6
Total M1	6.4 ± 0.9	6.8 ± 2.2
Total M2	8.7 ± 0.8	8.2 ± 1.7
Total M3	7.6 ± 0.8	7.2 ± 1.8
Sum	59 ± 7	58 ± 11

CONCLUSION

TLC has been demonstrated to be suitable for the assay of nomifensine in urine. In our laboratory, this technique has proved to be reliable, selective, sensitive and efficient method suitable for routine assay, and may be employed in most instances whenever radioimmunoassay is not available or all metabolites have to be determined separately.

Further improvements are expected in the future. New spotting devices, for instance the Fenimore Contact Spotter or the Camag Automatic TLC Sampler, should allow more efficient use of the TLC layer. Automation of measurements by Ebel's system [16] will certainly alleviate operator requirements. With a minimum of instrumental expense, new and selective reagents for derivatization of compounds will help to increase sensitivity and selectivity. In our experience, this is of utmost importance whenever interferences from unknown or undeclared biological materials or co-medications occur.

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Note

Lipophilic ion exchangers for group separation of conjugated metabolites of xenobiotics

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Xenobiotics undergo a variety of metabolic reactions in the mammalian organism. In many cases the metabolic end products are compounds coupled to glucuronic or sulphuric acid or to amino acids in thioether or amide linkage. Analysis of the mixtures of metabolites involves extraction and chromatographic fractionation prior to identification and quantitation. Usually differences in charge and acidity between metabolites are not utilized in the isolation scheme, and the mixture of metabolites is often hydrolysed first to remove conjugating substituents. This results in loss of information regarding individual conjugated metabolites, specificity of the conjugating enzymes and further metabolic transformation of conjugated compounds.

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The metabolism of xenobiotics shows several analogies with the metabolism of endogenous compounds such as bile acids and hormonal steroids. Groups of metabolites are formed differing in charge and acidity. In order to separate these groups, lipophilic ion exchangers have been synthesized that can be used in organic solvents and that give little or no non-ionic interaction with amphiphilic compounds [1—4]. Methods for the analysis of metabolic profiles of steroids and bile acids have included group fractionation on these ion exchangers prior to gas chromatography—mass spectrometry [3—9].

Because of the physico-chemical analogies between metabolites of less polar xenobiotics and endogenous steroids, we have investigated the use of lipophilic ion exchangers for group separation of metabolites of xenobiotics. Propachlor (2-chloro-N-isopropylacetanilide) was selected as a model compound as its metabolism has been well characterized [10] and is typical of many chlorinated aromatic pollutants.

EXPERIMENTAL

Solvents were of analytical-reagent grade and distilled in an all-glass apparatus. [^{14}C]Propachlor (2-chloro-N-isopropyl-[1-^{14}C]acetanilide) and its conjugated metabolites (cysteine and glutathione conjugates) were compounds used in previous investigations [10]. A germ-free rat (250 g), reared by the methods of Gustafsson [11, 12], was given a single oral dose of [^{14}C]propachlor (1.0 μCi, 2.5 mg) and urine was collected for 48 h.

Lipidex 1000 and Lipidex-DEAP were from Packard (Downers Grove, IL, U.S.A.). Other lipophilic ion exchangers were synthesized as described previously: TEAP-LH-20 (triethylaminohydroxypropyl Sephadex LH-20) [8]; SP-LH-20 (sulphohydroxypropyl Sephadex LH-20) [4]; SPHA-LH-20 (hydroxyalkylated SP-LH-20) [4]. All the lipophilic gels were washed before use [13]. Lipidex-DEAP was used in the acetate form [5], TEAP-LH-20 in the hydroxide form [8] and SP-LH-20 and SPHA-LH-20 in the acid form [4]. SP-Sephadex was from Pharmacia Fine Chemicals (Uppsala, Sweden) and was used in acid form.

Beds of the ion exchangers were prepared in 70% methanol in glass columns of I.D. about 4 mm [3, 8]. Column heights were 40 or 80 mm except for Lipidex-DEAP, which was 250 mm. The solutions of acid, base or buffer used for elution were all made in 70% aqueous methanol.

Beds of Lipidex 1000 were prepared in methanol and were washed with water prior to use [14]. A glass column of 8—10 mm I.D., equipped with a PTFE stopcock, was used. The bed volume was about 2—4 ml. Extraction experiments were made with solutions of the conjugated metabolites in water or in a 0.3 *M* phosphate buffer (pH 7) containing 0.03 *M* decyltrimethylammonium bromide as ion-pairing agent [15]. Flow-rates were about 1 ml/min.

Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.) and were washed with methanol and water prior to use [16].

RESULTS AND DISCUSSION

Extraction

Two methods for extraction of the glutathione and cysteine conjugates of propachlor from aqueous solution were studied. Lipidex 1000 did not retain these metabolites from water or a buffered solution containing 0.03 *M* decyltrimethylammonium bromide as ion-pairing agent. As conjugated bile acids and their sulphates are extracted in the presence of decyltrimethylammonium ions [15, 17], endogenous compounds of this type can be separated from S-conjugated metabolites of propachlor and similar xenobiotics by filtration through Lipidex 1000.

Sep-Pak C_{18} cartridges quantitatively extracted the two conjugated metabolites of propachlor from water with or without additon of decyltrimethylammonium ions. The conjugates were recovered by elution of the cartridge with methanol.

Cation exchange

Both conjugates were sorbed from aqueous methanol by sulphonic acid derivatives of cross-linked dextran. The lipophilic SP-LH-20 and its less polar derivative SPHA-LH-20 were equally effective, while some loss in the neutral fraction was seen with the more hydrophilic SP-Sephadex. Sorption from 70% methanol or 0.1 *M* acetic acid in 70% methanol was more effective than sorption from 30% methanol. The conjugates were quantitatively eluted with 0.3 *M* ammonia in 70% methanol. The results obtained with SP-LH-20 are shown in Fig. 1. The radioactivity not sorbed by the column represents impurities and not leakage of the labelled conjugates, as shown by rechromatography of this material. No attempts were made to separate the two conjugates on the cation exchangers.

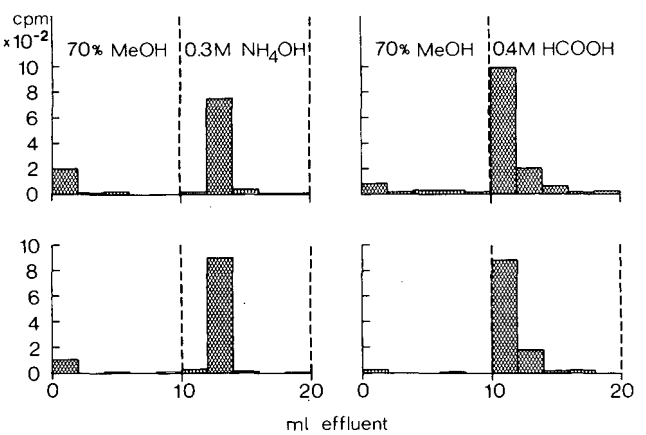

Fig. 1. Chromatography of the cysteine (lower panels) and glutathione (upper panels) conjugated metabolites of propachlor on SP-LH-20 (left panels) and TEAP-LH-20 (right panels). Columns: about 80 × 4 mm I.D. All solvents were 70% with respect to methanol.

Anion exchange

Both conjugates were sorbed from aqueous methanol by the lipophilic strong anion exchanger TEAP-LH-20 in base form (Fig. 1). They were quantitatively eluted with 0.4 *M* formic acid in 70% methanol, which will also elute monoglucuronides of aromatic and aliphatic alcohols [5, 8]. Further separation studies were made with the weaker anion exchanger Lipidex-DEAP, which is less likely to produce artifacts with alkali-labile compounds. However, TEAP-LH-20 in base form can be used when phenolic unconjugated metabolites have to be isolated as a separate group [3, 8].

Lipidex-DEAP was used in the acetate form. The glutathione conjugated metabolite was sorbed by this ion exchanger, while the cysteine conjugate was not taken up from either 70% methanol or 0.01 *M* acetic acid, sodium acetate or ammonia in 70% methanol (Fig. 2). The glutathione conjugate was not eluted by 0.25 *M* acetic acid in 70% methanol but appeared after elution with 2—3 column volumes of 0.25 *M* formic acid in 70% methanol. This is about the same position as that of monoglucuronides of neutral and phenolic steroids [5]. Thus, the cysteine and glutathione conjugates can be separated in this sytem, while a glucuronide with no other charged substituents would mix with the glutathione conjugate. However, separation of glucuronides from glutathione conjugates can be readily achieved on the cation exchanger. Further, the system can probably be refined by variations of eluting electrolyte and concentration of water in the solvent. This is illustrated by the group separation of monoglucuronides of neutral from those of phenolic steroids which can be achieved on DEAE-Sephadex when non-aqueous methanol is used as solvent [18].

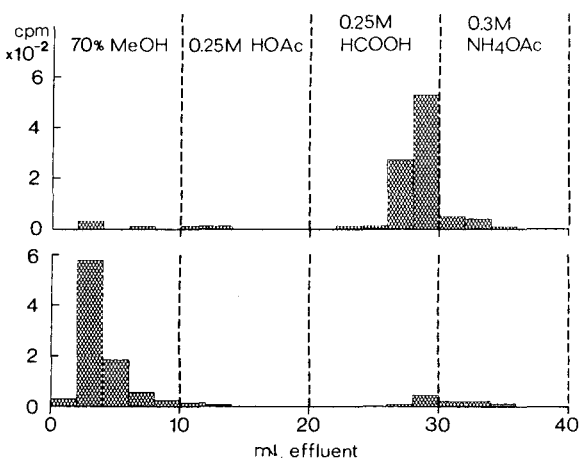

Fig. 2. Chromatography of the cysteine (lower chromatogram) and glutathione (upper chromatogram) conjugated metabolites of propachlor on Lipidex-DEAP in acetate form. Column: 250 × 4 mm I.D. Solvents were 70% with respect to methanol. The radioactivity appearing with formic acid and ammonium acetate in the lower chromatogram represents an impurity.

Fractionation of urine from a germ-free rat given ^{14}C propachlor

Based on the studies of reference compounds, the scheme shown in Fig. 3 was used for extraction and fractionation of urine from a germ-free rat given [^{14}C]propachlor. The yields of radioactivity in different fractions are shown in Table I. The pH of the effluent from SP-LH-20 was routinely checked and adjusted to about 7 with sodium hydroxide before application to Lipidex-DEAP to avoid losses of weak acids in the neutral fraction.

It may be noted that with the distribution of radioactivity found, it was not necessary to evaporate solvents at any point until the final fractions had been collected from Lipidex-DEAP. The volume of urine was 3 ml and the fractions containing more than 90% of the radioactivity were 4 ml, i.e., a volume increase of little practical importance. The capacity of the system has not been determined; however, in work with steroids and bile acids it is possible to process at least 20 ml of urine on columns of the size used. The limiting factor is the amount of organic ions in the sample in relation to the capacity of the ion exchangers.

The distribution of radioactivity in the different fractions permits the following tentative conclusions: (1) all metabolites are polar, as they are not extracted by Lipidex 1000; (2) they do not seem to contain basic substituents, as they are not sorbed by SP-LH-20; (3) they are acidic, as they are sorbed by Lipidex-DEAP; (4) they are stronger acids than glucuronic acid or glutathione conjugates.

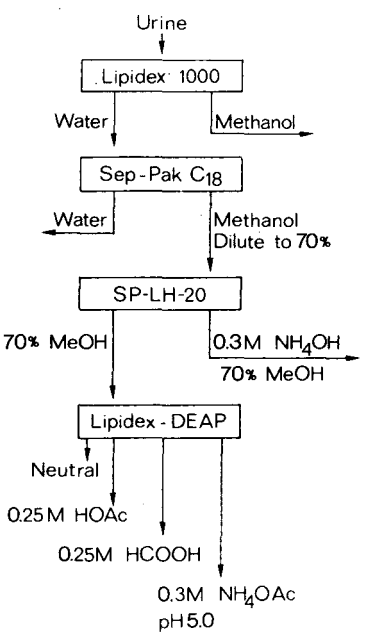

Fig. 3. Proposed scheme for group fractionation of metabolites of xenobiotics in urine. Metabolites eluted in methanol fractions indicated by an arrow at right angles can be subfractionated on the appropriate ion exchanger(s) after evaporation of the solvent. Alternative extraction procedures have to be tested for metabolites not sorbed by Lipidex 1000 or Sep-Pak.

TABLE I

DISTRIBUTION OF RADIOACTIVITY BETWEEN FRACTIONS IN THE SEQUENTIAL EXTRACTION AND GROUP SEPARATION OF METABOLITES IN URINE FROM A GERM-FREE RAT GIVEN [^{14}C]PROPACHLOR

Fractionation step	Fraction	Per cent of radioactivity
Extraction with Lipidex 1000	Water effluent	99.5
	Methanol eluate	0.5
Extraction with Sep-Pak C_{18}	Water effluent	1.6
	Methanol eluate	98.4
Cation exchange on SP-LH-20	70% methanol	99.8
	0.3 *M* ammonia	0.2
Anion exchange on Lipidex-DEAP	70% methanol, 0.25 *M* acetic acid, 0.25 *M* formic acid	0.2
	0.3 *M* ammonium acetate:	
	pH 5.0	95.5
	pH 6.0	1.1

The material in the fractions containing more than 90% of the radioactivity was treated with *n*-butanol—hydrochloric acid as described previously [19]. Gas chromatography—mass spectrometry, carried out on an LKB 2091 instrument, showed that by far the most predominant compounds was the butyl ester of the N-acetylcysteine conjugated metabolite. This was expected from previous characterization of metabolic pathways of propachlor in the germ-free rat [10].

Our study indicates that an extraction and group fractionation procedure of the type described can simplify the purification and analysis of mixtures of metabolites of several types of xenobiotics. While the model case studied is simple and gives only one fraction of metabolite(s), it is evident that the presence of other types of metabolites would have been detected if present. Cysteine and glutathione conjugates would have been sorbed by SP-LH-20 and appeared in the 0.3 *M* ammonia fraction, from which they could be separated on Lipidex-DEAP. Metabolites with a phenolic hydroxyl group as the only acidic substituent would have appeared in the neutral fraction from Lipidex-DEAP. Further purification of this group may be achieved on TEAP-LH-20 [3]. A monoglucuronide would have been eluted with 0.25 *M* formic acid from Lipidex-DEAP [5, 8] i.e., separated from the mercapturic acid. A monosulphate would also be separated from this conjugate, being eluted with acetate buffer pH about 6 [5]. Double conjugates with several acidic groups would also require higher pH for elution [5, 6].

The systems described are intended for group fractionation. Thus, separation of components of different polarity within the groups is negligible. Individual compounds in the fractions may be separated by high-performance liquid chromatography or gas chromatography and analysed by mass spectrometry after suitable derivatization, before or after hydrolysis of conjugates.

It is a disadvantage that non-volatile buffers sometimes have to be used for elution from ion exchangers. These have to be removed by redissolving the sample in water and extracting with Sep-Pak C_{18} cartridges. Advantages of the systems are the high capacity and inertness of the lipophilic ion exchangers, absence of irreversible adsorption and possibilities to select suitable solvents.

Finally, it should be pointed out that similar principles may be applied to analysis of metabolites in plasma, bile and milk. The main differences lie in the extraction procedures, which are influenced by protein binding and presence of lipids. Modified extraction procedures which can be used for plasma and bile have been described for different types of steroids [3, 14, 20, 21].

ACKNOWLEDGEMENTS

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Note

Simple method for increasing the life-time of 3-μm particulate columns for reversed-phase liquid chromatography

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Microparticulate columns for high-performance liquid chromatography (HPLC) are becoming increasingly popular. Short columns (10 cm) packed with 3—5 μm ODS material are often superior with respect to performance to the conventional columns (25—30 cm) containing 10 μm particle size material. These short columns carry the advantage of rapid, efficient analysis and low solvent consumption [1—3]. One disadvantage is the high back pressure obtained with the columns containing 3—5 μm packing material [4].

The microparticulate columns for reversed-phase chromatography are often mechanically fragile, which may partly be explained by the high back pressure [5]. We observed peak broadening and double peaks when using 5-μm ODS columns from commercial sources and 3-μm ODS columns packed in our own laboratory. Attempts have been made to avoid this problem by reducing the pressure surge on the top of the column by modification of the valve injector [6].

The present report describes procedures employed in our laboratory to stabilize short (10 cm) 3-μm ODS columns for HPLC. This was obtained by mounting a short guard column packed with 40-μm pellicular material as an extension of the analytical column. The guard column was subjected to mechanical compression using a stainless-steel piston, followed by refilling.

EXPERIMENTAL

Materials

ODS Hypersil 3-μm packing material for HPLC, and empty stainless-steel columns (10 × 0.5 cm), guard columns (2.5 × 0.5 cm), column adaptor for valve

0378-4347/83/$03.00

injection and accessories (seals, mesh and distributor) were purchased from Shandon Southern Products, Cheshire, Great Britain. Packing material for guard columns, Pelliguard LC-18, 40 μm, was from Supelco, Houston, TX, U.S.A. Adenosine, S-adenosyl-L-homocysteine and 2′-deoxyadenosine were obtained from Sigma, St. Louis, MO, U.S.A.

Instruments

A Spectra-Physics SP 8700 solvent delivery system was connected to a Perkin-Elmer ISS 100 autosampler for HPLC. The effluent from the column was monitored at 254 nm using a fixed-wavelength detector from Beckman, Model 160. The time constant was set at 0.2 sec. The chromatographic profiles were recorded using a reporting integrator from Hewlett-Packard, Model HP 3390 A.

Packing of the analytical column and guard column.

The analytical column was slurry packed with 3-μm ODS Hypersil material at 60 MPa, using a Shandon column packer. The solvents were changed in the sequence isopropanol, methanol, and the procedure was as recommended by Shandon.

The pellicular material for the guard column was dry-poured into the pre-column.

Preparation of samples

Tissues (liver, kidney, brain, spleen and thymus from mice) were homogenized in 0.8 *N* perchloric acid (1:4, w/v), and the precipitated protein removed by centrifugation. The perchloric acid extract was either subjected directly to HPLC analysis, or the acid was neutralized to pH 7.0 by the addition of 1.44 *N* KOH—1.2 *N* $KHCO_3$ [7]. The insoluble potassium perchlorate was allowed to precipitate for 30 min at 0°C, and then removed by centrifugation.

Column efficiency

The number of theoretical plates of the column (N) was calculated according to the equation [8]

$$N = 5.54 \left(\frac{t'_R}{W_h} \right)^2$$

The test mixture used for assessment of column performance contained benzamide, biphenyl, acetophenone and benzophenone, and the mobile phase was 70% methanol in water. The flow-rate was 1 ml/min. The efficiency of the 3-μm ODS columns was 90,000—110,000 theoretical plates per meter.

RESULTS

Protection of the analytical column by guard column

Samples (25 μl) of neutralized tissue extract were injected into a 3-μm ODS Hypersil column eluted with 15 m*M* acetate buffer, pH 4.5, containing 4.9% methanol. After 20—60 injections peak broadening and double peaks were

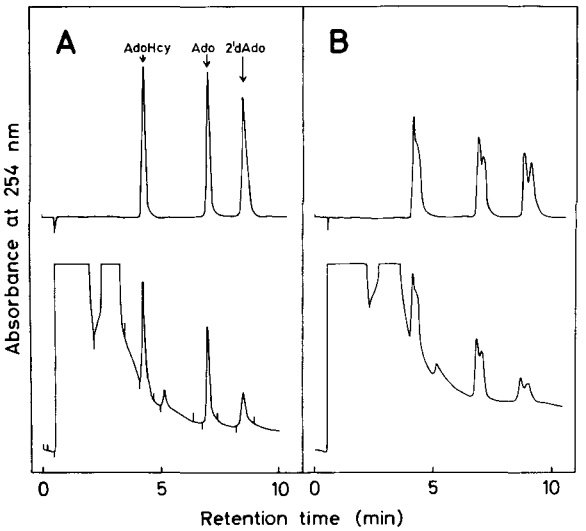

Fig. 1. Chromatograms of standards and liver extract before (A) and after (B) damage of the column. (A) Samples of 25 μl were analyzed on a 3-μm ODS Hypersil column (10 × 0.5 cm). The mobile phase was 15 m*M* acetate buffer, pH 4.5, containing 4.9% methanol, and the flow-rate was 2 ml/min (18.4 MPa). The upper panel shows the elution profile of standards, and the lower panel the chromatogram of neutralized extract from mouse liver. (B) The same samples were analyzed on the column damaged after 43 injections. AdoHcy = S-adenosylhomocysteine; Ado = adenosine; 2'dAdo = 2'-deoxyadenosine.

observed (Fig. 1). The performance of the column was often partly restored after elution of the column for 5—10 min in the opposite direction, but peak splitting soon (after 5—10 injections) reappeared.

The analytical column was equipped with a guard column (2 cm, Supelco), which was connected to the top of the analytical column by a 2-cm tube (0.254 mm I.D.). The performance of the system was markedly reduced (the number of theoretical plates decreased to about 60%). Only a slight protective effect on the analytical column was observed, i.e. after 40—70 injections peak broadening and splitting was observed. The performance was not restored by replacement of the guard column, showing that the damage was localized to the analytical column.

The guard column (2.5 cm, Shandon) was mounted on the top as an extension of the analytical column, and the two columns were separated by a thin (0.15-mm) mesh (2 μm). Broad peaks were observed after about 100 injections. After replacement (or compression, see below) of the guard column, the performance of the system was restored. The guard column extended the life-time of the analytical column to more than 1000 analyses. A defective guard column was not repaired by replacement of only the upper (0.5 cm) part of the packing material.

Mechanical compression of the guard column

Attempts were made to inject samples (25 μl) containing 0.8 *N* perchloric acid into ODS Hypersil columns equipped with guard column (end to end). The mobile phase contained 100 m*M* ammonium formate buffer, pH 3.5,

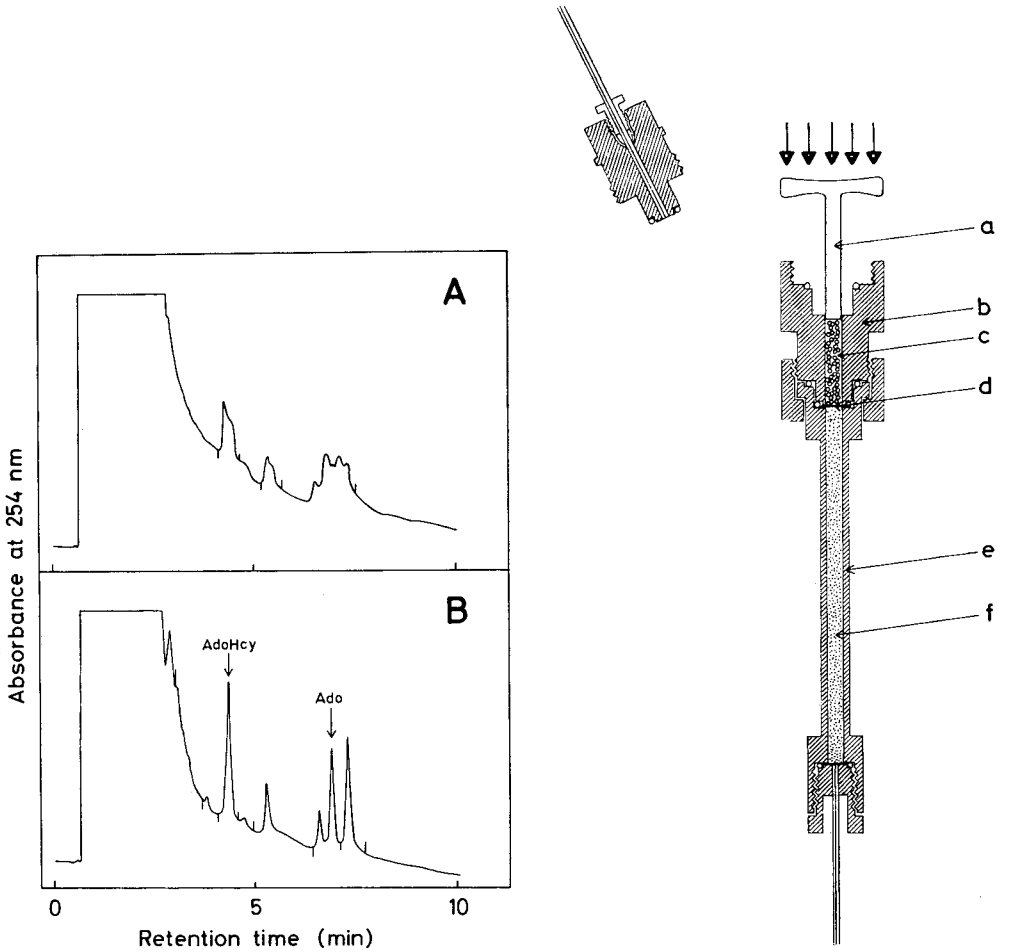

Fig. 2. Chromatograms of liver extract before (A) and after (B) compression of the guard column. (A) Liver extract (in 0.8 *N* perchloric acid) was analyzed on a 3-μm ODS Hypersil column equipped with a guard column forming an extension of the analytical column. The guard column was damaged prior to analysis by injecting ten samples. The mobile phase was 100 m*M* ammonium formate, pH 3.5, containing 1% acetonitrile, and the flow-rate was 2 ml/min. (B) Chromatogram of the same extract after compression of the guard column with a stainless-steel piston followed by refilling, as described in the text.

Fig. 3. Column design and compression of the guard column. a = Stainless-steel piston; b = guard column; c = 40-μm pellicular ODS material; d = mesh (0.15 mm), e = analytical column; f = 3-μm ODS material.

to prevent the detrimental effect of low pH on the bonded phase [9]. Peak splitting was observed after about ten injections (Fig. 2). The guard column was then compressed using a stainless-steel piston, fitting the internal diameter of the precolumn (Fig. 3), and the dead-volume created was filled with 40-μm pellicular material. The performance of the system was restored (Fig. 2). After a further 75 analyses (corresponding to 1.5 l of mobile phase) band broadening reappeared, but the compression was successfully repeated 5—10 times. The

life-time of the analytical column corresponded to 500—800 injections under these conditions.

Comments on column design

The procedure involving repetitive compressions and refilling of the guard column required opening of the guard column inlet. The use of a swaged connection at the column head was unsatisfactory because it was often damaged after only 10—20 dismantlings. The columns used in the present experiments (Fig. 3) were tightened by hand and could be opened repeatedly without damage. Eventually, only the O-ring needed to be replaced.

DISCUSSION

A guard column which forms a direct extension of the analytical column greatly improves the mechanical stability of the 3-μm ODS column. This may be explained by the protection given to the inlet of the analytical column against the high-speed inlet jet of the mobile phase and/or the pressure surge on the top of the column during injection.

When the microparticulate ODS column equipped with a guard column was loaded with samples containing strong acid and eluted with a mobile phase of high ionic strength, the guard column rapidly deteriorated. This resulted in the appearance of peak broadening and peak splitting. The mechanical nature of this phenomenon is indicated by the fact that the guard column was repaired by compression and refilling (Fig. 2). The damage of the guard column may be explained by formation of microchannels leading to multiple flow paths.

The performance of the guard column was restored several times by compression, and the life-time of the column was limited by the chemical stability of the packing material.

Attempts were made to slurry pack the precolumn at 60 MPa. This procedure showed no advantage to dry packing. This may be related to the low flow resistance offered by the 40-μm material used for the guard column.

Repetitive compression of the guard column forms the basis for the HPLC method developed in our laboratory for measurement of purines (adenosine, 2′-deoxyadenosine and S-adenosylhomocysteine) in several tissues and cell types from mammals [7, 10, 11]. The biological material was extracted in perchloric acid. Neutralization of the extract is not required, which ensures simple sample processing, and no further dilution of the sample. Furthermore, neutralization of perchloric acid with potassium hydroxide leads to a continuous precipitation of insoluble potassium perchlorate at low temperature. The precipitate is trapped in the column inlet filter, leading to a progressive increase in back pressure.

The highly efficient 3-μm ODS columns connected to a UV detector with low noise (2×10^{-5} a.u.f.s.), rapid response (time constant of 0.2 sec) and 1 cm light path, makes possible the detection of less than 0.2 pmol of these purines. Furthermore, mechanical compression of the guard column allows the unattended analysis of 75 samples.

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CHROMBIO. 1711

Note

Direct determination of D-[U-^{14}C]glucaric acid in urine by ion-exchange high-performance liquid chromatography

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The measurement of D-glucaric acid, and other metabolites (Fig. 1) of the D-glucuronic acid pathway, in urine has been used [1, 2] to monitor the levels of activity of hepatic microsomal enzymes, which are responsible for the metabolism of both endogenous compounds and environmental xenobiotics in the liver [3]. Whilst earlier studies were conducted using colorimetric methods [4, 5], more recent work has concentrated on the gas—liquid chromatographic analysis of urinary metabolites of D-glucuronic acid, after derivatisation [5—8].

It has been suggested [9] that D-glucaric acid may undergo further metabolism in mammals, as it does in microorganisms [10]. Since D-glucaric acid is present as an endogenous constituent of normal urine, making small variations in excretion difficult to detect by traditional methods, any possible metabolism would best be investigated by the use of ^{14}C-radiolabelling techniques, for which a non-destructive separation of D-glucaric acid and metabolites from urine was required.

Although the literature contains many methods for the measurement of sugars and organic acids [11, 12] no suitable separations of sugar acids could be found. This paper describes the development of a high-performance liquid chromatographic (HPLC) technique for the direct determination of D-glucaric acid and other metabolites of D-glucuronic acid in urine by both radioactivity monitoring and UV absorption.

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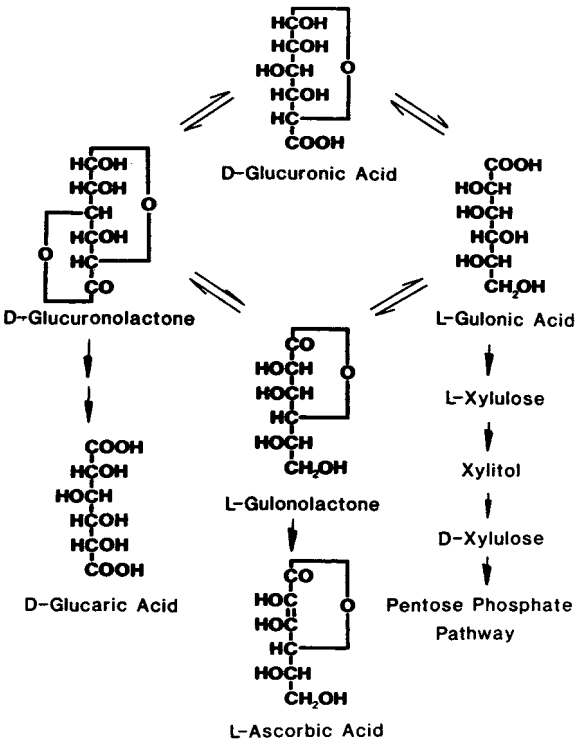


Fig. 1. The D-glucuronic acid pathway.

EXPERIMENTAL

Equipment

An Applied Chromatography Systems (Luton, Great Britain) LC750 high-performance liquid chromatograph was used for all separations. Columns, 250 × 4.6 mm I.D., were slurry-packed using a Haskel MCP-71 pump and packing reservoir (HPLC Technology, Macclesfield, Great Britain). Samples were applied via a Rheodyne 7120 valve and the effluent monitored either by UV absorbance at 200 nm using a CE2012 variable-wavelength monitor (Cecil Instruments, Cambridge, Great Britain) or by liquid scintillation counting [13] of collected fractions [14] in aqueous Tritosol [15] scintillant.

Materials

D-[U-^{14}C]Glucaric acid (1.3 μCi/mg) was prepared from D-[U-^{14}C]glucose (Amersham, Great Britain) by the method of Truchan [16] and purified by rapid recrystallisation from hot water [17].

D-Glucaric acid, D-glucaric acid-1,4-lactone, D-gulonic acid, D-glucuronic acid (Sigma, Poole, Great Britain), D-glucurono-3,6-lactone (Aldrich, Poole, Great Britain), L-γ-gulonolactone (Phase Separations, Queensferry, Great Britain) and L-ascorbic acid (Fisons, Loughborough, Great Britain) were all used as supplied. Ion-exchange stationary phases were Partisil-10 SAX (Whatman Labsales, Maidstone, Great Britain) and Calbiochem BA-X4 (C.P. Labo-

ratories, Bishops Stortford, Great Britain), packed in-house. All other chemicals were reagent grade or better. HPLC grade methanol was obtained from Rathburn Chemicals (Walkerburn, Great Britain) and water used was glass distilled.

Prepared mobile phases were sparged with helium and passed through 2-μm filters before use. Columns were flushed with water each night after use.

Sample treatment

Solutions of chromatographic standards were prepared and injected in the appropriate mobile phases. Urine samples were also directly injected without pre-treatment other than centrifugation to remove particulate matter.

Method development

Initial attempts at separation by reversed-phase chromatography with ion suppression, previously used for the determination of organic acids and esters in urine [18, 19] proved unsuccessful, with the sugar acids all virtually unretained in 1% aqueous sulphuric acid. Ion-pairing with tri-*n*-butylamine, successfully applied to the separation of L-ascorbic acid and its oxidation products [20] failed to resolve glucaric acid from UV-absorbing urinary material and gave consistently poor recoveries of radio-labelled material. Pre-column benzoylation, used for carbohydrate analysis [21] also failed to produce a useful separation in our hands.

Our attention was therefore directed towards anion-exchange chromatography. Partisil-10 SAX, a 10-μm silica based packing with a bonded quaternary amine functionality, was found to give a useful separation of D-glucaric acid from D-glucuronic acid, L-ascorbic acid and γ-gulonolactone (Fig. 2a), using an elution buffer of 0.05 *M* KH_2PO_4, adjusted to pH 6.0 with sodium hydroxide, at a flow-rate of 2.0 ml/min.

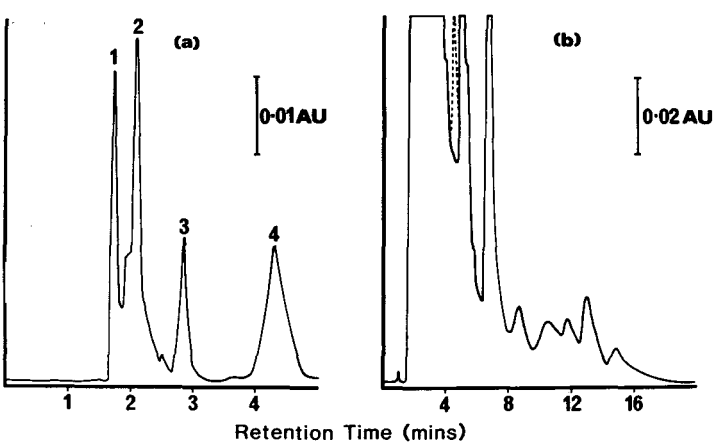


Fig. 2. Separations on Partisil-10 SAX. (a) 1 = γ-gulonolactone (25 μg) + D-glucuronic acid (25 μg), 2 = L-ascorbic acid (25 μg), 3 = unknown, 4 = D-glucaric acid (50 μg). (b) Rat urine: control and spiked with D-glucaric acid (1 mg/ml, shown dotted). See text for conditions.

Urine, spiked with D-glucaric acid showed only a partial resolution of the acid from other UV-absorbing material (Fig. 2b) although ^{14}C-monitoring showed only a single radiolabelled peak, corresponding to D-glucaric acid. Recovery of D-[U-^{14}C]glucaric acid from the column, however, was found to be directly dependent on the amount of unlabelled D-glucaric acid spike present in the sample. Even on saturation of a urine sample with D-glucaric acid, reproducible recoveries of better than 85% could not be achieved.

This problem was attributed to non-specific absorption onto the silica base of the packing, so a resin-based microparticulate anion exchanger was sought. Calbiochem anion-exchange resin BA-X4, a 7—10 μm polystyrene—divinylbenzene resin with 4% crosslinking and quaternary amine functional groups [22], appeared to suit our requirements. Preliminary trials with D-[U-^{14}C]glucaric acid gave 99.8% recoveries of label, independent of spiking. Although the actual column efficiency was relatively low, optimum performance was obtained at a flow-rate of 0.7 ml/min and column temperature of 60°C. The addition of a small amount of methanol to the mobile phase marginally improved peak shape and eliminated the tendency for the pump seals to leak at high buffer concentrations.

Optimum separation of D-glucaric acid from urine was achieved using an eluent of 0.67 *M* KH_2PO_4 in 6.7% methanol—water, adjusted to pH 5.50 with sodium hydroxide (Fig. 3a). Under these conditions, D-glucaric acid was resolved from its lactone with most of the other metabolites eluting much closer together, earlier on (Fig. 3b). L-Ascorbic acid gave a peak which was very sensitive to pH and methanol concentration, presumably through variations in its ionic state, and could not be accurately determined; increasing pH improved the L-ascorbic acid peak shape considerably, but correspondingly destroyed the resolution of D-glucaric acid and its lactone, as well as increasing the absorbance of the mobile phase and thus reducing sensitivity.

The early-eluting metabolites could readily be separated by reducing the buffer concentration to 0.067 *M* KH_2PO_4, keeping pH and methanol con-

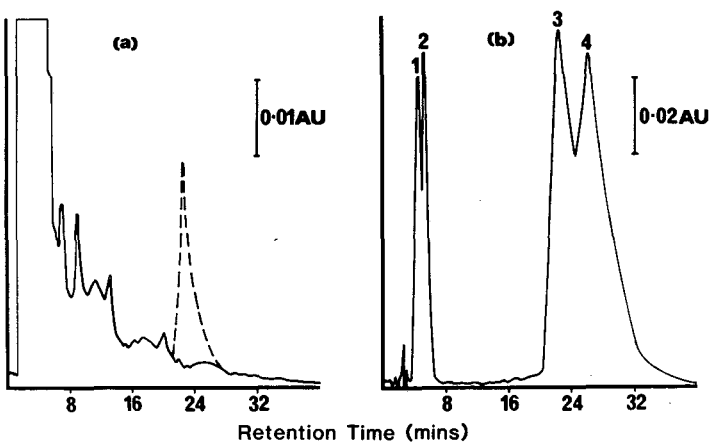
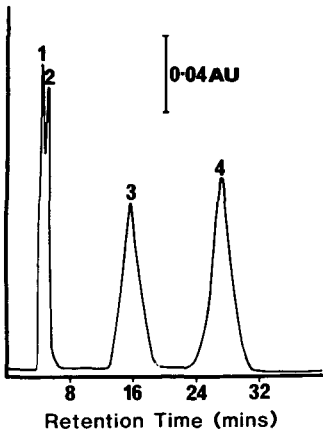


Fig. 3. Separations on BA-X4 resin. (a) Rat urine: control and spiked with D-glucaric acid (1 mg/ml, shown dotted). (b) 1 = D-glucuronolactone (0.10 mg), 2 = D-glucuronic acid (0.06 mg), 3 = D-glucaric acid (0.17 mg), 4 = D-glucaric acid lactone (0.29 mg). See text for conditions.



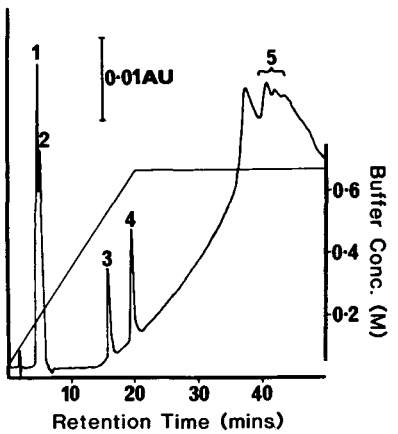


Fig. 4. Separation of early eluting peaks on BA-X4. Peaks: 1 = γ-gulonolactone; 2 = D-glucuronolactone; 3 = D-gluconic acid; 4 = D-glucuronic acid (0.5 mg each). See text for details.

Fig. 5. Gradient elution of D-glucuronic acid metabolites. Peaks: 1 = γ-gulonolactone; 2 = D-glucuronolactone; 3 = D-gluconic acid; 4 = D-glucuronic acid; 5 = L-ascorbic acid + D-glucaric acid + D-glucaric acid lactone (0.5 mg each).

centration the same (Fig. 4). Gradient elution between these two buffer compositions, with detection at 220 nm to overcome baseline intervention, albeit at the cost of sensitivity, gave excellent resolution of the early peaks but ran together L-ascorbic acid, D-glucaric acid and its lactone (Fig. 5). Isocratic elution at intermediate concentrations gave increased sensitivity over the gradient system but resulted in very badly tailed peaks and poor resolution.

RESULTS AND DISCUSSION

Detection by UV monitoring

Determination by UV absorbance at 200 nm is restricted by low sensitivity to the compounds of interest, the detection limit for D-glucaric acid being of the order of 20 μg in the isocratic BA-X4 system in Fig. 3, with a linear response up to around 1 mg. The sensitivity in gradient elution is still lower, due to the effects of baseline drift, though this might be eliminated by the addition of some unretained, UV absorbing compound to the initial mobile phase [23].

Determination by ^{14}C-monitoring

Gradient elution on the BA-X4 column was found to be a useful rapid screening process for the presence of radiolabelled D-glucuronic acid metabolites in urine. Isocratic elution, as in Fig. 3, has been used for the routine analysis of the urine of rats, mice, guinea pigs and marmosets treated with D-[U-^{14}C]glucaric acid. The mean recovery of label from all urine samples analysed was 100.3 ± 0.8% (S.E.M., n = 49) with detection down to 1000 dpm/ml urine, equivalent to 0.3 μg/ml glucaric acid. Greater sensitivity can be achieved by freeze-drying the urine to concentrate the radio-label or by increasing the specific activity of the administered material.

ACKNOWLEDGEMENTS

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Note

Application of anion-exchange column chromatography for determination of alkaline phosphatase activity using 2′-AMP or 3′-AMP as substrate

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In our previous paper we described a simple automated ion-exchange column chromatography method using AG MP-1 resin for the determination of adenosine [1]. This has been applied to the assay of the activity of 5′-nucleotidase which catalyzes the hydrolysis of 5′-AMP to adenosine and inorganic phosphate. Alkaline phosphatase which catalyzes the hydrolysis of AMP at high pH can therefore be determined by a similar procedure. However, in order to discriminate the activity from that of 5′-nucleotidase, 2′-AMP or 3′-AMP is used as substrate. The optimal conditions for the assay, and a modification of the procedure are described in this communication.

EXPERIMENTAL

Materials

AG MP-1 anion-exchange resin was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Adenosine monophosphates were obtained from Sigma (St. Louis, MO, U.S.A.). The purity and concentration of 2′-AMP and 3′-AMP were determined by a modification of the column chromatography [2, 3] as described below. A normal serum from the Blood Bank at Long Island Jewish—Hillside Medical Center was used.

Column chromatography

The same column packed with AG MP-1 resin (50 × 9 mm) was used for the analysis of adenosine as well as AMP throughout the entire experiment. Adenosine peak was eluted out from the column with distilled water in 8 min,

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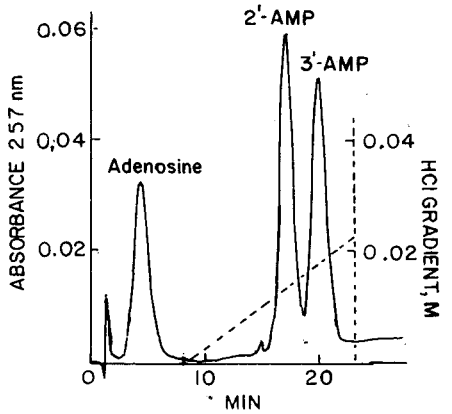


Fig. 1. Chromatogram of separation of adenosine and adenosine monophosphates (AMP). Adenosine is eluted out with distilled water, while AMP's can only be eluted out with hydrochloric acid. A mild acid gradient resolved 2'-AMP and 3'-AMP. For determination of adenosine only, the column is continuously eluted with distilled water while samples are consecutively injected into the column every 8 min. AMP's will remain in the column without interfering with the assay.

then 2'-AMP and 3'-AMP were separated by eluting the column with a linear hydrochloric acid gradient established by using a Gilson Mixograd gradient former from distilled water and 0.024 *M* hydrochloric acid in 15 min. The amounts were calculated from the area of each peak according to the equation previously described [2]. A chromatogram of their separation is shown in Fig. 1.

Assay for serum alkaline phosphatase activity

A general procedure for the assay of serum alkaline phosphatase was as follows: A substrate in 0.9 ml of various alkaline buffer solutions was preincubated to 37°C. The reaction was started by the addition of 100 μl of serum. Aliquots (200 μl) of the reaction mixture were withdrawn at 10-min intervals and injected into the column for the quantitation of adenosine produced according to the automated column chromatography described previously [1]. A unit of the activity is defined as the amount of the enzyme that will catalyze the production of 1 μmol of adenosine per min at 37°C under specified conditions.

RESULTS AND DISCUSSION

Purity and stability of adenosine monophosphates

The commercial 2'-AMP and 3'-AMP contained less than 0.04% of adenosine. 2'-AMP contained no detectable amount of 3'-AMP while 3'-AMP contained 0.28% of 2'-AMP.

Storage of AMP's in various alkaline buffer solutions at pH 10.6 at room temperature revealed that both 2'-AMP and 3'-AMP were stable in diethylamine or triethylamine, while they were slightly hydrolyzed in 2-amino-2-methyl-1-propanol. It is estimated that about 0.17% was hydrolyzed to ade-

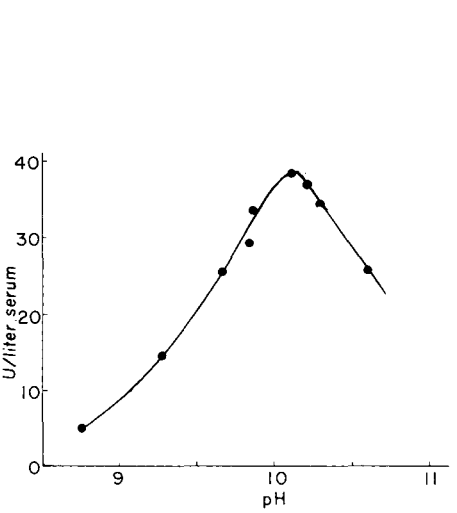
nosine in a week. Presence of magnesium chloride at 10 mmol/l tended to accelerate the non-enzymatic hydrolysis.

Effect of buffers and pH optimum

Serum alkaline phosphatase gave similar reaction rates in the buffers of 2-amino-2-methyl-1-propanol as well as of diethylamine. In triethylamine, the rate was reduced about 20% of the rate in diethylamine. Therefore, diethylamine was used as the buffer in subsequent experiments.

Fig. 2 shows the pH optimum of serum alkaline phosphatase using diethylamine as buffer. Since the range of the plateau is less than 0.1 pH, a sufficient buffering capacity at that range is required to yield a constant reaction rate.

Diethylamine at a concentration higher than 0.2 *M* is somewhat inhibitory. However, the variation in the pH is considered as a main factor causing the error in the rate measurement. As shown in Fig. 3, although diethylamine buffer was initially adjusted to pH 10.4, the pH values varied after the addition of serum and during the incubation. In 0.1 *M* diethylamine, a linear reaction rate versus time was obtained. At 0.05 *M* diethylamine, the reaction was reduced due to the lack of the buffering capacity. On the other hand, diethylamine at high concentration is somewhat inhibitory. This is evident from the fact that a non-linear reaction rate was obtained at a concentration higher than 0.2 *M*. Consequently the optimal buffer concentration at 0.1 *M* and pH at 10.2 were used.



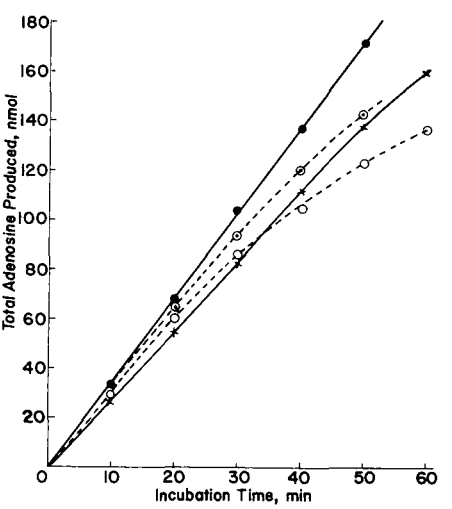


Fig. 2. pH optimum of serum alkaline phosphatase. The activity was measured in the presence of 10 m*M* 2'-AMP and 0.1 *M* of diethylamine at various pH values.

Fig. 3. Effect of buffer concentration on the reaction rate of serum alkaline phosphatase. The total amount of adenosine produced by 100 μl of serum was determined in the reaction mixture containing 100 μl of serum per ml, 10 m*M* 2'-AMP, and various concentrations of the diethylamine as indicated: ×, 0.05 *M* (pH 9.86—9.80); •, 0.1 *M* (pH 10.14—10.11); ⊙, 0.2 *M* (pH 10.31—10.20); ○, 0.3 *M* (pH 10.38—10.34). The values in parentheses are the initial and final pH values.

Optimum substrate concentration

Fig. 4 shows the relationship between the initial reaction rate and the substrate concentration. The reaction was carried out in a mixture containing 0.1 *M* diethylamine, pH 10.2, 100 μl of serum, and various concentrations of substrates. From the curves, it was estimated that K_m and V_{max} for 2′-AMP are 1.46 m*M* and 40.8 units/l, respectively; and for 3′-AMP, 3.32 m*M* and 38.9 units/l. The results indicated that serum alkaline phosphatase has a higher affinity for 2′-AMP than for 3′-AMP; however it catalyzes the hydrolysis of both substrates equally well. Thus, at pH 10.2, serum alkaline phosphatase requires a lower concentration of 2′-AMP than that of 3′-AMP to give a maximum activity. It can be seen from Fig. 4 that a maximum reaction rate was obtained at 8 mmol/l of 2′-AMP. The concentration beyond this did not lead to a significant increase in activity. For practical purposes, 10 mmol/l of 2′-AMP is considered optimum to yield a constant maximal rate. Variation of serum amount from 10 μl to 250 μl in a 1-ml reaction mixture containing 0.1 *M* diethylamine, pH 10.2, and 10 m*M* 2′-AMP showed that the alkaline phosphatase activity was linearly related to the amount of serum.

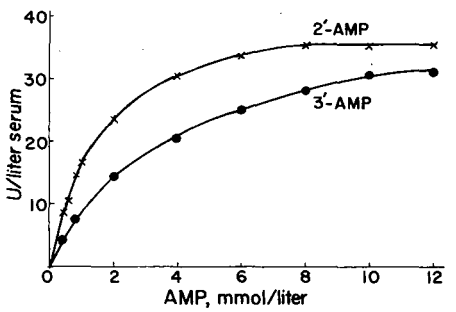


Fig. 4. Effect of substrate concentration on reaction rate of serum alkaline phosphatase. The activity was measured in the presence of 0.1 *M* diethylamine, pH 10.2, and various concentrations of 2′-AMP or 3′-AMP as indicated on the figure.

CONCLUSION

For a routine assay of serum alkaline phosphatase, 50 μl of serum are added into a 200-μl solution containing 12.5 m*M* 2′-AMP and 0.125 *M* diethylamine, pH 10.2. The mixture is incubated at 37°C, and after 10 min, 100 μl of the reaction mixture are injected into the column for chromatography. Since the reaction mixture is injected directly into the column without pretreatment, the whole procedure can be automated.

An assay method using HPLC for acid and alkaline phosphatases in serum has been published [4]. Our system can thus be similarly applied to the assay of acid phosphatase. Using 2′-AMP or 3′-AMP as substrates can eliminate the requirement of employing nickel chloride to inhibit 5′-nucleotidase activity when 5′-AMP is used as substrate. The distinctinve advantage of our method is that it is much simpler.

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Note

Separation and quantitation of plasma lipoproteins by high-performance liquid chromatography

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The identification and follow-up of individuals at risk for cardiovascular disease require a better evaluation of the distribution of lipid and protein components in the various lipoproteins. The quantitation of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, and of the apoproteins AI and B, requires a rapid separation of the lipoprotein classes and the preservation of their antigenic properties.

The separation and quantitation of plasma lipoproteins has mainly been performed by ultracentrifugal flotation, either stepwise or in a density gradient [1, 2]. Electrophoretic separation in a gel gradient and column chromatography have also been applied to the separation of the lipoprotein fractions [3, 4]. Precipitation techniques using polyanions and divalent cations have been used for the selective fractionation of VLDL, LDL, HDL and the subclasses HDL_2 and HDL_3 [5, 6].

Most of these techniques are time consuming, require 2—5 ml of serum and cannot be easily applied to the routine analysis of the lipid and protein constituents of plasma lipoproteins.

High-performance liquid chromatography (HPLC) on a gel exclusion column appears to be a suitable alternative for lipoprotein separation [7, 8]. In this

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paper we report on the application of this technique to the separation of lipoproteins from purified mixtures, from newborn sera and from normal and pathological adult sera. The isolated lipoprotein fractions were subsequently analyzed for their cholesterol and apoprotein content and the results evaluated by comparison with those obtained by density gradient ultracentrifugation of the same samples.

EXPERIMENTAL

Samples

In adults, fresh serum was drawn after 16 h fasting from individuals with normal lipids, Fredrickson type IIB and IV patients and from a patient with nephrotic syndrome. Cord blood was obtained immediately after delivery and blood was drawn by venipuncture after 6 h fasting in 7- and 30-day-old infants [9].

Separation of lipoproteins by ultracentrifugation

VLDL, LDL and HDL were obtained by stepwise ultracentrifugation using the method of Havel et al. [1]. For the density-gradient ultracentrifugation, 0.3 and 0.5 ml plasma was spun in an SB 283 swinging-bucket rotor of an International B 60 ultracentrifuge, for 66 h at 180,000 *g* [10].

The gradient consisted of sucrose, NaBr and NaCl, spanning the density range 1.02–1.20 g/ml. Fractions of 0.5 ml were collected after reading the absorbance at 280 nm through the 8-μl flow-cell of a Pye-Unicam spectrophotometer.

Total lipoproteins were isolated by flotation of 170 μl serum in an air-driven ultracentrifuge (Beckman Airfuge) after spinning at 100,000 *g* at d = 1.21 g/ml for 4 h [11].

Lipid and apoprotein quantitation

Cholesterol was assayed in total plasma and in lipoprotein fractions using an enzymatic–fluorimetric assay [12], at a sensitivity of 1 μg of cholesterol, with a precision of ± 4%.

Aproproteins AI and B were assayed by immunonephelometry, using either 50 μl of serum diluted 150-fold or 100 μl of the ultracentrifugal or chromatographic fractions, diluted two- to ten-fold [13, 14]. The precision of these assays was 7%.

Separation of lipoproteins by HPLC

The separation of the lipoproteins was performed on a Spectra-Physics SP 8000 liquid chromatograph equipped with a Schoeffel SF 770 variable-wavelength UV detector. For gel permeation an Ultro Pac TSK-G 4000 SW (600 × 7.5 mm I.D.) column (particle size 10 ± 2 μm) from LKB preceded by an Ultro Pac LKB TSK-G SWP (75 × 7.5 mm I.D.) guard column (particle size 10 ± 2 μm) was used. The column was equilibrated in 0.2 *M* Na_2HPO_4 containing NaN_3 (0.5 g/l). A 50-μl sample was injected and eluted at room temperature with the 0.2 *M* phosphate buffer at a flow-rate of 0.5 ml/min.

The lipoprotein fractions were monitored by the absorbance at 280 nm and collected in 0.5-ml fractions with a Gilson Microcol TDC 80 fraction collector.

RESULTS

A mixture of VLDL, intermediate density lipoprotein (IDL), LDL and HDL prepared by stepwise ultracentrifugation was applied to a single TSK-G 4000 SW column. The elution profile depicted in Fig. 1 shows a baseline resolution for the major lipoprotein classes. The first peak which eluted at the exclusion volume of the column, contains the VLDL fraction and the chylomicrons. IDL (fraction 3) elutes in a well-separated peak between VLDL and LDL (fraction 4). The HDL peak (fractions 5 + 6) could not be subfractionated into HDL_2 and HDL_3 using the TSK-G 4000 SW column. Under the experimental conditions used all lipoproteins were eluted within 50 min.

The sensitivity of the fluorimetric and immunonephelometric assay used for cholesterol and apoprotein analysis is sufficient to analyze the lipoprotein fractions isolated from 20 μl of serum. The composition of the lipoprotein fractions was not altered by fractionation through the TSK-G 4000 SW column. Due to protein adsorption to the matrix, the recovery of the lipids and apoproteins lies between 70% and 80% and is similar for all fractions.

In Fig. 2, the UV pattern (dotted lines) corresponding to the total serum is compared to the UV pattern (full line) of the $d > 1.21$ supernatant of the same serum after flotation in the Beckman Airfuge. The flotation step in the Airfuge is sufficient to separate the lipoproteins from most of the plasma proteins, though some albumin is still present in the supernatant.

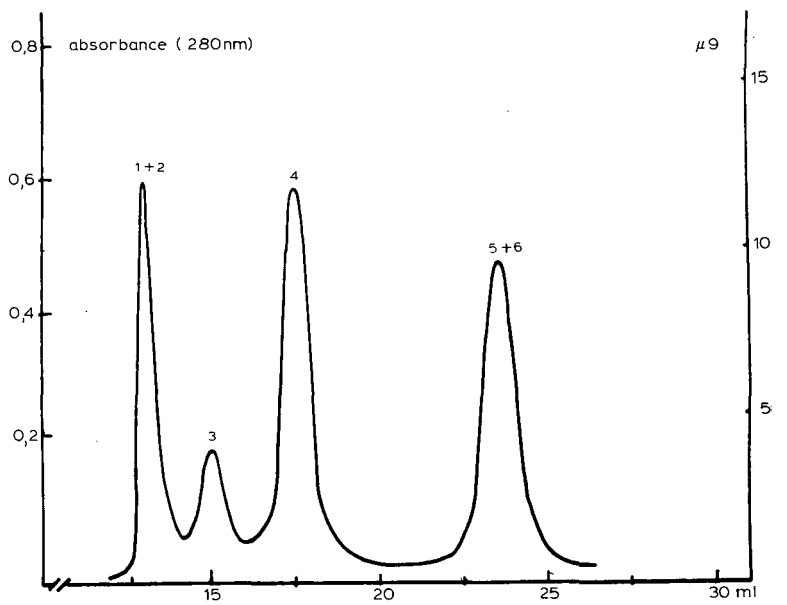

Fig. 1. Elution curve of a mixture of lipoprotein fractions on an Ultro Pac TSK-G 4000 SW column (600 mm × 7.5 mm I.D.). Eluent, sodium phosphate buffer, 0.2 *M*, pH 6.8; flow-rate, 0.5 ml/min; T, 25°C; load volume, 50 μl. Peaks: 1 + 2 = chylomicrons + VLDL; 3 = IDL; 4 = LDL; 5 + 6 = HDL_2 + HDL_3.

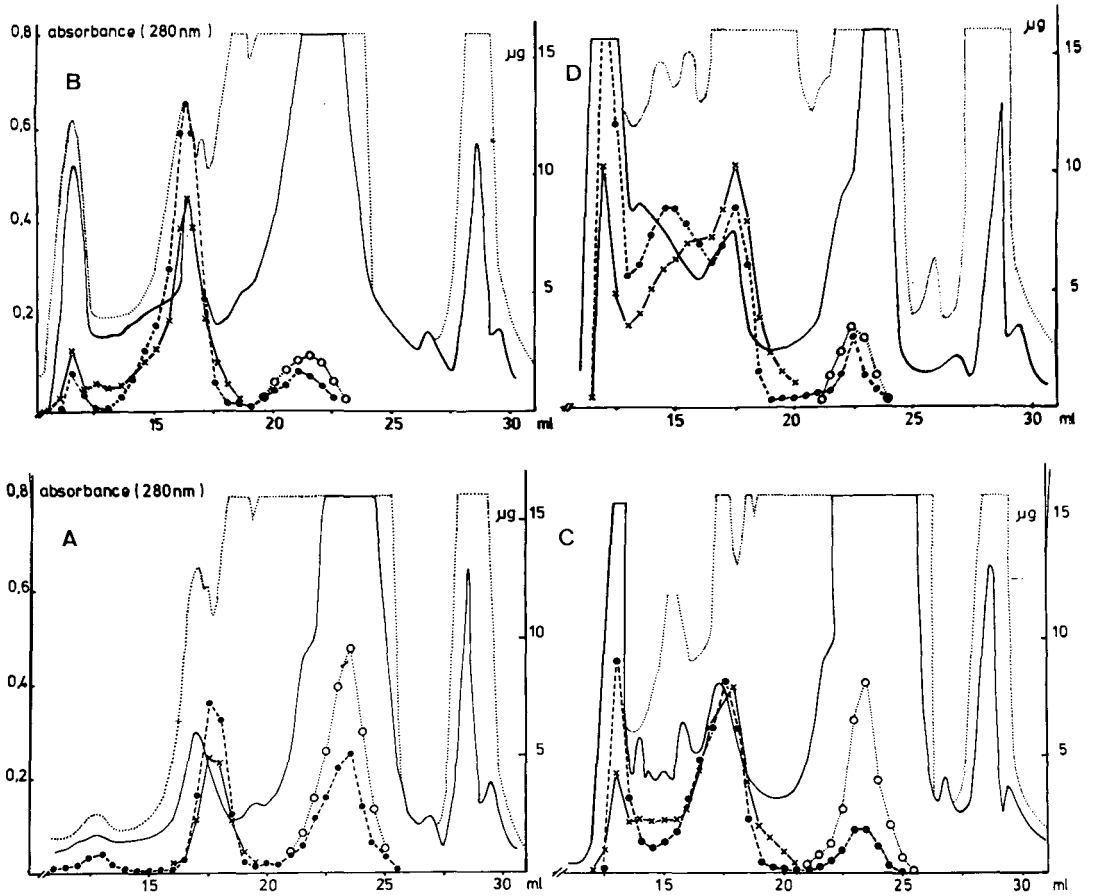

Fig. 2. Elution curves of lipoproteins in total serum (. . . . .) and ultracentrifugal Airfuge fractions (——): (A) normolipemic female; (B) Fredrickson type IIA patient; (C) Fredrickson type IV patient; (D) patient with nephrotic syndrome. HPLC conditions as in Fig. 1. (•), Cholesterol; (×), apo B; (○), apo AI.

The lipoprotein separation from serum by HPLC was completed in 60 min with a total elution volume of 30 ml. Fig. 2A is representative of the serum of a normal individual, whereas Fig. 2B and C represents the elution patterns obtained for sera of Fredrickson type IIA and type IV patients, respectively. Fig. 2D shows the results for a patient with nephrotic syndrome.

The cholesterol, apo AI and apo B contents of the eluted fractions are also plotted in Fig. 2A–D, showing that the UV maxima of the VLDL, LDL and HDL peaks coincide with those of cholesterol and apoproteins. The lipoprotein distribution in a normolipemic individual (Fig. 2A) is characterized by a low VLDL fraction and a comparable concentration of LDL and HDL. The type IIA individual has normal VLDL, an elevated LDL and a decreased HDL peak. The cholesterol/apo B ratio in the LDL fraction is decreased compared to that of the normal individual (Table I).

The pattern of the congenital type IV patient (Fig. 2C) shows an elevated

TABLE I

DISTRIBUTION AND COMPOSITION OF PLASMA LIPOPROTEINS AFTER FRACTIONATION BY HPLC AND BY DENSITY-GRADIENT ULTRACENTRIFUGATION

Patient	Technique	VLDL-C* (%)	IDL-C (%)	LDL-C (%)	HDL-C (%)	VLDL-C/ apo B	LDL-C/ apo B	apo AI/ apo B
Baby, 0 days	HPLC	<3	<3	43	57	—	0.9	1.5
	UCF**	8.1		37	56	1.2	1.4	1.7
Baby, 7 days	HPLC	3.2	5.0	50.0	42.0	—	1.2	1.4
	UCF	26.4		42.6	30.9	1.5	0.9	1.4
Baby, 30 days	HPLC	2.2	9.9	51.5	36.4	1.6	1.0	1.3
	UCF	25.2		47.6	27.5	—	1.1	1.2
Normolipemic individual	HPLC	<3	<3	61.1	38.9	—	1.5	0.8
	UCF	3.1	—	60.3	37.4	1.8	1.7	1.0
Type IIA patient	HPLC	6.9	—	79.6	13.4	1.3	1.2	0.33
	UCF	9.1	—	73.8	17.1	1.8	1.5	0.38
Type IV patient	HPLC	25.3	18.2	43.6	12.8	2.1	0.9	0.53
	UCF	29.0		58.7	11.2	1.8	1.2	0.55
Patient with nephrotic syndrome	HPLC	24.2	41.2	27.0	7.6	1.8	1.2	0.14
	UCF	58.0		35.0	7.0	2.0	1.5	0.18

*C = cholesterol.
**Density-gradient ultracentrifugation.

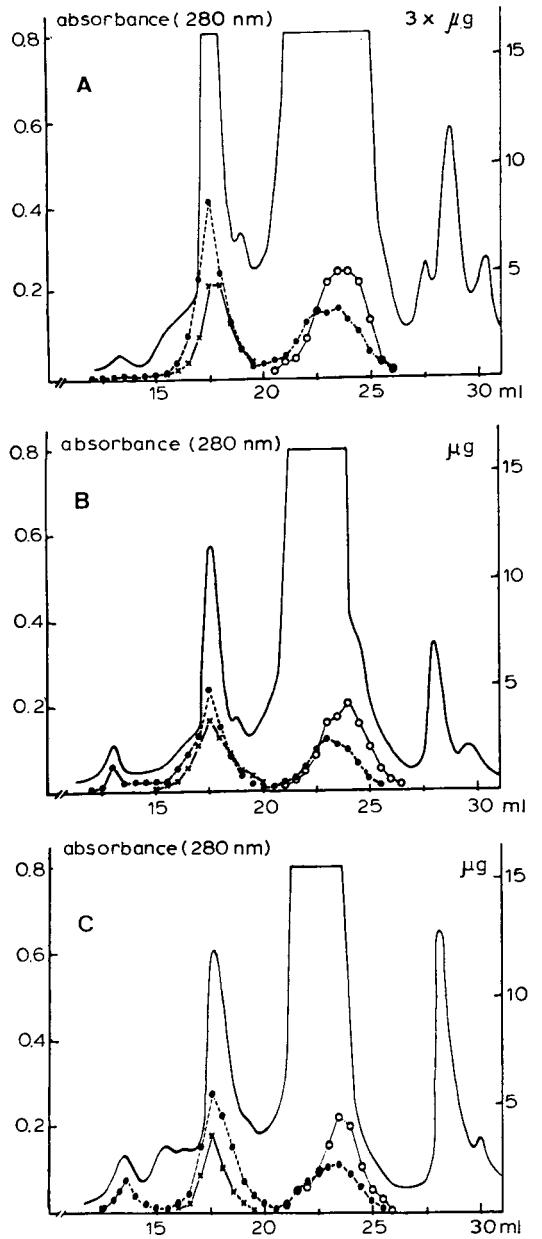

Fig. 3. Elution curves of lipoproteins in Airfuge fractions: (A) cord blood; (B) 7-day-old infant; (C) 30-day-old infant. HPLC conditions as in Fig. 1. (——), UV absorption at 280 nm; (•), cholesterol; (×), apo B; (○), apo AI.

VLDL concentration and the presence of an IDL fraction, characterized by an cholesterol/apo B ratio intermediate between those of VLDL and LDL. The concentrations of HDL cholesterol and apo AI are decreased in this patient.

The patient with nephrotic syndrome (Fig. 2D) is also characterized by an elevated VLDL and shows an IDL fraction eluting between VLDL and LDL which is highly heterogeneous in its cholesterol and apo B content. The HDL fraction is also lower in this patient than in the normal individual. Similar patterns were observed by density-gradient ultracentrifugation [15].

HPLC was also applied to the study of lipoproteins in cord serum and in the serum of 7- and 30-day-old infants (Fig. 3A—C). The HPLC pattern obtained after injection of 50 μl of cord serum, concentrated three-fold using a Minicon concentrating system, is depicted in Fig. 3A. This chromatogram demonstrates that newborns have very low VLDL at birth, low LDL and relatively high HDL, in agreement with the patterns obtained by density-gradient ultracentrifugation [10]. At 7 and 30 days VLDL and especially LDL levels have increased to a value about half of that measured in adults. The HDL concentration remains elevated at both 7 and 30 days.

The measurement of the cholesterol and apoproteins in the various fractions enabled calculation of the lipoprotein composition and distribution. These data are summarized in Table I and compared to those obtained by density-gradient ultracentrifugation of the same samples. The results indicate that the percentages of the lipoprotein fractions obtained by the two techniques are comparable. The resolution of the density-gradient ultracentrifugation did not enable efficient separation of VLDL and IDL, while HDL_2 and HDL_3 were well resolved [10]. The sensitivity of the lipid and apoprotein assay was insufficient for accurate analysis of the low VLDL levels in cord blood.

DISCUSSION

The data obtained by HPLC separation of the lipoproteins, present either in a mixture or in total serum, illustrate the potentiality of the HPLC technique for this type of separation. In agreement with other authors [7], we observed a good separation of VLDL, LDL and HDL, and were also able to separate IDL in a well-resolved peak. HDL_2 and HDL_3 could not be resolved using the TSK-G 4000 SW column. After injection of 50 μl of either infant or adult serum, the cholesterol and the apoproteins AI and B assays in the various lipoproteins were satisfactory. The patterns were in good agreement with those obtained by density-gradient ultracentrifugation of the same samples and illustrate the variability of the lipoprotein distribution in normal and dyslipemic individuals.

The cholesterol/apoprotein ratio in the various lipoproteins was also calculated, as Sniderman et al. [16] have suggested that the apo B/cholesterol ratio in LDL is significantly increased in patients with coronary artery disease. The HPLC technique provides a new approach to a rapid and more exact quantitation of these parameters, which are of clinical relevance.

The resolution of the particular column in the VLDL—IDL—LDL range enables the detection and quantitation of several subclasses within this heterogeneous distribution of particles whose size and composition are correlated with their atherogenicity index [17]. The advantage of HPLC for

analyzing the lipoprotein distribution and composition in infants appears clearly from the above data. The use of a minimal sample volume (50 μl), if necessary after concentration, and a reduced separation time, shows its applicability to the screening and follow-up of newborns at risk for atherosclerosis. Such a separation could be combined with the existing screening procedure based on apoprotein quantitation in plasma [18].

Previous authors [8] had proposed the use of a pre-staining procedure for the quantitation of cholesterol in the various lipoprotein fractions. In our experience this approach has several disadvantages. The lifespan of the column can be drastically shortened due to the absorption of the dye; the recovery of the fractions is decreased, and finally pre-staining does not enable a subsequent immunological quantitation of the apo AI and B proteins.

In conclusion, HPLC represents a potentially useful technique for the separation and isolation of lipoproteins from serum, and the further analysis of their lipid and apoprotein components. It should be applicable to the detection and follow-up of individuals at risk for atherosclerosis and the monitoring of patients with primary and secondary dyslipoproteinemia during diet and drug therapy.

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Note

Simultaneous determination of hippuric acid and *o*-, *m*- and *p*-methylhippuric acids in urine by high-performance liquid chromatography

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Toluene and xylene are widely used as organic solvents in industry. The solvents inhaled by workers are mainly eliminated unchanged via the lungs or excreted as metabolites in urine. Major metabolites of toluene and xylene are hippuric acid (HA) and methylhippuric acid (MHA), respectively [1]. Recent study showed that *o*-MHA as well as *m*- and *p*-MHA are major metabolites of the corresponding xylene isomers in humans [2]. Concentrations of the metabolites in urine of workers quantitatively reflect the solvent vapour concentrations in their work-place, and have been regarded as indices of exposure to these solvents [3—8].

In 1977, high-performance liquid chromatography (HPLC) was introduced for the quantitative determination of HA and *m*- or *p*-MHA in urine [9, 10]. The methods, however, can not separately determine each component in a mixture of *m*- and *p*-MHA. Although efforts for separation of *m*- and *p*-MHA were made with HPLC [11] as well as with gas chromatography [12, 13], those methods require complicated pretreatment of urine to form the derivative of each metabolite. A simple method for the separation of xylene metabolites has not been reported yet. We previously reported the determination of total *m*- plus *p*-MHA in urine by HPLC without any pretreatment [14].

The work published so far indicates that the chromatographic application of inclusion compounds such as cyclodextrins allows the solution of specific analytical problems [15]. Here we describe that the use of cyclodextrin as a component of the mobile phase in reversed-phase HPLC leads to effective separation of isomers of MHA, and that simultaneous determination of HA and *o*-, *m*- and *p*-MHA in urine serves as a useful index of solvent exposure.

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EXPERIMENTAL

Chemicals

MHA isomers (*o*-, *m*- and *p*-MHA) and β-cyclodextrin (CD) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). HA, acetonitrile, and acetic acid were purchased from Wako (Osaka, Japan). Acetonitrile was of HPLC grade and other chemicals were of analytical grade.

Automated HPLC

A Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of a pump (LC-3A), an automatic sample injector (SIL-2AS), a column oven (CTO-2AS), a variable-wavelength spectrophotometer (SPD-1), and an integrator (C-R1A) was used. The column used was Zorbax C8 (250 × 4.6 mm, particle size 5 μm, Dupont Instruments, Wilmington, DE, U.S.A.). A guard column (50 × 4.6 mm) was packed with Zorbax ODS (particle size 7—8 μm, Dupont). The mobile phase was the mixture of 200 ml of acetonitrile, 800 ml of distilled water, 15 ml of acetic acid, and 20 g of CD. The mixture without CD was also used for the separations. The flow-rate and column temperature were set at 1.2 ml/min and 40°C, respectively. Detector wavelength was set at 272.4 nm where *m*- and *p*-MHA showed identical molar absorption [14]. Although *m*- and *p*-MHA were not separated when CD was not added to the mobile phase, *m*- and *p*-MHA standards and their mixture gave the same peak area which was independent of the ratio of the isomers in the mixture. For calculations of urinary HA and MHA concentrations, we used a mixture of HA and *o*-, *m*- and *p*-MHA in equal amount as standards (Fig. 1A).

Urine samples

Samples used here were those obtained from solvent-workers at their work-place and from non-exposed control subjects. Urine (10 μl) was directly injected into the HPLC system without any pretreatment.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic separation of HA, and *o*-, *m*- and *p*-MHA. In the present HPLC system, four metabolites of solvents inhaled are completely separated from urine constituents in less than 10 min. Standard curves were linear in the wide range of concentration of each acid (0—2000 mg/l for MHA and 0—6000 mg/l for HA). The results in Table I show the recoveries of the four acid standards added to six urine samples. Recoveries are close to 100%. Within-run coefficients of variation (C.V.) are listed in Table II. The data indicate good precision of the method. Detection limits for HA and each MHA were found to be 50 mg/l urine and 10 mg/l urine, respectively. In the range of low concentrations the signal-to-noise ratio was more than 3, although the precision was decreased to some extent (C.V. = 17.6%). Since an atmospheric concentration of toluene corresponding to a urinary HA concentration of 8100 mg/l, and that of xylene corresponding to a urinary MHA (*m*- plus *p*-isomer) concentration of 700 mg/l are estimated to be about

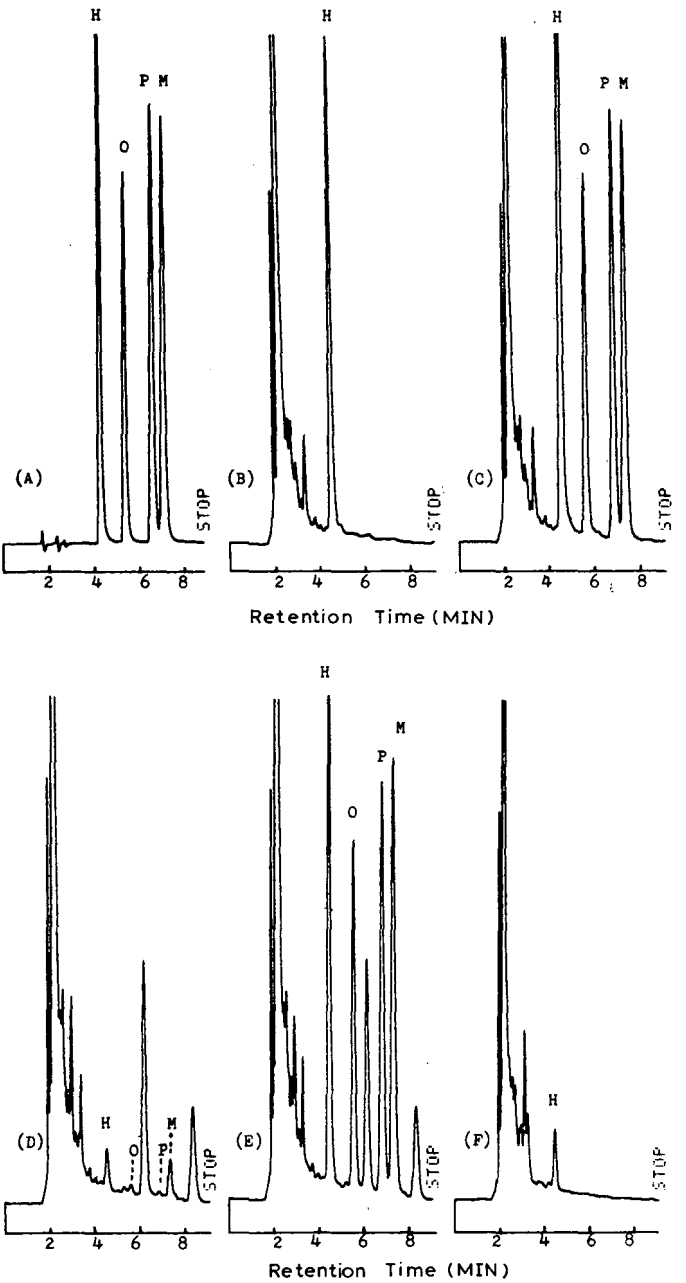

Fig. 1. Chromatographic separations of four metabolites from urine constituents. (A) Four acid standards (H = HA, O = *o*-MHA, P = *p*-MHA, and M = *m*-MHA), 1 g/l of each. (B) Urine from a person exposed to toluene. (C) Sample B plus four acid standards. (D) Urine from a person exposed to xylene. (E) Sample D plus four acid standards. (F) Urine from a control subject not exposed to the solvents.

TABLE I

RECOVERY OF FOUR ACID STANDARDS ADDED TO URINE FROM WORKERS (A—F) EXPOSED TO TOLUENE AND/OR XYLENE

Samples	HA			*o*-MHA			*p*-MHA			*m*-MHA		
	Original (mg/l)	Added (mg/l)		Original (mg/l)	Added (mg/l)		Original (mg/l)	Added (mg/l)		Original (mg/l)	Added (mg/l)	
		1000	2500		250	1000		250	1000		250	1000
		Recovery (%)			Recovery (%)			Recovery (%)			Recovery (%)	
A	186	95.5	100.6	62	95.5	97.0	20	99.6	97.6	210	98.5	98.6
B	1822	96.0	101.8	0	103.0	99.0	15	103.0	99.4	32	101.9	100.5
C	3005	104.1	102.8	22	99.9	99.6	21	99.3	99.5	151	100.2	97.6
D	3162	99.2	99.2	27	95.0	98.8	24	100.5	99.8	23	104.0	100.2
E	885	100.9	102.5	0	98.2	99.8	0	97.1	99.6	0	95.1	100.4
F	2025	97.2	101.9	0	97.5	98.9	0	100.5	100.0	0	100.0	100.3
Mean		98.8	101.5		98.2	98.9		100.0	99.3		100.0	99.6

TABLE II

PRECISION OF THE METHOD

$n = 10$.

	Mean ± S.D. (mg/l)	C.V. (%)
HA	57 ± 0.86	1.5
	478 ± 3.5	0.7
	3366 ± 23.7	0.7
o-MHA	11.1 ± 1.63	14.7
	92 ± 1.4	1.5
	364 ± 4.0	1.1
p-MHA	9.8 ± 1.72	17.6
	152 ± 1.8	1.3
	361 ± 2.2	0.6
m-MHA	9.5 ± 1.08	11.4
	258 ± 6.3	2.3
	600 ± 7.2	1.2

225 ppm and 15 ppm, respectively [14], the method is sufficiently accurate to detect solvent exposure.

Some samples from control and exposed subjects were analyzed by the present method (with CD in the mobile phase) and by the previously reported method (without CD) [14] (Fig. 2). HA and MHA concentrations determined by the present method agree well with those obtained by the previously reported method. The data indicate that the four acids can be determined simultaneously by the addition of CD to the mobile phase with little change in results. Using the present method we determined urinary HA concentrations in 49 healthy male subjects not exposed to the solvent. Fig. 3 shows the frequency distribution of HA concentrations in non-exposed subjects. Arithmetic and geometric means of urinary HA concentrations are 344.2 and 216.8 mg/l, respectively. The percentile (95th) is 1135 mg/l in non-exposed subjects. MHAs were not detected in the non-exposed subjects.

For the evaluation of occupational exposure to xylene, we have determined the sum of *m*- and *p*-MHA in urine because the mixture of *m*- and *p*-xylene is actually used in industry and the rate of metabolism of *m*-xylene is reported to be similar to that of *p*-xylene [7, 11]. However, the separate determination of *m*- and *p*-MHA is necessary if there is a difference in the toxicity of the isomers [1, 11].

In the method previously reported [14], we did not determine *o*-MHA concentrations because *o*-MHA had been known as a minor metabolite of *o*-xylene [1]. Recent study showed that *o*-MHA was a major metabolite of *o*-xylene in humans [2]. Xylene used in industry is composed of 15—20% *o*-xylene besides *m*- and *p*-isomers. So the determination of *o*-MHA is also necessary for the evaluation of xylene exposure. There are many factories where both xylene

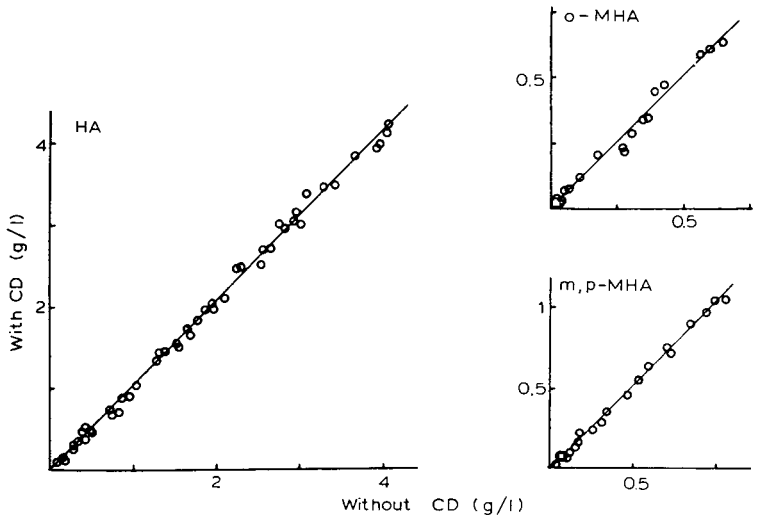

Fig. 2. Comparison of the present method (with CD) with the previously reported one (without CD). HA: $Y = 1.030X - 6.242$ ($n = 49$, $r = 0.999$). *o*-MHA: $Y = 0.996X + 4.442$ ($n = 20$, $r = 0.993$). *m,p*-MHA (*m*- plus *p*-MHA): $Y = 1.049X - 27.94$ ($n = 24$, $r = 0.998$).

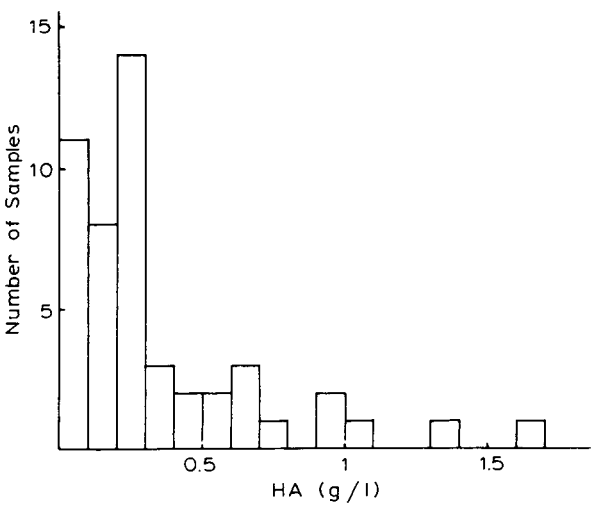

Fig. 3. Frequency distribution of HA concentrations in normal male subjects ($n = 49$).

and toluene are used in a mixture or simultaneously, and xylene often occurs as a contaminant of toluene in industry [16]. Hence the method of simultaneous determination of urinary HA and MHAs would give indicative data from which occupational exposure to either toluene or xylene or both can be monitored.

CONCLUSIONS

A simple method is described for the simultaneous determination of urinary HA, and *o*-, *m*- and *p*-MHAs concentrations by HPLC. The four metabolites were separated from urine components in less than 10 min without any

pretreatment of samples. The detection limits were found to be 50 mg/l and 10 mg/l for HA and MHAs, respectively. The method is sufficiently accurate to detect abnormal exposure to either toluene or xylene, or both.

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Note

Rapid high-performance liquid chromatographic method for detection of interindividual differences in carcinogen metabolism

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Interindividual variation in metabolism of carcinogens [mostly polycyclic aromatic hydrocarbons (PAH)] has been studied using various human tissues and cells [1—9]. The observed variation seems to be primarily under genetic control [10]. Differences in metabolic capacity to activate environmental carcinogens may result in differences in susceptibility to these carcinogens. However, all the studies indicated above have used biopsy material that is not convenient for screening of populations because of practical reasons (e.g. with bronchus) or because of considerable different metabolic capacities compared to tissues susceptible to chemical carcinogenesis.

Human hair follicles have been suggested as a convenient biopsy tissue for screening individual differences in carcinogen metabolism since: (1) They are of epithelial origin, which is important in view of the fact that 90% of human cancers arise in epithelial cells (i.e. are carcinomas). (2) They are available from a large number of volunteers without any risk. (3) They have been shown able to activate and metabolise benzo[*a*]pyrene (BP), a widely distributed carcinogen in our environment and a possible health hazard to humans [11]. (4) Metabolism of BP to dihydrodiol derivatives, the direct precursors of the suspected carcinogens, the diol-epoxides, has been shown to be genetically determined for a large part in hair follicles [12]. (5) Human hair follicle keratinocytes can be brought in culture [13], which enables the study of the effect of inducers and inhibitors of carcinogen-metabolising enzymes on the metabolite pattern of carcinogens [14]. (6) The response of BP metabolism in cultured hair follicle keratinocytes towards pre-exposure to PAH is comparable to that in cultured epithelial cells of the human bronchus [15], the target tissue for PAH-induced neoplasia.

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In the present report the high-performance liquid chromatographic (HPLC) analysis of the whole spectrum of organic solvent-soluble metabolites of BP in freshly isolated hair follicles and in cultured hair follicle keratinocytes is described. This methodology gives the opportunity to detect individual differences in carcinogen metabolism, using an epithelial biopsy tissue.

EXPERIMENTAL

Chemicals

[G-^{3}H]BP was purchased from The Radiochemical Centre (Amersham, Great Britain). BP was from Aldrich (Beerse, Belgium). NADPH was obtained from Boehringer (Mannheim, G.F.R.) and gentamycin sulfate from Schering (Kenilworth, Great Britain). Fetal calf serum, Minimal Essential Medium (with Earle's salts, MEM) and glutamine were purchased from Gibco (Glasgow, Great Britain). Hydrocortisone was from Sigma (St. Louis, MO, U.S.A.), insulin from Organon (Oss, The Netherlands), and epidermal growth factor from Collaboratorive Research (Waltham, MA, U.S.A.). LiChrosob RP-18 was obtained from Merck (Darmstadt, G.F.R.). Synthetic BP derivatives were kindly provided by the NCI Chemical Repository at the IIT Research Institute (Chicago, IL, U.S.A.). Aquasol was obtained from New England Nuclear (Boston, MA, U.S.A.).

Collection of tissue and cell culture

Human hair follicles were obtained from the scalp of healthy volunteers using a pair of tweezers. Only hair follicles with visible bulb and sheath were used. Human hair follicle keratinocytes were cultured as described earlier [13] using a natural basement membrane-like extracellular matrix as growth substrate (bovine eye lens capsules) as described [16]. Lens capsules and culture dishes (Epicult) were obtained from Sanbio B.V. (Nistelrode, The Netherlands). In short, hair follicles were placed on the lens capsules in the Epicult dishes and one drop of medium (MEM containing 15% fetal calf serum, 0.4 μg/ml hydrocortisone, 4 μg/ml bovine insulin and 10 ng/ml epidermal growth factor) was added. The cultures were placed in a humidified atmosphere of 5% carbon dioxide in 95% air. After three days when initial outgrowth started to appear, 0.3 ml of fresh medium was added. From then on the medium was changed twice a week. After 2—3 weeks the cultures had grown to confluency (about $2 \cdot 10^5$ cells/dish) and experiments were started.

Equipment

Throughout the study the following equipment was used: a liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a U6K universal injector, two pumps (Model 6000A), a solvent programmer (Model 660), a UV—visible variable-wavelength detector (Model 450), a reversed-phase LiChrosorb RP-18 (5 μm) column (120 × 4.6 mm) and an Omniscribe recorder (Houston Instruments, Houston, TX, U.S.A.). Fractions were obtained with a programmable fraction collector $FRAC_{3000}$ (Pharmacia, Uppsala, Sweden) and the radioactivity in the samples was analysed with an LKB_{1215} Rackbeta liquid scintillation counter (LKB, Stockholm, Sweden).

Analysis of [^{3}H] BP metabolism

[^{3}H]BP (5 μCi/ml culture medium) was purified by thin-layer chromatography [11], dissolved in ethanol, diluted with unlabeled BP and added to the cultures in a final concentration of 0.5 μM. In the case of freshly isolated hair follicles, incubation was performed with 60 hair follicles in 1 ml of 50 mM Tris—HCl, pH 8.5, containing 0.1 M sucrose, 3 mM $MgCl_2$, 10 μg/ml gentamycin sulfate, 2 mM NADPH and 0.5 μM [^{3}H]BP (5 μCi). After the incubation period (1 h for freshly isolated hair follicles and 24 h for the cultures) cells or hair follicles and medium were extracted three times with an equal volume of ethyl acetate. For this purpose cultured cells were scraped in the medium with a bent Pasteur pipette and transferred to an Eppendorf tube. The collected organic phases were evaporated to dryness under a nitrogen stream, dissolved in 50 μl of methanol and loaded on the HPLC column. From the time of injection the column was eluted with a linear gradient of 65—100% methanol in water. The gradient change was completed in 20 min. The constant flow rate was 0.8 ml/min. Eighty fractions of 0.4 min each (0.32 ml) were collected in minivials. As a consequence the last 30 fractions were obtained with the elution gradient in the end condition (100% methanol). The radioactivity in each fraction was determined with Aquasol as counting medium. After the collection of fractions background radioactivity could be washed out of the column within 10 min, and the column could be equilibrated for the next chromatographic analysis. A mixture of synthetic BP derivatives was used for the determination of the retention times of the various metabolites. For this purpose detection was carried out by UV spectroscopy at 254 nm. Identification of the [^{3}H]BP metabolites was achieved by comparison with the position of the authentic standards.

DNA assay

DNA in cultured hair follicle cells and freshly isolated hair follicles was determined by the mithramycin technique. After the metabolite extraction the tubes were centrifuged and the medium changed for distilled water. Then the cells or hair follicles were treated with pronase and the DNA measured as described earlier [17]. Metabolite formation was expressed as fmol metabolite per μg DNA per h.

RESULTS AND DISCUSSION

Fig. 1 represents the separation of a mixture of BP and synthetic BP derivatives under the conditions described. The fraction numbers and the corresponding retention times of these BP metabolites are listed in Table I. Fig. 2 shows the HPLC profile of organic-solvent-soluble [^{3}H]BP metabolites after incubation of 60 freshly isolated hair follicles for 1 h (A) and of cultured hair follicle keratinocytes incubated for 24 h (B). In addition to diols, quinones and phenols, two early eluting components can be identified, especially in the cultured cells. The first one is a polar compound which hardly has any retention delay and which possibly is BP-3-yl hydrogen sulfate, an ethyl acetate extractable sulfate conjugate. This metabolite has been identified in, for example, human and rodent lung cultures [18]. However, certain tetrols can

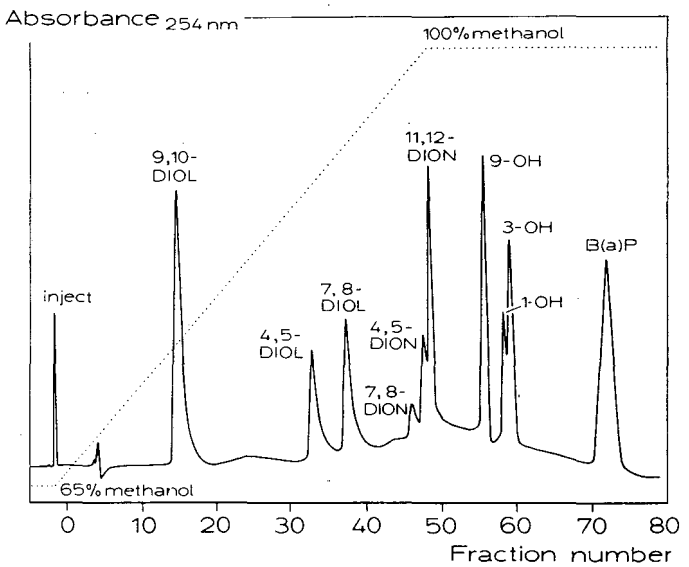

Fig. 1. Separation of a mixture of BP and synthetic BP derivatives under the conditions described in Experimental. Abbreviations: diol = *trans*-dihydro-dihydroxy-BP; OH = hydroxy-BP.

TABLE I

PEAK FRACTION NUMBERS AND CORRESPONDING RETENTION TIMES OF VARIOUS REFERENCE BP METABOLITES IN THE HPLC METHODOLOGY DESCRIBED

Compound*	Peak fraction number	Corresponding retention time (min)
9,10-Diol	16	6.4
4,5-Diol	33	13.2
7,8-Diol	38	14.8
7,8-Dion	46	18.8
4,5-Dion	48	19.2
11,12-Dion	49	19.6
9-OH	57	22.4
1-OH	59	23.6
3-OH	60	24.0
BP	72	28.8

*Abbreviations as in Fig. 1.

also elute in this region. The second early eluting component (fraction number 10—13) has been reported frequently in various tissues [7, 19, 20] and probably is one or more tetrol derivatives of BP. However, since absolute proof of the identity of this component is not available, we have denoted this peak "pre-9,10-diol". It should be noted that tetrols and triols are evidence for the formation of diol-epoxides, the proposed ultimate carcinogens of PAH [21].

Although several quinone and phenol isomers of BP can be separated under the conditions described (Fig. 1), it is difficult to identify each peak in

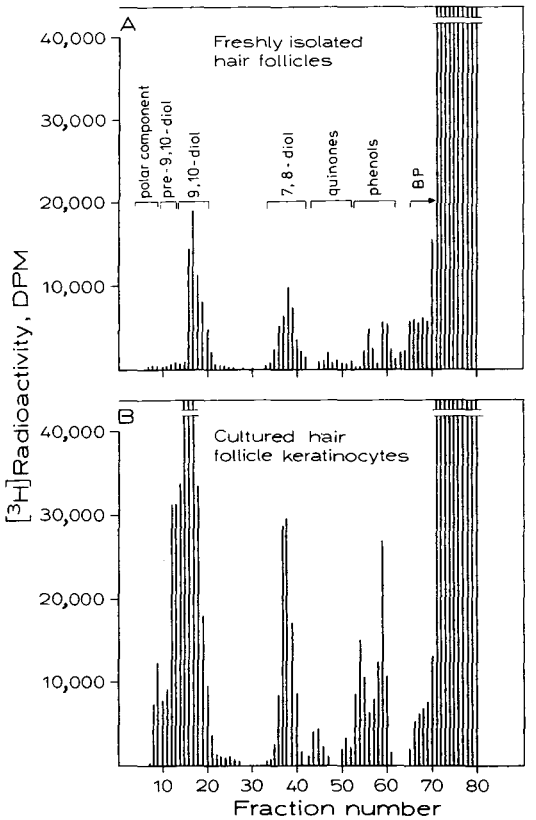

Fig. 2. HPLC profile of organic-solvent-soluble [^{3}H]BP metabolites of freshly isolated hair follicles (incubated for 1 h) (A) and cultured hair follicle keratinocytes (incubated for 24 h) (B). Fractions 4—9 contain a polar component, possibly BP-4-yl hydrogen sulfate. Fractions 10—13 probably represent one or more tetrol metabolites of BP, called "pre-9,10-diol". The *trans*-9,10- and 7,8-dihydrodiol derivatives of BP elute between fractions 14 and 20, and 34 and 42, respectively. Quinones appear between fractions 44 and 52, and phenols between fractions 53 and 62. Unmetabolised [^{3}H]BP elutes from fraction 65.

the quinone and phenol region exactly as one specific isomer. Therefore, all the metabolites which elute between fractions 44 and 52 have been taken together as quinones and the peaks between fractions 53 and 62 as phenols. The HPLC methodology described results in good separation of all the three dihydrodiol metabolites which have been isolated and characterized from various sources [21] as the (—)-*trans*-4,5-dihydrodiol, (—)-*trans*-7,8-dihydrodiol and the (—)-*trans*-9,10-dihydrodiol. Fig. 2A shows that in freshly isolated hair follicles more than 70% of the organic-solvent-soluble metabolites are represented by the 7,8- and 9,10-dihydrodiol metabolites. In contrast, freshly isolated hair follicles hardly metabolise BP to the 4,5-dihydrodiol derivative. Two other important groups of organic-solvent-soluble BP metabolites formed by freshly isolated hair follicles are represented by quinones and phenols although they are formed to a lesser extent than the dihydrodiols.

We have analysed BP metabolism in freshly isolated hair follicles from a

TABLE II

RANGE OF FORMATION OF VARIOUS [^{3}H]BP METABOLITES AND GROUPS OF METABOLITES IN FRESHLY ISOLATED HAIR FOLLICLES OF A NUMBER OF VOLUNTEERS

n = 4. Abbreviations as in Fig. 1. For further explanation see text.

Compound	Range of variation*	Mean ± S.D.
Polar component	0.6—1.5	1.1 ± 0.5
Pre-9,10-diol	1.3—2.6	1.8 ± 0.7
9,10-Diol	41.1—46.3	44.4 ± 2.6
7,8-Diol	29.1—31.5	29.9 ± 1.4
Quinones	3.6—8.5	5.3 ± 2.7
Phenols	10.7—23.1	17.5 ± 6.3

*Expressed as the percentage of the total amount of organic-solvent-soluble metabolites.

number of volunteers. The range of formation of each of the metabolite groups is illustrated in Table II. It is obvious that the variation in dihydrodiol formation is much smaller than the range in variation in phenol formation. Since dihydrodiols result from epoxide hydratase activity and phenols reflect aryl hydrocarbon hydroxylase activity, these findings suggest that the interindividual variation in epoxide hydratase is lower than that for aryl hydrocarbon hydroxylase. In fact, low interindividual variation for epoxide hydratase and large interindividual differences in aryl hydrocarbon hydroxylase activity have been reported for various human tissues (see, for example, ref. 22 and references therein) including human hair follicles [23]. Interindividual variation in total BP metabolism to organic-solvent-soluble metabolites was about three-fold as analysed in the present study.

The metabolite pattern of cultured human hair follicle keratinocytes is qualitatively comparable with freshly isolated hair follicles with dihydrodiols representing the major metabolite group, phenols and quinones the minor ones. Due to the longer incubation time the total level of metabolism is higher than in freshly isolated hair follicles (Table III). However, the mean rate of BP metabolism in cultured cells is somewhat lower than in freshly isolated hair follicles. This can be ascribed to the greater formation of dihydrodiols, quinones and phenols in freshly isolated hair follicles. In contrast, the amount of the more polar organic-solvent-soluble metabolites is greater in cultured hair follicle keratinocytes than in freshly isolated hair follicles. The observation that at longer incubation times there is about the same amount of 9,10-dihydrodiol and much more tetrols indicates that 9,10-dihydrodiol is an end-point in BP metabolism and 7,8-dihydrodiol can be further metabolised to the diol-epoxides.

Cultured hair follicle keratinocytes offer the opportunity to investigate BP metabolism after induction of the enzyme aryl hydrocarbon hydroxylase. High levels of induced activity of this enzyme have been correlated with genetic susceptibility to PAH-induced neoplasia in some studies [9, 24], while other authors failed to confirm this [25, 26]. One of the sources of controversy can be the choice of human biopsy tissue, peripheral lymphocytes. In

TABLE III

FORMATION OF [^{3}H]BP METABOLITES OR METABOLITE GROUPS IN FRESHLY ISOLATED HUMAN HAIR FOLLICLES AND CULTURED HAIR FOLLICLE KERATINOCYTES FROM THE SAME DONOR

Abbreviations as in Fig. 1. The data represent the mean values for three persons and are expressed as fmol product per μg DNA per h.

Compound	Freshly isolated hair follicles	Cultured hair follicle keratinocytes
Polar component	4 ± 1	22 ± 5
Pre-9,10-diol	4 ± 1	30 ± 5
9,10-Diol	130 ± 20	96 ± 16
7,8-Diol	92 ± 17	33 ± 6
Quinones	13 ± 3	10 ± 3
Phenols	73 ± 27	31 ± 12
Total	316 ± 43	222 ± 35

view of the prevalence of carcinomas, the use of human keratinocytes seems to be a more appropriate alternative. It has already been shown that the response of BP metabolism towards pre-exposure to benz[*a*]anthracene in cultured hair follicle keratinocytes, is qualitatively comparable with that in cultured human bronchial epithelial cells, the target tissue of PAH-induced neoplasia [15]. In contrast, murine epidermal cells, a frequently studied cell type in chemical carcinogenesis, do respond differently to pre-exposure to benz[*a*]anthracene as compared with human epidermal keratinocytes [14].

The method presented here offers the advantage of rapid analysis of BP metabolism in an easily available human biopsy tissue of epithelial origin, the hair follicle. Both differences in the profile of the various BP metabolites and variations in the rate of BP metabolism can be monitored easily. The application of the methodology on cultured hair follicle cells gives the opportunity to measure inducibility of BP metabolites after pre-exposure to PAH. Together with the recently developed assays for carcinogen-metabolising enzymes in hair follicles [27, 28], the method may contribute to identification of high-risk populations.

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Note

Measurement of 2-amino-N-(1,1-dimethylhexyl)acetamide (A643C), an investigative antidepressant, in plasma by electron-capture gas chromatography

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2-Amino-N-(1,1-dimethylhexyl)acetamide (A643C; structure I, Fig. 1) has a pharmacological profile in animal models indicative of a potential antidepressant. To allow the investigation of the pharmacokinetics of this compound in laboratory animal species and man, a selective and sensitive analytical method was necessary. This report describes conditions for submicrogram derivatisation of A643C from plasma with pentafluoropropionic anhydride and for determination of the resulting pentafluoropropionamide by electron-capture gas chromatography.

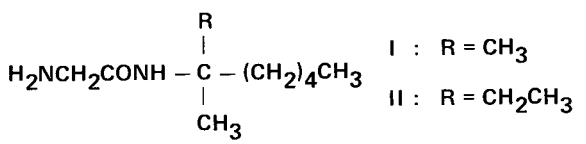

Fig. 1. Structures of A643C (I) and A404C (II).

MATERIALS AND METHODS

Reagents and materials

A643C hydrogen succinate and the homologue A404C hydrogen oxalate (2-amino-N-(1-methyl-1-ethylhexyl)acetamide; structure II, Fig. 1) used as internal standard were synthesised at the Chemical Research Laboratories, Wellcome Research Laboratories (Beckenham, Great Britain).

Other chemicals used were: pentafluoropropionic anhydride (PFPA; Pierce and Warriner, Chester Great Britain); *n*-hexane (Rathburn Chemicals, Walkerburn, Great Britain); OV-225 (Phase Separations, Queensferry, Great Britain);

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and Chromosorb W HP 100—120 mesh (Analabs, North Haven, CT, U.S.A.). All other chemicals and reagents were analytical grade and were obtained from BDH (Poole, Great Britain). Plasma used for the development of the assay was obtained from blood taken by venipuncture from healthy volunteers who had not received any drug treatment for at least one month before blood withdrawal.

Glassware

Screw-capped 20-ml glass tubes were used for the extraction and solvent evaporation.

Instrumentation

A Perkin-Elmer F30 instrument, modified to allow sample injection from a Hewlett-Packard 7670A autosampler, was used with a ^{63}Ni electron-capture detector. Operation of the autosampler and processing of data were carried out using a Hewlett-Packard 3352B minicomputer-based laboratory automation system.

Extraction procedure

A643C and A404C were prepared as stock solutions of their respective salts in water. They were prepared weekly and stored at 4° C. Depending upon the concentration of A643C expected and the volume of sample available, replicate 0.1—1 ml portions of plasma were taken for extraction. A404C was added (250 ng in 0.1 ml) followed by 1 ml of 4 *M* sodium hydroxide. The mixtures were extracted on a tumbler for 15 min (15 rpm) with 10 ml *n*-hexane. After centrifugation at 2000 *g* for 10 min, the organic layers were transferred to clean tubes. A 2-ml volume of 2 *M* hydrochloric acid was added and the tubes shaken vigorously on a mechanical shaker at approximately 275 strokes min^{-1} for 15 min. The phases were separated by centrifugation at 2000 *g* for 10 min and the supernatants removed by aspiration and discarded. The aqueous layers were made basic by the addition of 0.8 ml of 10 *M* sodium hydroxide, 10 ml *n*-hexane were added and the mixture extracted on a box tumbler for 10 min. The phases were separated by centrifugation at 2000 *g* for 10 min and the supernatants transferred to clean tubes. PFPA (30 μl) was added and the tubes placed on a box tumbler for 15 min. Excess reagent and solvent were removed under a stream of nitrogen gas at room temperature. Care was taken to remove tubes as soon as they were dry. The residues were redissolved in 0.4 ml cyclohexane, using a Vortex mixer to wash the sides of the tubes. Samples were transferred to Hewlett-Packard microvials for analysis by gas chromatography; sample injection volume was 5 μl.

Gas chromatography

After preliminary evaluation of a number of stationary phases, it was concluded that 5% OV-225 on Chromosorb W HP (100—120 mesh) in a 4 m × 3 mm I.D. glass column was optimal in terms of separating the drug and internal standard from extraneous biological material. The injection port of the gas chromatograph was maintained at 225° C, the detector at 350° C and the column at 210° C. Argon was used as carrier gas at a flow-rate of 40 ml min^{-1}.

Quantitation and calibration

The Hewlett-Packard 3352B data system identified A643C and A404C by retention time, measured their peak areas and calculated the peak area ratios. Calibration curves were constructed by a weighted linear regression of peak area ratios against the concentrations added. A weighting factor of $1/(\text{concentration})^2$ was used. Concentrations of unknowns were calculated from peak area ratios by interpolation of the computed calibration curve. Calibration curves were linear with zero intercepts. Precision and accuracy were essentially constant with a relative standard deviation below 3.5% over the range 10—250 ng ml^{-1} (Table I).

TABLE I

PRECISION AND ACCURACY OF A643C ANALYSIS IN DOG PLASMA

Known value (ng ml^{-1})	Assayed value (ng ml^{-1})	Standard deviation ($n = 6$)	Relative standard deviation (%)
10	10.1	0.3	2.6
50	48.7	1.5	3.2
100	101.0	3.4	3.4
250	254.1	2.6	1.0

RESULTS AND DISCUSSION

A typical gas chromatogram of a derivatised extract from dog plasma is shown in Fig. 2. Studies with radiolabelled A643C show that the overall extraction efficiency is 61 ± 3% ($n = 6$). Although not giving the highest extraction efficiency of the range of solvents initially investigated, hexane was chosen as extracting solvent because it gave an efficiency which was more than adequate for the levels of A643C encountered and because the resultant chromatograms were free from any interfering peaks. The minimum detectable quantity of pure drug (signal-to-noise ratio = 2) is 100 pg injected on column. The lowest measurable concentration in plasma is approximately 10 ng ml^{-1}.

In addition to problems with adsorption onto glassware used in pre-chromatographic manipulations, the analysis of free amines by gas chromatography is frequently unsatisfactory due to peak tailing and low sensitivity caused by adsorption of the sample onto the chromatographic support. In most cases derivatisation of the amine is necessary to achieve optimal chromatographic characteristics. At a very early stage in the development of an assay for A643C it became apparent that, whilst there were no problems with adsorption onto glassware, the formation of a suitable derivative would be essential both to improve chromatography and to provide adequate sensitivity. Perfluoroalkyl anhydrides are commonly used for the derivatisation of amines and amides [1, 2]; for example trifluoroacetic (TFAA) [1, 3, 4] pentafluoropropionic (PFPA) [5, 6] and heptafluorobutyric (HFBA) [7—9] anhydrides. Preliminary experiments showed the TFAA and HFBA derivatives of A643C to be unsuitable because of instability and excess reagent removal difficulties,

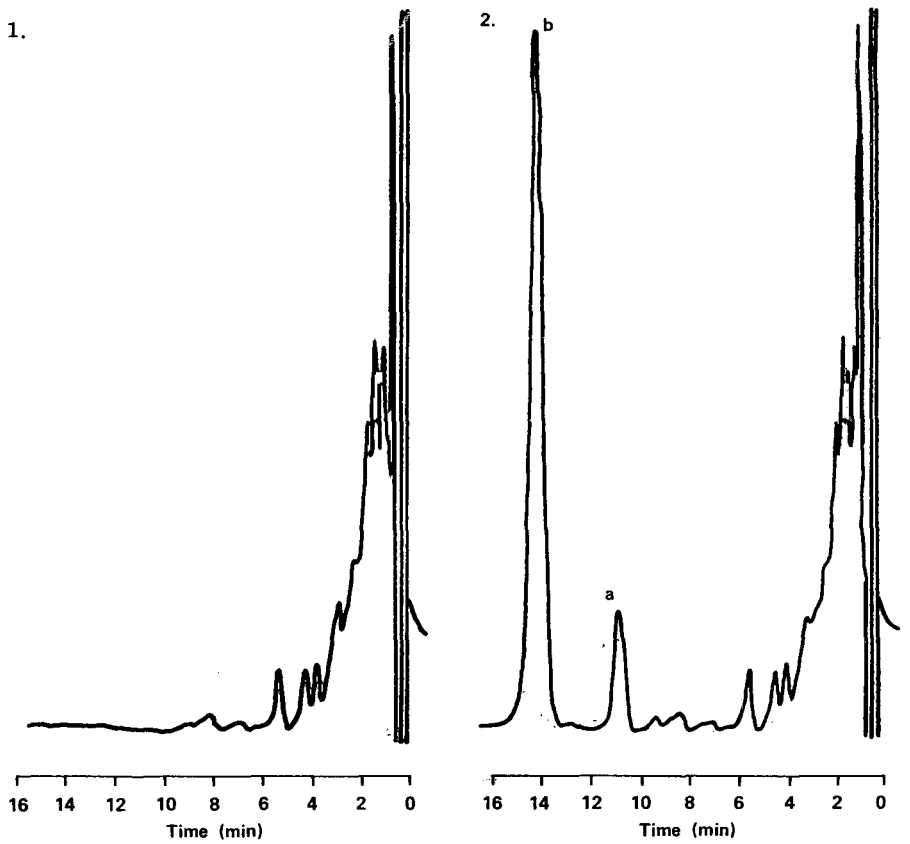

Fig. 2. Gas chromatograms of dog plasma samples extracted and analysed for A643C. (1) Control plasma; (2) plasma containing 50 and 250 ng ml^{-1} of A643C and A404C, respectively. Peaks: a = A643C pentafluoropropionamide; b = A404C pentafluoropropionamide.

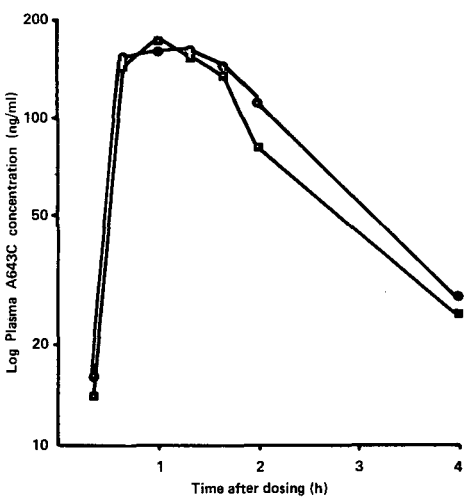

Fig. 3. Plasma concentration—time profile of A643C in two male beagle dogs dosed orally with A643C hydrogen succinate at 10 mg kg^{-1}.

respectively. No attempts were made to overcome the reagent removal problems with HFBA as an additional step in the assay procedure was undesirable. Although perfluoroamide derivatives of amines are frequently sensitive to hydrolysis [1, 2, 10] the stability of the pentafluoropropionamides of A643C and A404C is very good. A decrease in peak area ratios is noticed after two weeks. After three days storage at 4°C a decrease in peak areas of only a few percentage units is noted but with peak area ratios remaining constant. As a routine, samples are analysed within two days of extraction and derivatisation. The improved stability of a PFPA derivative over that of the corresponding TFAA derivative has been previously reported [7, 10].

The reaction of A643C and A404C with PFPA at room temperature is rapid and results in a quantitative conversion of the primary amines to their respective monopentafluoropropionamide derivative. The identities of these derivatives were established by gas chromatography—mass spectrometry. Reactions at elevated temperatures (60°C+) resulted in a non-reproducible mixture of di- and monopentafluoropropionamides. Three derivatives of each compound were identified: the mono-amine and amide derivatives and the di-amine/amide derivatives.

The method described is selective, sensitive and reproducible. Although described for dog plasma, it can also be applied to the analysis of A643C in human plasma and urine with no modifications. The suitability and application of this method to pharmacokinetic studies have been established (Fig. 3) and will be reported elsewhere.

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Note

Morphine analysis by high-performance liquid chromatography

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Several techniques for the analysis of the low concentrations of morphine (< 200 ng/ml) in biological samples have been reported including gas—liquid chromatography with electron-capture detection (GLC—ECD) [1, 2], high-performance liquid chromatography (HPLC) with amperometric (electrochemical) detection (HPLC—AD) [3—5], thin-layer chromatography [6, 7], and radioimmunoassay [8]. However the use of these techniques is not without difficulties. The necessity of sample volatility for GLC analysis requires that morphine be derivatized, commonly by acylation of the hydroxyl functions with a polyfluorinated anhydride. This procedure enables the quantitation of morphine by electron-capture detection, a process which is essentially halogen specific. Radioimmunoassay techniques for morphine analysis are exquisitely sensitive (50 pg/ml plasma [8]), but suffer from a potential lack of selectivity. Morphine-3-glucuronide, the major metabolite of morphine has approximately 10% the potency of morphine in displacing radiolabelled ligand from the antibody binding site [9], thus generating a potential source of error. HPLC, on the other hand, requires no derivatization step for either the chromatographic separation or detection of morphine. Although several reported methods utilize ultraviolet light absorbance (UV) detection the minimum detectable quantity of morphine by this technique is quite large [10]. Alternatively the use of HPLC—AD increases the sensitivity to morphine by 100-fold over UV detection techniques. By this method morphine is quantitated amperometrically by the electrochemical oxidation of the phenolic hydroxyl group of morphine [5].

Irregardless of the chromatographic technique employed for the analysis of morphine in biological samples, an initial separation of morphine from endogenous interfering compounds is necessary. Commonly the biological sample is extracted with a mixture of a chlorinated hydrocarbon or toluene

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and an alcohol [1, 2]. Unfortunately these methods frequently result in emulsion formation which renders further sample processing difficult [2].

In this paper we describe a morphine extraction and HPLC—AD assay capable of detecting 1 ng morphine per ml plasma, and which obviates the necessity of sample derivatization and commonly encountered extraction problems. The results obtained for plasma samples containing morphine with this HPLC—AD method and a previously reported GLC—ECD morphine assay are compared.

EXPERIMENTAL

Materials

Reagent-grade chemicals and solvents were used throughout. The derivatizing agent, pentafluoropropionic anhydride (PEPA) was purchased from Pierce (Rockford, IL, U.S.A.). Morphine sulfate for the preparation of plasma standards and normorphine free base were obtained from Health and Welfare Canada (Ottawa, Canada). The internal standard for the GLC—ECD assay, N-ethyl normorphine, was synthesized from normorphine by the procedure of Ebbighausen et al. [11].

All glassware was siliconized with 10% Surfasil® (Pierce) in hexane followed by three rinses in 95% ethanol and oven drying (60°C, 12 h).

Apparatus

GLC—ECD morphine analysis was performed using a Model 5713 gas—liquid chromatograph equipped with a pulsed ^{63}Ni electron-capture detector (Hewlett-Packard, Mississauga, Canada). The sample was separated on a 1.8 m × 2 mm silanized glass column packed with 3% OV-17 on Gas-Chrom Q, 100—120 mesh (Chromatographic Specialties, Brockville, Canada) at 210°C with a detector and injector temperature of 250°C. The carrier gas (5% methane in argon) flow-rate was 30 ml/min.

For the HPLC—AD separation of morphine a liquid chromatograph was assembled of the following components: M-6000 constant flow pump; 25 cm × 4 mm C_{18} reversed-phase (μBondapak) column (10 μm particle size); a WISP Model 710B automatic sample injector (Waters Assoc., Mississauga, Canada) and a Model 4A electrochemical detector with a glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.) operated at + 0.65 V with respect to an Ag/AgCl reference electrode. The mobile phase was a mixture of methanol—water—ammonium hydroxide (50:50:0.1) degassed by continual stirring, and delivered at a flow-rate of 1.3 ml/min. Chromatogram peak areas and heights were determined by an electronic integrator (HP3353 data system, Hewlett-Packard).

GLC assay

The GLC—ECD assay for morphine was a modification of the method of Dahlstrom et al. [1]. The alterations involved the use of 5.0 ml 25% *n*-butanol in toluene as the extraction solvent instead of 3.0 ml toluene—butanol (9:1). In addition all extractions were performed twice with a second 5.0-ml aliquot of extraction solvent. All solvent evaporations were carried out in vacuo on a centrifugal evaporator (Savant Instruments, Hicksville, NY, U.S.A.).

HPLC analysis

To a PTFE-lined screw-capped test-tube were added plasma (0.5 ml) and acetonitrile (2.0 ml) containing the internal standard normorphine (100 ng/ml). The tube was capped, shaken (15 min) and centrifuged (250 *g*, 15 min). The morphine-containing supernatant fluid was decanted into a second test-tube containing extraction solvent (3.0 ml 10% *n*-butanol in chloroform) and 0.1 *M* hydrochloric acid (1.0 ml). The tube was capped, shaken and centrifuged as before. The aqueous phase (upper) was removed and added to a third tube containing extraction solvent (3.0 ml), 1 *M* sodium hydroxide solution (0.1 ml), ammonium chloride buffer (1 ml of 1 *M* ammonium hydroxide solution titrated to pH 9.0 with 2 *M* hydrochloric acid), capped, shaken (15 min) and centrifuged (250 *g*, 15 min). The organic phase was removed and evaporated in a 5-ml Reacti-Vial (Pierce) under a stream of nitrogen. The residue was redissolved in methanol (200 μl) and 20 μl injected onto the HPLC column.

Statistical analyses

Standard curves were determined by a least-squares linear regression procedure. Comparisons between data were performed by a 2-tail Student *t*-test.

RESULTS AND DISCUSSION

Sample chromatograms for plasma containing no morphine and for plasma with 15 ng/ml morphine by the HPLC—AD method are shown in Fig. 1. For the HPLC—AD morphine assay standard curves obtained by plotting either the ratio of morphine to normorphine peak areas or peak heights against known sample concentrations of morphine were linear over the concentra-

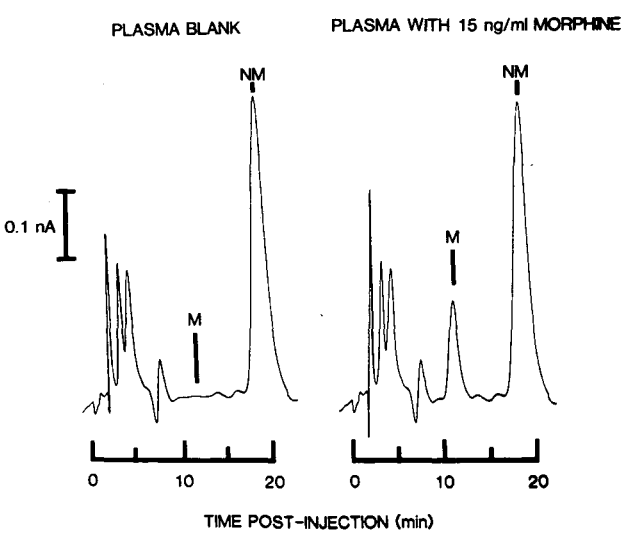

Fig. 1. Representative chromatograms for the elution of morphine (M) and normorphine (NM) by high-performance liquid chromatography with electrochemical detection. Patient samples after a drug dose yielded chromatograms essentially the same as that seen by addition of morphine to drug-free plasma.

TABLE I

COMPARISON OF GLC AND HPLC FOR THE QUANTITATIVE ESTIMATION OF MORPHINE IN PLASMA

Method	Known concentration (ng/ml)	Observed concentration (ng/ml)		
		Mean	Standard deviation	Coefficient of variation (%)
GLC—ECD	80	80.3	4.1	5.1
	20	21.0	2.3	9.1
	8.0	7.6	1.4	18.4
	2.0	2.6	2.0	76.9
HPLC—AD	100	100.6	4.1	4.1
	25	24.8	2.8	11.3
	8.0	8.2	1.4	17.2
	2.0	2.4	0.75	31.5

tion range examined (1.6—400 ng/ml) with intercepts not significantly different from zero. For the GLC—ECD morphine assay separate standard curves were constructed for plasma samples containing both high (160—20 ng/ml) and low (20—2 ng/ml) concentrations of morphine. In each case a plot of the ratio of morphine to N-ethyl normorphine peak areas against known standard concentrations was linear and not significantly different from zero in intercept. The between-day reproducibility over eight separate occasions of the estimation of morphine contained in four plasma samples of known concentration by each method is indicated in Table I. It is apparent from this table that both methods accurately assessed the concentration of morphine in the samples over a wide range. For the analysis of those samples containing morphine at concentrations equal to and in excess of 8 ng/ml plasma the precision of morphine quantitation, as indicated by the coefficient of variation, was similar for the two methods. However the HPLC—AD morphine assay was more reliable for assessing plasma concentrations less than 8 ng/ml even though only 0.5 ml of plasma was used as opposed to 1.0 ml by the GLC—ECD method. The coefficient of variation for the analysis of a 2 ng/ml plasma standard by HPLC—AD was 31.5% in comparison to 76.9% for the GLC—ECD technique. In this light the minimum detectable quantity of morphine, defined as a peak twice the height of the baseline noise, is in excess of 2 ng/ml plasma for the GLC—ECD assay and approximately 1 ng/ml for the HPLC—AD method.

The sample preparation for the HPLC analysis of morphine in plasma represents a departure from the procedures commonly used. Both halogenated hydrocarbon—alcohol and toluene—alcohol mixtures have been widely used for the extraction of morphine from biological samples. However, emulsions are frequently produced by shaking either of these solvent mixtures with plasma (and other protein-containing fluids) making phase separation difficult. To overcome this problem, column extraction of morphine following absorption of the aqueous phase on an inert support such as cellulose powder [12], silica [13], or gauze sponges [14] has been reported. The ex-

traction procedure described in this paper circumvents the problem of emulsion formation and the difficulties of column extraction techniques by an initial acetonitrile denaturation of plasma proteins prior to solvent extraction. Following the addition of acetonitrile to the plasma samples, the plasma proteins form a hard pellet on centrifugation, allowing the morphine-containing supernatant liquid to be decanted. Since acetonitrile is completely miscible with aqueous solutions there is no phase separation nor the associated loss of morphine following an extraction step.

For optimal extraction of morphine, an amphoteric compound, into an organic solvent, the pH of the aqueous phase must be adjusted to 8.96 [15]. An ammonium chloride buffer solution was selected over the more commonly employed carbonate buffer solutions [1, 6] due to the greater pH stability of the former on storage [2]. Although less polar solvents, such as toluene—butanol and benzene—butanol, provide cleaner extracts, the use of more polar solvents (chloroform—butanol) results in less critical pH adjustment, higher extraction efficiencies and reduced sample adsorption to glass surfaces [2]. The overall extraction efficiency of morphine for the HPLC—AD method (85%) is greater than that reported for the GLC—ECD method of Dahlstrom et al. (67%) [1], presumably due to the greater extraction recovery obtained through the use of chloroform—butanol.

In man, normorphine has been reported to be a minor metabolite not detectable in plasma and accounting for approximately 1% of the morphine dose excreted in the urine [16]. Hence endogenously produced normorphine causes little or no interference with the added normorphine internal standard (200 ng per sample). Other opiates could have been employed as an internal standard, e.g. nalorphine [4] or N-ethyl normorphine [1], provided they possess a free phenolic hydroxyl group necessary for electrochemical activity [4].

The method described for the HPLC—AD analysis of morphine presents several advantages over the GLC—ECD method described by Dahlstrom et al. [1]. It combines increased sensitivity for morphine quantitation (1 ng/ml plasma) with a simplified extraction procedure of a smaller (0.5 ml) sample and the absence of a derivatization process. The assay is sufficiently reliable and predictable that analytical runs of 50 h are routinely performed unattended with automatic sample injection and peak quantitation by electronic integrator. Prepared samples are stable at −20°C (no noticeable degradation over three months) such that samples may be processed in batches and stored for subsequent analysis. Over 1000 analyses have been performed on one reversed-phase column without significant deterioration.

ACKNOWLEDGEMENTS

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CHROMBIO. 1720

Note

High-performance liquid chromatographic determination of practolol in plasma

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(First received December 24th, 1982; revised manuscript received February 17th, 1983)

Practolol, 4-(2-hydroxy-3-isopropylaminopropoxy)acetanilide, is a cardioselective β-adrenergic blocking agent mainly used for the emergency treatment of cardiac arrhythmias. It is given by a slow intravenous injection of 5 mg, repeated if necessary according to the patient's response. Few methods have been reported for the determination of practolol in biological fluids. They include fluorimetric [1] and gas chromatographic methods [2—4], both requiring derivatization before analysis. Only one high-performance liquid chromatographic (HPLC) method [5] has been published to date utilizing reversed-phase separation and UV detection at 254 nm. This paper describes a new method using a reversed-phase radial compression column with UV detection at 248 nm. Its use in monitoring practolol therapy is also demonstrated.

EXPERIMENTAL

Chemicals and reagents

Liquid chromatography grade methanol, ethyl acetate and analytical reagent-grade ammonia were purchased from BDH (Poole, Great Britain). Practolol and acebutolol hydrochloride were received from ICI (Macclesfield, Great Britain) and May & Baker (Dagenham, Great Britain), respectively.

Apparatus

A reversed-phase column 10 X 0.8 cm, particle size 10 μm, Radial-Pak C-18 (Waters Assoc., Hertford, Great Britain) and a radial compression module, Model RCM-100 (Waters Assoc.), was used in conjunction with a septumless injector, Model U6K (Waters Assoc.), a variable-wavelength UV detector, Model 450 (Waters Assoc.), a pump, Model 110A (Altex Scientific, Berkeley, CA,

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U.S.A.) and a recorder, Model RE 571-20 (Smiths Industries, London, Great Britain).

Procedure

To a stoppered 10-ml tube 1.0 ml plasma sample, 0.2 ml 5 *M* sodium hydroxide, 0.2 ml internal standard (10 μg/ml solution of acebutolol hydrochloride in water) and 5.0 ml ethyl acetate were added. The contents were vortexed for 1 min followed by centrifugation at 750 *g* for 5 min. The ethyl acetate phase was transferred to a tapered tube and evaporated on a sample concentrator at 80°C under a stream of nitrogen. The residue was reconstituted with 200 μl of mobile phase and 80 μl were injected onto the chromatograph.

The following HPLC conditions were used. Mobile phase, methanol–water (75:25) containing 0.03% ammonia (sp. gr. 0.88); flow-rate, 1.8 ml/min; wavelength, 248 nm; detector range, 0.02 a.u.f.s.; injection volume, 80 μl; chart speed, 120 mm/h; temperature, ambient.

Standard solutions of practolol ranging from 0.1 to 1.0 μg/ml were prepared in drug-free plasma and 1.0 ml of each standard was assayed according to the procedure described. The peak height ratio of drug to internal standard was plotted against concentration and the calibration graph was used for measuring the concentration of practolol in the samples.

RESULTS AND DISCUSSION

Chromatograms of spiked plasma as well as a patient's plasma sample of practolol are shown in Fig. 1. The retention times for practolol and acebutolol hydrochloride are 5 and 13 min, respectively. The change in the concentration of ammonia in the mobile phase had a significant effect on the elution of the drug and internal standard (Fig. 2). A concentration of ammonia of 0.03% was found to be optimum under the chromatographic conditions employed. The calibration graph was linear within the above mentioned concentration range. The regression equation for the calibration graph is $y = 0.016 + 1.08x$, $r = 0.999$. Good reproducibility of the method was indicated when ten separate determinations of a 0.25 μg/ml sample of practolol gave a within-day coefficient of variation of 2.4% and a day-to-day coefficient of variation of 5.3%. The lower limit of determination was found to be 30 ng/ml. Percentage recoveries of practolol and acebutolol hydrochloride were calculated by comparing the peak heights of plasma sample (after extraction) with those of aqueous solution containing the same concentration of these compounds. The recovery of practolol at 0.25 μg/ml and acebutolol hydrochloride at 10.0 μg/ml was 75% and 95%, respectively. No peaks were observed at the retention times of the drug or internal standard from the blank plasma sample nor from lignocaine which may be coadministered with practolol. The retention time of lignocaine was 3 min. The presence of 0.03% ammonia in the mobile phase did not cause a reduction of the column efficiency. When stored at 4°C, the plasma standards (calibrators) were stable for four weeks. The method is currently being used to study the pharmacokinetics of practolol in patients with acute myocardial infarction. Fig. 3 shows plasma levels of practolol in a patient following incremental intravenous doses. A total of 20 mg was given in four

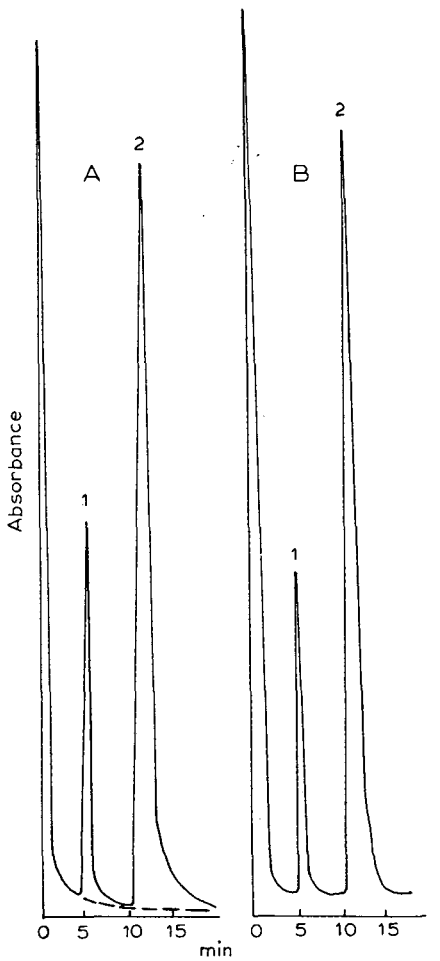

Fig. 1. (A) Chromatogram of plasma standard containing (1) 0.5 μg/ml of practolol and (2) 10 μg/ml of acebutolol hydrochloride. The broken line shows a trace from blank plasma. (B) Chromatogram of a patient's plasma sample containing (1) 0.37 μg/ml of practolol and (2) 10 μg/ml of acebutolol hydrochloride as internal standard.

divided doses every 15 min, the first two doses were 2.5 mg each followed by a 5-mg and a 10-mg dose.

In the previously published HPLC method for practolol [5], a reversed-phase stainless-steel column was used with a mobile phase of ethanol—water (1:9) and a wavelength of 254 nm. No internal standard was used. In the method proposed here the drug is analysed at the wavelength of maximum absorption in presence of internal standard thereby imparting maximum sensitivity and precision to the assay. The mobile phase contains relatively less water and seldom requires deaeration. The radial compression separation system presents a new concept in HPLC. In our opinion radial compression columns afford shorter analysis time, longer life and provide excellent baseline stability. They can be operated at lower pressures and are more economical than the conventional stainless-steel columns.

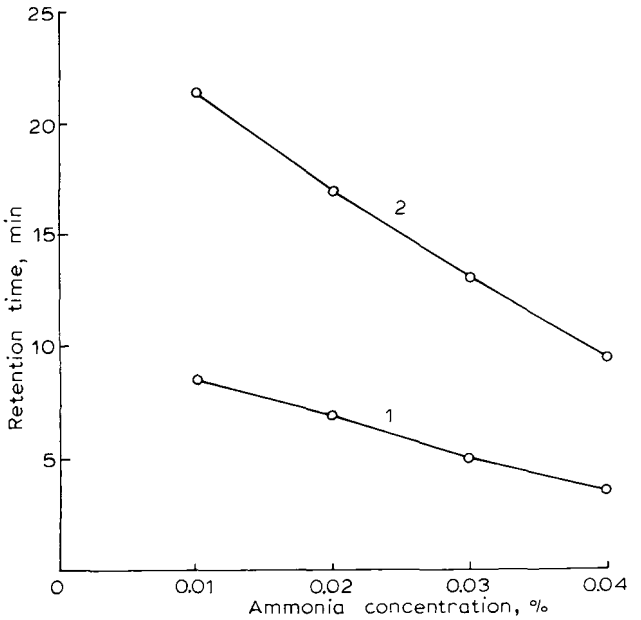

Fig. 2. Effect of ammonia concentration on retention times of (1) practolol and (2) acebutolol hydrochloride.

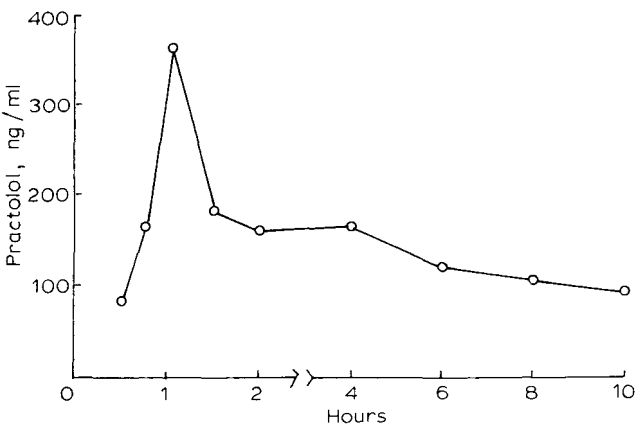

Fig. 3. Plasma profile of practolol from a patient who received repeated intravenous doses totalling 20 mg in 45 min.

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CHROMBIO. 1717

Note

Determination of lodoxamide in plasma using ion-pairing and reversed-phase high-performance liquid chromatography

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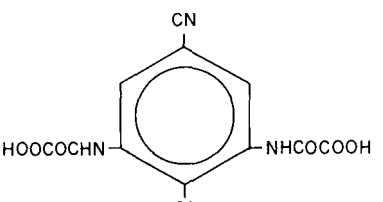
Lodoxamide (I), N,N′-(2-chloro-5-cyano-*m*-phenylene)dioxamic acid is currently under investigation as an orally and inhalation active antiasthmatic agent [1, 2]. An inhibitor of IgE-mediated allergic reactions, I is chemically different from disodium cromoglycate, a currently marketed antiasthmatic drug. Lodoxamide is 2500 times more active in rats than disodium cromoglycate when assayed by the passive cutaneous analphylaxis test [2].



Lodoxamide
I

In continuing efforts to apply high-performance liquid chromatography (HPLC) to pharmaceutical analysis [3], a HPLC procedure for the separation and quantitation of I in human plasma is reported. The separation is effected utilizing ion-pair formation with tris(hydroxymethyl)aminomethane on an octadecylsilane column. The chromatographic separation takes approximately 20 min. The overall analysis time is about 90 min, which includes extraction of the drug from plasma followed by HPLC separation and quantitation.

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EXPERIMENTAL

Materials

A powdered sample of lodoxamide tromethamine (Upjohn Company, Kalamazoo, MI, U.S.A.) was used in the preparation of standard solutions. Tris(hydroxymethyl)aminomethane (Sigma, St. Louis, MO, U.S.A.) was used as the ion-pair reagent in the mobile phase. All other chemicals and solvents were the highest grade of commercially available materials.

HPLC conditions

The HPLC analyses were performed on a Waters Assoc. (Milford, MA, U.S.A.) Model ALC202 equipped with an M-6000 pump, a U6K injector, and a 254-nm fixed-wavelength UV detector. The column was a 10-μm μBondapak C_{18} column (300 mm × 4 mm I.D.) (Waters Assoc.). The mobile phase used was absolute methanol–0.05 *M* aqueous tris(hydroxymethyl)aminomethane (10:90) and was degassed before use. The pH of the mobile phase was adjusted to pH 6 with concentrated phosphoric acid and the flow-rate was set at 1.0 ml/min (82.74 bar).

Standard solutions for calibration curve

A stock solution of lodoxamide tromethamine (74 μg/ml) was prepared by dissolving a weighed amount of the powder in distilled water. The resulting solution of the salt was equivalent to 40 μg/ml of lodoxamide free acid. In addition, an internal standard stock solution (115 μg/ml) of *p*-nitrocinnamic acid in absolute methanol–distilled water (50:50) was prepared.

Plasma calibration procedure

Into individual 15-ml centrifuge tubes were placed 1.0-ml quantities of drug-free human plasma. Accurately measured volumes of 10, 5, and 2.5 μl of lodoxamine tromethamine stock solution were added such that the final concentration of lodoxamide free acid was 400, 200, and 100 ng/ml, respectively. Internal standard stock solution (10 μl) and concentrated hydrochloric acid (100 μl) were added to each tube, followed by mixing on a vortex mixer (30 sec) and heating on a steam bath (1 min). After cooling to room temperature, ethyl acetate (5 ml) was added, and the mixture vortexed (45 sec) followed by centrifugation at 860 *g* (10 min). The organic phase (4 ml) was removed with the aid of a volumetric pipette, and transferred to a clean 15-ml centrifuge tube. Upon evaporation of the ethyl acetate to dryness on a water bath with the aid of a nitrogen stream, the residue was dissolved with shaking in 100 μl of distilled water–absolute methanol (50:50) containing 0.05 *M* tris(hydroxymethyl)aminomethane (pH 6). If the resulting solution was cloudy, the sample was centrifuged at 1239 *g* (5 min), the clear supernatant transferred to a clean tube with the aid of a disposable pipette, and a 25-μl aliquot was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

The HPLC analysis of I in plasma necessitated the examination of two

separate problems: (a) the development of HPLC operating parameters that would separate I from plasma components without interference, and (b) the detection and quantitation of I at expected plasma levels (100—400 ng/ml) following a 2—4 mg total oral dose.

Initially, the chromatographic process was investigated to determine if a non-extraction sample preparation method similar to the one recently reported by this laboratory for *p*-aminosalicylic acid in plasma would be feasible for the analysis of I [4]. In the method, an initial protein denaturation step is performed using equal volumes of plasma and an organic solvent such as acetonitrile or methanol. After centrifugation, an aliquot of the supernatant fluid is injected into the liquid chromatograph. Retention times and other chromatographic parameters were determined for I and/or I-tetrabutylammonium ion-pair on phenyl and octadecylsilane columns. Analysis of I using a phenyl column with mobile phases of methanol—water in the pH 2—4 range or acetonitrile—water containing 0.01 *M* tetrabutylammonium ion at pH 7 was unsuccessful either due to lack of suitable resolution of I from endogenous plasma components or unacceptable band broadening due to the presence of several ionic species of I. Mobile phases containing methanol—water and/or acetonitrile—water in differing ratios and pH values (4—7 range) with and without tetrabutylammonium ion were used to evaluate the retention of I and/or I—tetrabutylammonium ion-pair on the octadecylsilane column. Acetonitrile—water (30:70) containing 0.01 *M* tetrabutylammonium ion at pH 6 was the most suitable mobile phase on the octadecyl column since it provided adequate chromatographic resolution of I from endogenous plasma components and was compatible with the use of acetonitrile in the sample pretreatment step as plasma protein precipitant. However, I still exhibited excessive tailing.

Concurrent with these preliminary HPLC studies, it was determined that a non-extraction sample preparation method would not be sensitive enough to allow detection of I at the expected plasma levels due to lack of sufficient molar absorptivity of the drug at the concentration available in the injection volume. It was decided to utilize a sample preparation procedure involving solvent extraction followed by a sample concentration step in order to improve the UV detectability of I.

Further HPLC investigations revealed that ion-pairing of I with tris(hydroxymethyl)aminomethane on an octadecylsilane column was a more successful approach to the chromatographic separation of I. The effect of mobile phase composition and pH on the retention time of the ion-pair is shown in Table I. The ion-pairing mobile phase increased the capacity factor (k') for I so that the drug would be adequately resolved from any endogenous plasma components extracted in the assay procedure. Retention time of the ion-pair increased with increasing water content and decreasing pH (4—8 range).

The ion-pair was most propitiously separated using absolute methanol—distilled water (10:90) at pH 6. Fig. 1 shows a typical chromatogram of the separation of lodoxamide and *p*-nitrocinnamic acid (internal standard) in a spiked human plasma sample using tris(hydroxymethyl)aminomethane at pH 6. Under the chromatographic conditions chosen, endogenous plasma constituents do not interfere with the assay.

A flow-rate of 1.0 ml/min (82.74 bar) allowed the separation to be obtained

TABLE I

EFFECT OF MOBILE PHASE COMPOSITION AND pH ON RETENTION TIME OF LODOXAMIDE USING OCTADECYLSILANE COLUMN

Mobile phase*		pH				
		8	7	6	5	4
A		278**	478	684	798	825
		(40)***	(53)	(60)	(71)	(80)
	k'	0.54	1.8	2.8	3.4	3.6
B		192	366	400	441	488
		(30)	(39)	(36)	(36)	(50)
	k'	0.67	1.0	1.2	1.45	1.7
C		188	228	252	261	296
		(21)	(27)	(27)	(29)	(33)
	k'	0.04	0.30	0.40	0.50	0.60

*Solvent composition: A, absolute methanol—distilled water (10:90) containing 0.05 *M* tris(hydroxymethyl)aminomethane; B, absolute methanol—distilled water (20:80) containing 0.05 *M* tris(hydroxymethyl)aminomethane; C, absolute methanol—distilled water (30:70) containing 0.05 *M* tris(hydroxymethyl)aminomethane.

**Retention time expressed as seconds measured as elapsed time between injection and attainment of the chromatographic peak maximum. The eluted peaks were monitored using a 254-nm UV detector.

***Base peak width expressed as seconds.

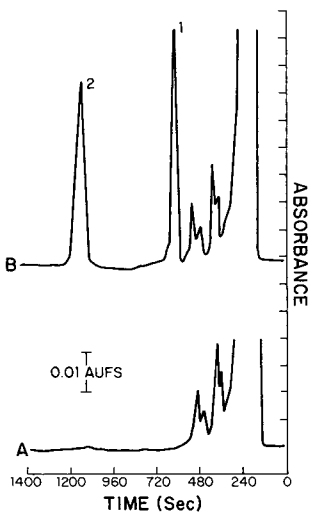

Fig. 1. Typical chromatogram of lodoxamide (1) and *p*-nitrocinnamic acid (2) (internal standard) in a human plasma sample spiked with 200 ng/ml of drug (B). For comparison, a chromatogram of blank plasma (A) is shown. Conditions: column, octadecylsilane (300 mm × 4 mm I.D.); eluent, absolute methanol—distilled water (10:90) containing 0.05 *M* tris(hydroxymethyl)aminomethane adjusted to pH 6 with concentrated phosphoric acid; flow-rate, 1 ml/min; UV detector set at 254 nm.

in approximately 20 min. The void volume of the column was 2.6 ml. A fixed-wavelength detector (254 nm) was suitable for the assay since I showed a broad UV absorption spectrum with a maximum at 249.5 nm. Thus, maximum

detector sensitivity was available for the low concentration levels present in the extracted plasma samples.

A calibration curve for lodoxamide in the anticipated therapeutic concentration range (100—400 ng/ml of plasma) based on a total oral dose of 2—4 mg was performed. The digital determinations of peak heights of drug and internal standard on the chromatograms were determined with an electronic integrator (Spectra-Physics Autolab Minigrator, San Jose, CA, U.S.A.). The ratio of lodoxamide peak height to the height of the internal standard (D/IS) was calculated for each chromatogram. Regression analysis of these data at the various concentrations of drug gave slope, 0.0702; intercept, 0.0037; and correlation coefficient, 0.9955 (n = 11). The minimum detectable quantity of lodoxamide that can be measured using this procedure is 20 ng/ml based upon extraction of drug from plasma. The percent recovery of lodoxamide using the procedure described herein was 98.90 ± 1.60% (mean ± S.D., n = 4).

TABLE II

ANALYSIS OF LODOXAMIDE IN SPIKED PLASMA SAMPLES

Initial concn. (ng/ml)	Conc. found* (ng/ml)	Relative standard deviation (%)	Relative error (%)
150	150.07 ± 4.79	3.19	0.05
300	306.23 ± 3.12	1.02	2.08

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

Human plasma samples containing spiked quantities of lodoxamide in the therapeutic concentration range were chromatographed concurrently with the calibration solutions and the ratios of drug peak heights to internal standard peak heights were calculated. The slope and intercept data from regression analysis for lodoxamide calibration solutions were used to calculate the concentration in the spiked samples: D/IS = (slope × concentration) + intercept. The data in Table II demonstrate the quantitative results obtained from these spiked plasma samples. The utility of HPLC in the assay of lodoxamide plasma levels using ion-pairing with tris(hydroxymethyl)aminomethane is clearly demonstrated with relative error < 5%.

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CHROMBIO. 1708

Note

Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in plasma

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(Received January 21st, 1983)

Diminazene (4,4′-diamidinodiazoaminobenzene diacetamidoacetate) (Berenil; Hoechst, Frankfurt a.M., G.F.R.) is used extensively in the treatment of trypanosomiasis and babesiosis in animals [1—5]. It has also been used in clinical trials in early cases of human sleeping sickness [6].

Several techniques have been reported for the quantitative extraction and determination of diminazene in plasma. These methods include biological assay [7], paper and thin-layer chromatography [8], colorimetry [9], high-performance liquid chromatography (HPLC) [10], gas chromatography—chemical ionization mass spectrometry (MS) [11] and radiometry using the ^{14}C-labelled drug [12]. The bioassay, colorimetric and radiometric methods lack sufficient specificity as they determine the total of all biologically active or radioactive components, and normal plasma may give a significant colour reaction when diazotized. The paper and thin-layer chromatographic methods are not sufficiently sensitive to detect submicrogram concentrations, while the HPLC and MS methods, although sensitive, involve tedious and protracted sample preparation steps, including the reduction of diminazene to 2 mole of 4-aminobenzamidine prior to extraction, derivatization and analysis [10, 11].

In animals treated with diminazene, there may be metabolites which are structurally unknown or closely related to the parent drug or the reduction products. An analytical method suitable for pharmacokinetic studies of diminazene must include an efficient extraction of the parent compound from plasma and other body fluids. The ion-pair extraction method and assay by reversed-phase HPLC described in this paper is specific for the intact diminazene molecule and is suitable for its pharmacokinetic evaluation.

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EXPERIMENTAL

HPLC instrumentation

A Waters Assoc. (Milford, MA, U.S.A.) modular HPLC system was used; it consisted of a constant-flow solvent delivery pump (Model M-6000A) and a WISP injector (Model 710B); separation of injected compounds was achieved by a Radial-PAK CN column (10 cm × 5 mm I.D., 10 μm particle size) fitted with a CN guard column. The column effluent was monitored with a 254-nm detector (Model 440) operated at 2×10^{-2} absorbance units full scale (a.u.f.s.); areas and concentrations of peaks were determined by an on-line computer (Waters Data Module M-730). Mobile phase was pumped at a rate of 0.8 ml min^{-1}. The cartridge (Sep-Pak C_{18}) which was used to extract diminazene from plasma was supplied by Waters Assoc.

Reagents

Berenil and diminazene diaceturate were supplied by Hoechst and imidocarb dipropionate by Burroughs Wellcome (London, Great Britain). Methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, Great Britain) and were of HPLC grade. Triethylamine was supplied by Fluka (Buchs, Switzerland). Orthophosphoric acid was supplied by Koch-Light (Colnbrook, Great Britain). 1-Heptanesulphonic acid was supplied as Pic B-7 reagent by Waters Assoc.

HPLC mobile phase

The mobile phase used for isocratic elution of diminazene and the internal standard consisted of acetonitrile and glass-distilled deionized water (50:50, v/v) to which was added 0.2% of triethylamine. The pH was adjusted to 4.20 with orthophosphoric acid.

Standard solutions

Stock solutions of diminazene diaceturate and the internal standard, imidocarb dipropionate (100 μg ml^{-1} each), were prepared in glass-distilled water. A series of standard solutions (2.5—250 ng of diminazene containing 15 ng of the internal standard per 50 μl injected) were then prepared in methanol–heptanesulphonic acid for use in establishing the linearity of the assay procedure.

Extraction procedure

General concept. The method utilized a cartridge with C_{18} reversed-phase packing material. After applying the sample to the cartridge it was washed under conditions not eluting diminazene and the internal standard. An ion pair was then formed with a suitable counter ion and eluted from the cartridge with a solvent with moderate solvating ability. The HPLC analysis was carried out according to the concept of internal standardization in the plasma sample. The internal standard, imidocarb [3,3′-di(2-imidazolin-2-yl)carbanilide] was selected as it is closely related chemically to diminazene (Fig. 1) and was readily extracted and quantitated by the same method.

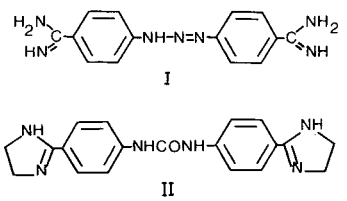

Fig. 1. Diminazene (I) and the internal standard, imidocarb (II).

Sample treatment. To 1.0 ml of plasma were added internal standard solution equivalent to 6 μg of imidocarb base and an aliquot of the stock diminazene solution equivalent to 0.05—5.0 μg ml^{-1} of plasma. The sample was mixed on a Vortex mixer and then half the volume was passed through the Sep-Pak C_{18} cartridge fitted to a Luer Lock syringe. The cartridge was pre-washed with 2 ml of methanol and 5 ml of distilled water. After washing with 2 ml of each of 20% methanol in distilled water and methanol, 1 ml of 0.025 *M* 1-heptanesulphonic acid in 90% methanol in distilled water was used to elute diminazene and imidocarb. The solvent was allowed to drip through the cartridge from the syringe without applying pressure, except the last few drops, which were forced through by air applied from the syringe. The effluent was vortexed and 50 μl were injected into the HPLC system.

Quantitative evaluation and sensitivity

The recovery of diminazene from plasma was determined by comparing the amounts resulting from spiked plasma samples to those obtained from direct injection of similar concentrations of diminazene in aqueous solution.

RESULTS

Chromatograms

Sample chromatograms of blank plasma, of plasma collected from a goat 1 h after an intravenous dose of diminazene (2.0 mg kg^{-1}), of plasma fortified with both diminazene and imidocarb and of diminazene are presented in Fig. 2. Diminazene and imidocarb are well separated with retention times (t_R) of 5.08 and 6.60 min, respectively, and a separation factor (α) of 1.475. Neither the solvent front nor coextracted endogenous plasma compounds interfered with the measurement of either compound. About 2.0 μg ml^{-1} of diminazene and 1.0 μg ml^{-1} of the internal standard gave about 50% full-scale response at 2×10^{-2} a.u.f.s. The peaks were well resolved with peak resolutions (R_s) of 5.32 and 4.38 for diminazene and imidocarb, respectively.

Statistical validation of the method

The results of the intra-assay linearity, precision and sensitivity limit of the method are summarized in Table I. The response of diminazene was linear over the range of concentrations studied (0.05—5.0 μg ml^{-1}) ($r^2 > 0.998$). The recovery from plasma was 92.0 ± 7.8% (S.D.) with a mean coefficient of variation of 6.5%. The limit of quantification was 50 ng ml^{-1} in plasma, without any concentration step.

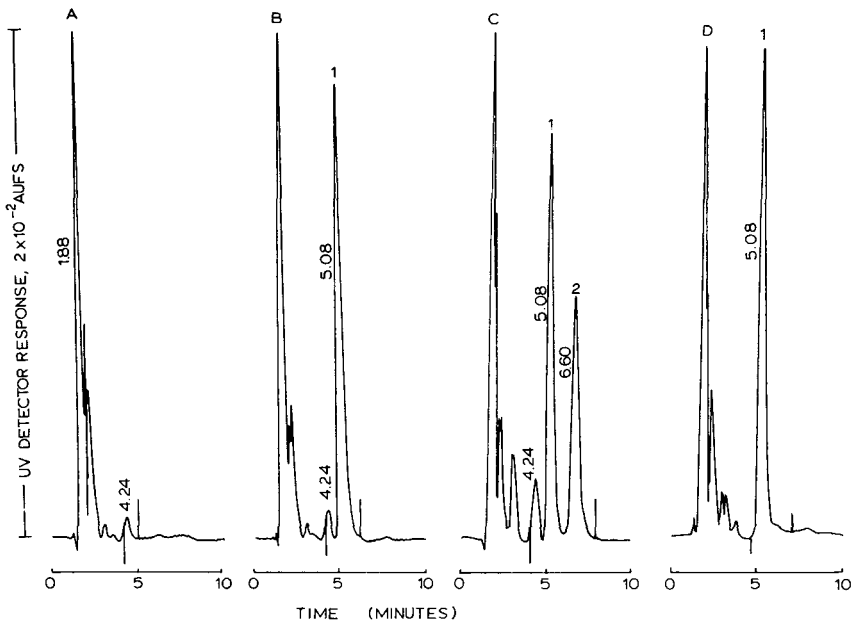

Fig. 2. HPLC traces of (A) blank plasma; (B) goat plasma 1 h after a 2.0 mg/kg intravenous dose of diminazene; the peak corresponds to a concentration of 3.4 μg/ml; (C) plasma fortified with authentic diminazene (3.0 μg/ml) and imidocarb (1 μg/ml); (D) aqueous solution of authentic diminazene (3.8 μg/ml) injected directly into the HPLC system. Peaks: 1, diminazene; 2, internal standard (imidocarb).

TABLE I

LINEARITY OF THE ASSAY PROCEDURE AND RECOVERY OF DIMINAZENE FROM PLASMA

Spiked concentration (μg)	Number of assays	Recovery (mean ± S.D.) (μg)	Recovery (%)
0.05	3	0.039 ± 0.005	77.3
0.10	5	0.079 ± 0.010	79.4
0.20	3	0.18 ± 0.021	91.7
0.30	3	0.27 ± 0.031	88.9
0.40	3	0.36 ± 0.020	90.4
0.50	5	0.47 ± 0.046	93.0
1.0	7	0.97 ± 0.077	96.0
2.0	3	1.94 ± 0.065	96.8
3.0	7	2.92 ± 0.105	97.2
4.0	3	3.94 ± 0.021	98.6
5.0	5	5.13 ± 0.153	102.7
Mean ± S.D.			92.0 ± 7.8

Application of the method

The method is sufficiently accurate to permit the determination of approximately 1/150th the peak plasma level following single therapeutic doses of Berenil in goats. The method has also been used to follow milk and urine levels

of diminazene and its partition between plasma and erythrocytes following single intravenous injections in goats. Commercially available Berenil was dissolved in the sulphonic acid—methanol solution to a nominal concentration, i.e., the manufacturer's value of 3.0 μg ml^{-1}, and injected into the HPLC system for the quantitation of its diminazene content. The amount of diminazene in the formulation, as established by the present method, was 2.97 ± 0.05 μg ml^{-1}. Diminazene (t_R = 5.08 min) was well separated from the phenazone (t_R = 2.84 min) content of Berenil.

DISCUSSION

Sensitive HPLC and MS systems have been utilized by Fouda [10, 11] for determining diminazene, but the intact drug could not be extracted from plasma and had to be split into 2 mole of 4-aminobenzamidine, which were extracted, derivatized and analysed. In the present method, the extraction of intact diminazene from plasma using a cartridge packed with reversed-phase material was found to be simple and quantitative. The plasma extract was sufficiently clean to be injected directly into the HPLC system. Each cartridge was used 7—9 times without loss of efficiency.

Diminazene exhibited a high affinity for the stationary phase of the cartridge as it could not be eluted by water or organic solvents such as methanol, acetonitrile, diethyl ether, or dichloromethane. However, it was readily eluted by a small volume of methanol to which a counter ion was added. This indicated that diminazene is present in aqueous solutions mainly in the ionized form, as can also be predicted by the presence of both primary amine and imino groups in its structure. The traditional approach of solvent extraction in the uncharged form may therefore not succeed, making ion-pair extraction a good alternative [13]. Three counter ions were found to be suitable: (1) a mixture of perchloric acid (0.2 *M*) and sodium perchlorate (0.8 *M*); (2) tetrabutylammonium hydrogen sulphate (0.02 *M*); and (3) 1-heptanesulphonic acid (0.025 *M*) in 90% methanol in water. Eluates of the perchlorate ion pair could not be concentrated by evaporation to dryness at 60° C as they appeared to form volatile derivatives since no degradative products could be detected. The sulphonate ion pair was selected as it produced higher recovery (81% at 5 μg ml^{-1} drug plasma concentration) than did the tetrabutylammonium hydrogen sulphate (74%) when both were compared at a concentration of 0.01 *M* in 90% methanol. The paired-sulphonate eluates could also be concentrated 5—10 times by evaporation to dryness at 60° C without any loss. This concentration step permitted the determination of the diminazene level (0.042 μg ml^{-1}) in goat plasma 2 weeks after an intravenous dose of 2 mg kg^{-1}.

However, recovery at submicrogram concentrations was lower than that at higher concentrations (77.3% at 0.05 μg ml^{-1} compared with 102.7% at 5 μg ml^{-1}). This is attributable to dissociation of the ion pair, which occurs typically at low concentrations [14]. However, the system is still sensitive and flexible enough as the recovery was increased from 40% at 0.05 μg ml^{-1} concentration to 77% by increasing the concentration of the ion pair from 0.01 to 0.025 *M*.

CONCLUSION

The procedure described provides a rapid and sensitive method for quantitating intact diminazene in biological fluids and in pharmaceutical preparations. The extraction step using ion-pair extraction on Sep-Pak C_{18} is short and simple and eliminates the need to split the diminazene molecule. Recovery from plasma is high (92%) and reproducible (mean assay coefficient of variation is 6.5%). Heptanesulphonic acid, perchloric acid—sodium perchlorate or tetrabutylammonium hydrogen sulphate may be used as pairing ions to effect extraction with a solvent with moderate solvating ability. The limit of quantitation in plasma is 50 ng ml^{-1}. The method has been used to follow diminazene levels in goat plasma, whole blood, milk and urine, for 2 weeks after an intravenous dose of 2.0 mg kg^{-1} body weight.

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One of us (Y.O.A.) thanks the entire staff of the Department for the tremendous cooperation he has enjoyed, Drs. Sverre Weberg Teigen and Janneche Utne Skaare for their valuable contribution, and the Vice-Chancellor of Ahmadu Bello University, Professor Ango Abdullahi, for granting him sabbatical leave. The project was supported by the Norwegian Agency for International Development (NORAD) and the Department of Pharmacology and Toxicology.

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CHROMBIO. 1705

Note

High-performance liquid chromatographic method for determination of phenylbutazone in bovine milk with special reference to the fat content in milk

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From the aspect of food hygiene drug monitoring in products from food producing animals is very important, as residue levels might be a potential hazard to the consumer. In Sweden, the nonsteroidal antiinflammatory agent phenylbutazone (PBZ) is used for treating cows suffering from arthritis and laminitis, although knowledge of PBZ residue levels in milk is lacking. Methods for determination of PBZ in different body fluids are reported [1—6], but not in milk from lactating dairy cows. Attempts have been made to study PBZ concentrations in human milk [7, 8] by a method which is well documented for detection of PBZ in plasma or serum [1]. However, this method is inexpedient for determinations of PBZ in cows' milk, due to matrix problems and poor sensitivity. We here describe a method which allows the determination of minute amounts of PBZ in cows' milk by a reversed-phase high-performance liquid chromatographic (HPLC) technique and UV detection.

EXPERIMENTAL

Chemicals

PBZ was obtained form Lääke/Farmos Yhtymä Oy (Åbo, Finland). All other chemicals used were purchased from E. Merck (Darmstadt, G.F.R.). Diethyl ether and ethanol were of spectrographic grade, light petroleum, *n*-hexane and methanol of p.a. grade and ammonia was of suprapure grade.

Apparatus

The HPLC system consisted of a Constametric III pump, a SpectroMonitor

0378-4347/83/$03.00

III variable-wavelength UV detector (LDC, Riviera Beach, FL, U.S.A.) and a Rheodyne 7120 injector (Rheodyne, Berkeley, CA, U.S.A.) with a 100-μl loop. The output signal (0.005 a.u.f.s.) was integrated and calculated by a chromatograph Control Module II (LDC) and plotted on an LDC two-channel thermal printer plotter. A Shimadzu UV-210 A spectrophotometer was used to measure the UV spectra of PBZ.

Chromatographic technique

The column (150 × 4.0 mm I.D.) was packed with LiChrosorb RP-18, 5-μm particle size (E. Merck) and the pre-column (70 × 2.0 mm I.D.) with CO:Pell ODSC-18, diameter 30—38 μm (Whatman, Clifton, NY, U.S.A.).

The mobile phase was prepared from 550 ml of a 0.02 *M* phosphate buffer, pH 7.0, and 450 ml methanol. The flow-rate was set at 1.200 ml/min. Detection of PBZ was achieved at 264 nm. With the system operating at room temperature, the retention time of PBZ was 6 min.

To protect the columns and to keep the retention time constant the system was washed with 50 ml methanol after every 50 injections.

Extraction procedure

To 1 ml of milk were added 1.1 ml of ethanol (99.5%)—ammonia (25%) (10:1) and 2.4 ml of diethyl ether. The tubes were vigorously shaken for 1 min and left for 5 min. Thereafter 2.4 ml of light petroleum (b.p. 40—60° C) were added. The tubes were turned upside down ten times and left for 1 h. The ether phase was carefully sucked off and discarded. To the remaining aqueous phase were added 5 ml of *n*-hexane and 0.4 ml 3 *M* hydrochloric acid. The tubes were shaken for 30 min and centrifuged for 30 min. The organic phase was transferred to a new tube and then evaporated to dryness in a nitrogen stream. The residue was redissolved in 0.5 ml of mobile phase. Of this an aliquot of 100 μl was injected onto the column.

Calibration curve

The calibration curve for determination of PBZ by HPLC was prepared by dissolving PBZ in methanol to a concentration of 0.1 mg/ml. Appropriate amounts of this standard solution were added to milk samples to yield concentrations of 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5 μg/ml. These samples were then treated as described above. The peak areas were plotted versus the concentrations. The calibration curve was linear ($y = 0.977x + 0.011$). The correlation coefficient was 0.9987. From the slope of the calibration curve a reference factor was calculated which was used for automatic calculation of the concentrations of PBZ in the milk samples.

Distribution of PBZ between the fat and aqueous fractions in milk

From milk, spiked with 1 μg/ml PBZ, four whole milk samples were taken. The remaining milk was centrifuged in order to separate the fats from the aqueous fraction and four samples were collected from the aqueous fraction. The concentrations were compared in these two sets of samples.

Determination of PBZ in milk with different fat concentrations

PBZ to a concentration of 1 μg/ml was added to milk from six cows, with fat concentrations ranging from 3.5–4.4%, to Swedish standard milk (3%) and to Swedish low fat milk (0.5%). The PBZ concentrations were determined in the different milk samples and the correlation coefficient between the PBZ concentrations and the fat concentrations was calculated.

RESULTS AND DISCUSSION

As milk is a suspension of fat droplets in an aqueous phase, a drug may be unevenly distributed in the two phases depending on its physiological properties. The distribution of a drug between a complicated fat fraction and a water phase which also contains proteins, makes analysis of drugs in milk a difficult task. The high fat content in milk may interfere with the sampling, the extraction procedure and the HPLC separation. PBZ, which is a lipid-soluble weak acid (pK_a = 4.5), was found to be distributed both in the fat and in the water fraction. In our study, the concentration of PBZ in the water fraction was half the concentration found in the whole milk. Providing there is a fat content of 4% in the milk the concentration of PBZ is 25 times higher in the fat fraction. This means that to have representative samples the milk must be carefully mixed before collection of samples. Moreover, it is known that the fat concentration in milk increases during the course of milking [9] so the foremilk may contain less PBZ than the milk at the end of milking. To collect representative samples for quantitative calculations of PBZ, the udder must be emptied and the obtained milk carefully mixed before sampling.

The methods used by others [7, 8] for detection of PBZ in human milk starts with acidifying a 1-ml sample. When we used this application on cows' milk we found that acidification sometimes led to a gel formation which disturbed further extraction of PBZ. We also found that repeated injections onto the HPLC column of extracted but not fat-reduced samples increased the pressure drop across the columns and decreased the retention times, the separation efficiency and the peak height, probably due to accumulation of milk components on the columns. Similar effects are reported by Wiese et al. [10] after repeated injections of plasma samples only treated with methanol for precipitation of the serum proteins. To minimize the negative effects on the extraction procedure and to improve separation efficiency, the milk fats were reduced according to the initial steps of the international standard method for fat determination in milk [11]. The fats were dissolved and extracted to an ether phase under alkaline conditions leaving PBZ, which is ionised at high pH, in the aqueous phase. This procedure eliminated the problems with the gel formation. However, the defatting step was not sufficient to completely eliminate the components deteriorating the HPLC columns but the negative effects were almost negligible. However, to avoid an extensive pressure drop across the columns, it is recommended that the pre-column should be changed or the system washed after about 50 injections. The decrease in separation efficiency produced during 50 injections was not a problem as the peak area was constant. This was verified by measuring the peak area after injections of standard solutions. If the peak height instead of the area

is used for quantitation, the problems may be more pronounced, even if an internal standard is used, since the decreases in peak heights for substances with different retention times are not necessarily comparable — a fact which we have seen in our laboratory when simultaneously determining chloramphenicol and its monsuccinate in plasma from pigs by an HPLC technique [12]. Such disturbance phenomena must be considered when the internal standard is chosen.

Since the fat content in cows' milk varies among breeds, individuals, nutrition and lactation stages, it is important that an assay monitoring drugs in milk is independent of the fat content. When tested in the method described above, we could not find any correlation (r = 0.016) between the fat content and the measured PBZ concentrations in the milk. Thus, no corrections had to be made for different fat concentrations in the milk samples.

In recent publications [4—6] UV detection of PBZ has been performed at acidic pH and 240 or 254 nm. As can be seen in Fig. 1, PBZ has different absorption maxima at different pH values. The dashed line is the spectrum at pH 7.0 with a maximum at 264 nm ($\epsilon = 2.2 \cdot 10^4$ l mol^{-1} cm^{-1}) and the solid line is the spectrum at pH 3.0 with a maximum at 237 nm ($\epsilon = 1.3 \cdot 10^4$ l mol^{-1} cm^{-1}). This means that the sensitivity is increased if UV detection is performed at neutral pH and 264 nm compared to pH 3.0 and 240 nm.

By assaying ten in vitro samples containing 100 ng/ml PBZ, the recovery was calculated and found to be 89% with a coefficient of variation of 5.7%. A PBZ concentration in milk as low as 20 ng/ml could be readily detected.

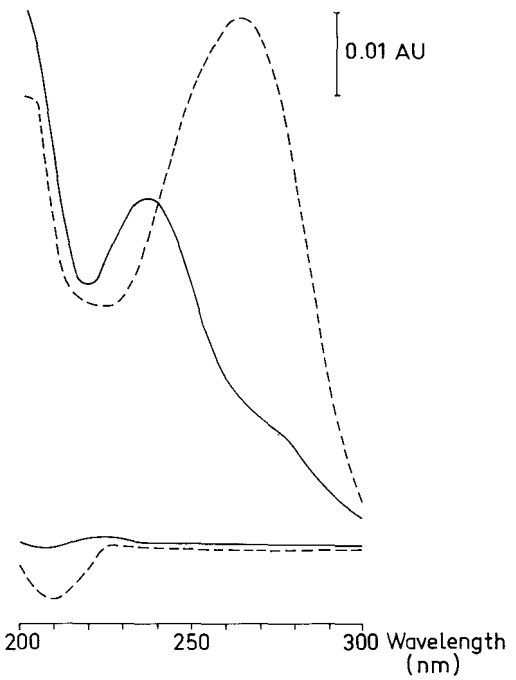

Fig. 1. UV spectra at different pH values for PBZ (1 μg/ml) dissolved in phosphate buffer—methanol (55:45). The horizontal lines show the UV spectra of the solvents. (- - -) pH = 7.0, (—) pH = 3.0.

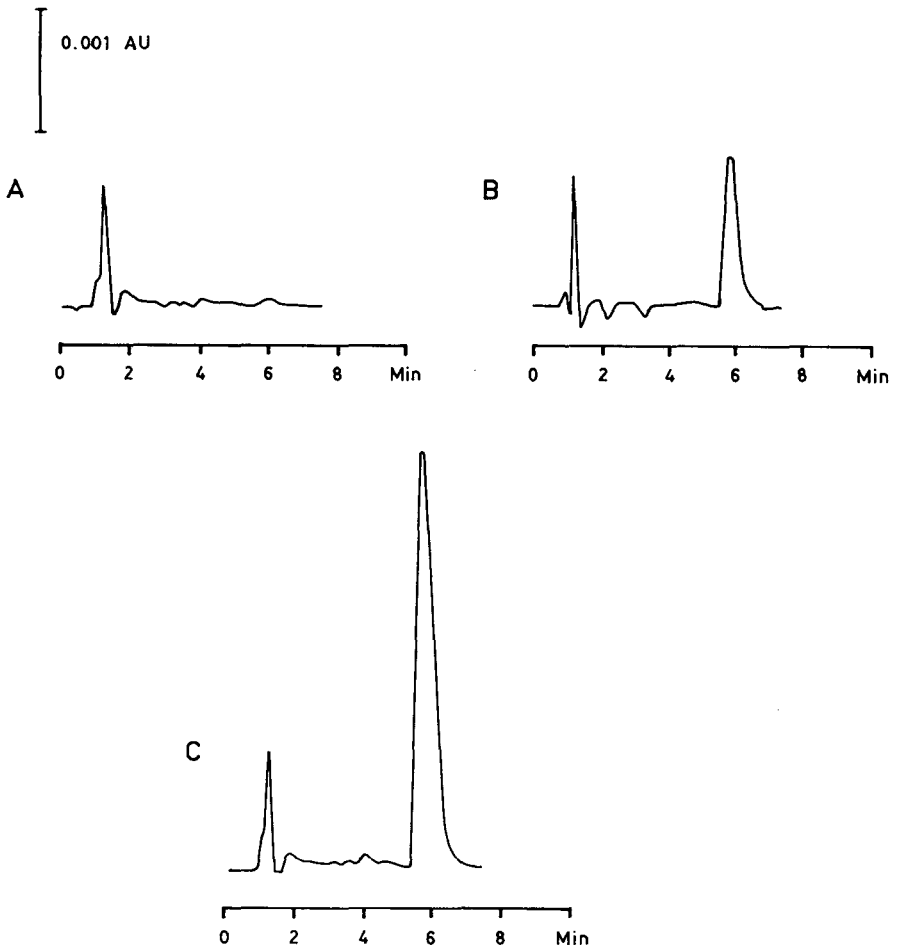

Fig. 2. Chromatograms of milk extract: (A) from blank milk; (B) from milk spiked with PBZ (50 ng/ml); (C) from milk of a cow after administration of 2.5 g of PBZ 16 and 24 h before sampling.

Fig. 2 shows chromatograms obtained by this method. Fig. 2A illustrates milk without PBZ, Fig. 2B milk to which was added 50 ng/ml PBZ and Fig. 2C milk from a cow after administration of 2.5 g of PBZ 16 and 24 h before sampling.

In summary, a method for quantitative determinations of PBZ in cows' milk is described. The extraction procedure used should be generally applicable as a first step in determinations of lipid-soluble weak acids in milk. The method is independent of the fat concentration of the milk samples and permits estimation of minute amounts of PBZ as the HPLC conditions are optimised for highest sensitivity.

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Note

Rapid and simple determination of carprofen in plasma by high-performance liquid chromatography with fluorescence detection

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Carprofen, *rac*-6-chloro-α-methylcarbazole-2-acetic acid (I) (Fig. 1) is a new non steroidal anti-inflammatory agent marketed in Europe as Imadyl [1, 2].

High-performance liquid chromatographic (HPLC) methods have been used for the determination of drugs in blood and plasma based on normal-phase [3] and reversed-phase ion-pair partition [4]; both methods involve extraction of the drug into diethyl ether, evaporation of the extracts to dryness and HPLC with fluorescence detection. HPLC has also been used to determine the stereoselective disposition of carprofen in humans [5].

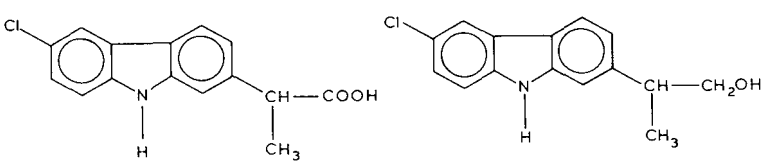

Fig. 1. Structures of compounds I and II.

In order to avoid time-consuming operations (transfer of extracts, evaporation, etc.), we have developed an HPLC method for the determination of I with the use of an internal standard, *rac*-2-(6-chloro-2-carbazolyl)-1-propanol (II) (Fig. 1), which allows the directs injection of the extracts without the evaporation step, the eluent mixture is similar to that described by Puglisi et al. [3].

EXPERIMENTAL

Materials and reagents

The water used for the preparation of solutions was of HPLC grade, produced by a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Methylene chloride and methanol (HPLC grade), acetic acid and sodium acetate (analytical-reagent grade) were obtained from E. Merck (Darmstadt, G.F.R.).

To prepare an acetate buffer (1 *M*), a solution of sodium acetate (1 *M*) was added to 1 *M* acetic acid solution to achieve a final pH of 2.8.

Carprofen (Ro 20-5720) (I) and the internal standard (Ro 21-0134) (II) were of pharmaceutical grade (Hoffmann-La Roche, Basle, Switzerland).

Standard solutions

Stock standard solutions of compounds I and II were prepared by dissolving 10 mg of each compound in 10 ml of methanol. Working standard solutions were prepared from the stock solutions by suitable dilutions with methanol (Table I), and 50 μl were added to plasma as described below. The stock solutions and standard solutions were stored at 0—5°C in amber-coloured volumetric flasks and prepared freshly each week or each day, respectively.

TABLE I

STANDARD SOLUTIONS USED FOR THE ANALYSIS

Standard solution No.*	Compound I (ng per 50 μl)	Compound II (ng per 50 μl)	Expected concentration range of I in unkown (ng/ml)
1	3000	3000	500—7000
2	500	500	100—2800
3	150	150	20—200
4	0	3000	500—7000
5	0	500	100—2800
6	0	150	20—200

*Solutions 1, 2 and 3 are mixed standard solutions to be added to control plasma for instrument calibration (working standard solutions). Solutions 4, 5 and 6 are internal standard solutions to be added to unkown samples.

HPLC equipment and operating conditions

The chromatographic system consisted of a Model 110 constant-flow pump (Altex, Berkeley, CA, U.S.A.), a Model SFM 23 spectrofluorimetric LC detector (Kontron, Zürich, Switzerland) with a flow cell of volume 20 μl, operating at 305 nm for excitation and 375 nm for emission, a Model 7120 sample injector (Rheodyne, Berkeley, CA, U.S.A.) with a loop capacity of 20 μl, an analytical column (25 × 0.4 cm I.D.) of the Hibar type filled with LiChrosorb Si 60 (5 μm) and a pre-column (3 × 0.4 cm I.D.) filled with LiChrosorb Si 60 (7 μm) (E. Merck).

The mobile phase, consisting of methylene chloride—methanol—acetic acid

(98:1:1), was filtered through a 0.5-μm filter before use. The procedure was carried out at a constant flow-rate of 1.5 ml/min.

The fluorescence detector was coupled to a chromatographic computer (Sigma 10; Perkin-Elmer, Norwalk, CT, U.S.A.) for the integration of peak areas and subsequent calculations, using the internal standard method. The chromatograms were recorded on a W+W 600 recorder (Kontron).

Under these conditions, the retention times of compounds I and II were about 4 and 5.5 min, respectively. The entire system was maintained overnight at a flow-rate of 0.2 ml/min of the mobile phase. Before a period of non-use (for example, a weekend) the column had to be treated first with 50 ml of methylene chloride—methanol (98:2), then 50 ml of methylene chloride and finally 50 ml of *n*-hexane, which was the solvent used for column storage.

Procedure for plasma samples

A 50-μl aliquot of internal standard solution (Table I; depending on the expected concentration of the unknown sample) was transferred into a screw-topped test-tube (PTFE-lined caps, Sovirel 13) for each unknown sample; 1 ml of plasma was added and mixed well. For concentrations greater than 5 μg/ml, 0.5 ml or less of plasma was taken, followed by dilution to 1 ml with water.

A separate set of standards was prepared by transferring 50-μl aliquots of mixed standard solutions (Table I) into separate screw-topped test-tubes; control plasma was then added and mixed well.

To all samples, 0.5 ml of acetate buffer was added, then homogenized by slow rotation; 2 ml of *n*-butyl acetate were added and the sample was shaken on a tumble extractor (5 min at 25 rpm).

The samples were centrifuged at 2000 *g* for 5 min at 5°C on a refrigerated centrifuge (Hermle Z 365-K; Kontron); 20 μl of the upper organic phase were then injected into the HPLC system.

Calculation

The Sigma 10 computed the peak areas and the corresponding concentrations of I were obtained according to the internal standard method. The response factor was checked daily from one or more working standard solutions prepared freshly each day according to the expected concentration range of I (Tables I and III).

RESULTS

Linearity

A linear correlation between the peak-area ratio of I:II versus concentration of I was observed in the range 0.02—6 μg/ml of plasma (n = 10). The linear regression analysis performed on theoretical data (amount of I added to control plasma) versus amount found gave the equation $y = 1.0557x - 0.0213$ with a correlation coefficient of 0.9998.

Accuracy and precision

Before performing accuracy experiments using the internal standard method, the absolute recoveries of I and II were obtained from plasma spiked with the

TABLE II

ACCURACY AND PRECISION RESULTS FOR PLASMA SAMPLES SPIKED WITH I

Amount added (ng/ml)	Accuracy (%)	n	Intra-assay precision (%)*	Instrument reproducibility (%)**
3000	95.4	5	0.75	0.65
500	96.2	5	2.20	1.78
250	97.0	5	2.10	2.60
150	97.0	5	2.25	2.50
50	95.7	5	4.80	5.20
20	89.0	5	4.55	4.00

*Refers to the standard deviation obtained after analysing five plasma samples having the same nominal concentration during one day.
**Refers to the standard deviation obtained on chromatographing one of the five plasma extracts six times over a one-day period.

TABLE III

INTER-ASSAY PRECISION EVALUATED FROM THE WORKING STANDARD SOLUTIONS ON DIFFERENT DAYS

Working standard solution No.*	Date**	RF***	mean RF (± S.D.)	C.V. (%)
1	8.23.82	1.020	1.0432 ± 0.01948	1.9
	8.24.82	1.011		
	8.26.82	1.019		
	8.31.82	1.062		
	9. 1.82	1.071		
	9. 3.82	1.054		
	9. 6.82	1.060		
	9. 8.82	1.045		
	9. 9.82	1.038		
	9.10.82	1.052		
3	8.23.82	0.993	1.041 ± 0.0378	3.6
	8.24.82	1.002		
	8.26.82	1.000		
	8.31.82	1.040		
	9. 1.82	1.001		
	9. 3.82	1.082		
	9. 6.82	1.049		
	9. 8.82	1.100		
	9. 9.82	1.071		
	9.10.82	1.074		

*No. 1 contains I and II, both at 3000 ng/ml in plasma. No. 3 contains I and II, both at 150 ng/ml in plasma. The control plasma used for preparing the working standard solutions is the pre-dose sample of the subject involved in the pharmacokinetic study. Therefore ten different pre-dose plasma samples were tested.
**Date of the assay (month, day, year).
***Response factor, $RF = \frac{\text{Peak area (II)} \cdot C\text{ (I)}}{\text{Peak area (I)} \cdot C\text{ (II)}}$; mean of two determinations.

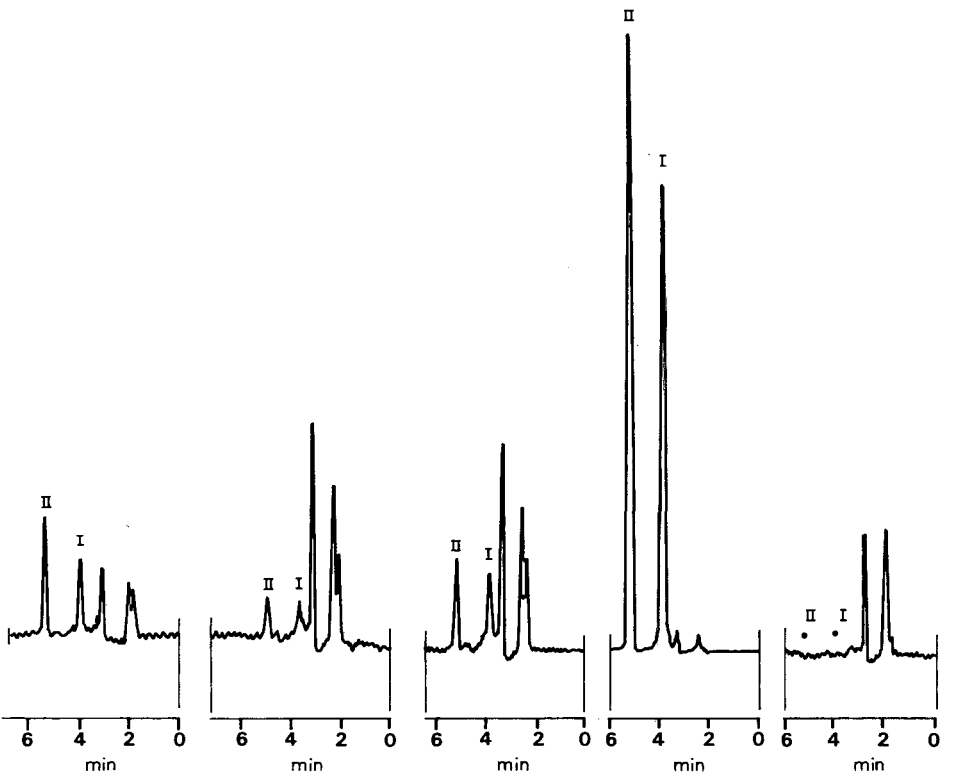

Fig. 2. Chromatogram of control plasma extract. Instrument settings: sensitivity adjustment (coarse) = High HV var; sensitivity adjustment (fine) = 10; variable photomultiplier high voltage adjustment = 4. Recorder range = 100 mV.

Fig. 3. Chromatogram of authentic standards recovered from control plasma, I and II, 3 μg/ml in plasma. Instrument settings: sensitivity adjustment (coarse) = High; sensitivity adjustment (fine) = 5.

Fig. 4. Chromatogram of authentic standards recovered from control plasma, I and II, 50 ng/ml in plasma. Instrument conditions as in Fig. 2.

Fig. 5. Chromatogram of authentic standards recovered from control plasma, I and II, 20 ng/ml in plasma. Instrument conditions as in Fig. 2.

Fig. 6. Chromatogram of plasma extract from a volunteer dosed rectally with 150 mg of carprofen. Plasma sample 36 h after the dose. Instrument settings: sensitivity adjustment (coarse) = High; sensitivity adjustment (fine) = 10. Concentration of I found: 0.21 μg/ml.

two compounds; it was about 96% for both substances over a wide range of concentrations.

Accuracy studies were performed on control plasma spiked with I. The accuracy, defined as (amount found/amount added) · 100, was found to be about 96% over a wide range of concentrations (Table II). Table II also reports intra-assay and inter-assay precision results (Figs. 2—4).

The inter-assay precision results, verifying the long-term reproducibility of the assay, are reported in Table III.

Sensitivity

The detection limit was about 20 ng/ml in plasma, with a signal-to-noise ratio of approximately 3:1 (Fig. 5).

Application of the method to biological specimens

The assay was applied to the quantitation of I in the plasma of volunteers dosed orally and rectally with carprofen (150 mg, single dose) in a cross-over study (Fig. 6).

The plasma concentration–time course curve in a volunteer is shown in Fig. 7. In this example, the high sensitivity of the method was not required, but it could prove necessary for drug determinations 48 h or more after drug administration, or in cases of lower doses (for instance, 50 mg).

The reported example refers to a bioavailability study performed during a drug product development (suppository). The relative bioavailability, in this case (the standard dosage form was oral tablets) was low (about 50%) because of critical factors of formulation; recently further biopharmaceutical studies

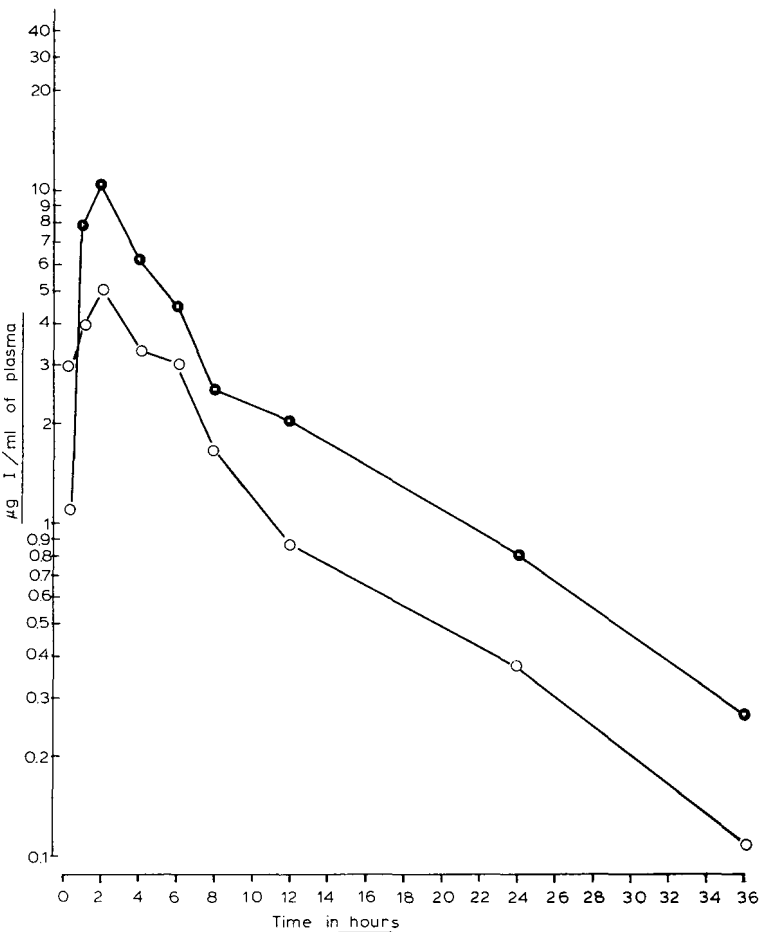

Fig. 7. Drug plasma profile of I in a volunteer dosed orally (•) and rectally (○) with 150 mg of carprofen (between the two administrations a 2-week "wash-out" period was allowed).

have led to a formulation with about 100% relative bioavailability. The pharmacokinetics of carprofen in humans was reported recently [6].

DISCUSSION

The main advantage of the procedure described is associated with the sample preparation stage. Time-consuming transfers of extracts and evaporation are avoided.

Carprofen and the internal standard could be quantitatively extracted from plasma with halogenated solvents, but this necessitated the removal of the upper aqueous layer prior to injection. The pH selected was that which allowed the minimum volume of *n*-butyl acetate to be used (2 ml).

The strong fluorescence of carprofen allowed the injection of diluted plasma extracts which, in turn, led to a relatively long column life.

The method was readily adapted to automatic injection and allowed the rapid analysis of large numbers of samples arising from clinical studies.

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Note

High-performance liquid chromatographic determination of 2-β-D-ribofuranosylthiazole-4-carboxamide in urine and plasma

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(First received October 4th, 1982; revised manuscript received March 16th, 1983)

2-β-D-Ribofuranosylthiazole-4-carboxamide (thiazole nucleoside) was synthesized [1] and evaluated as a potential compound against type I herpes virus, type 3 parainfluenza virus, type 13 rhinovirus and parainfluenza virus and is also reported as an active inhibitor of guanine nucleotide biosynthesis [2]. It was found that the compound exhibited antitumor activity against L1210 and P388 murine leukemias. Lewis lung carcinoma, a neoplasm refractory to many chemotherapeutic agents, was cured by treatment with the thiazole nucleoside over a broad range of doses (25—800 mg/kg) [3]. The drug was shown to arrest cells in the "S Phase" of the cell cycle and also inhibited the synthesis of RNA and DNA in P388 murine leukemia cells growing in culture [4]. The present study is aimed to measure the levels of the thiazole nucleoside in biological samples to be able to understand the fate of the drug in the biological system to permit pharmacological studies.

EXPERIMENTAL

Apparatus

Separation was achieved on a Model 6000A solvent delivery system, Model U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) and a Model SF-720 Spectroflow monitor (Schoeffel Instruments, Westwood, NJ, U.S.A.). Peak areas, retention time, and concentrations based on standards were calculated with a Model 720 system controller and a Model 730 data module (Waters Assoc.). Absorption spectra were obtained using a Gilford Spectrophotometer Model 250 (Gilford Instrument Laboratories, Oberlin, OH, U.S.A.).

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Column

The column used for reversed-phase high-performance liquid chromatography (HPLC) was 300 × 4 mm μBondapak C_{18} (Waters Assoc.). The column was prepacked with 10 μm (average diameter) porous silica particles to which octadecyl groups were covalently bonded through a Si—O—Si bond.

Reagents

The following reagents were used: ammonium formate (Sigma, St. Louis, MO, U.S.A.); trichloroacetic acid (Baker Chemical, Phillipsburg, NJ, U.S.A.); methanol (HPLC grade, Fisher Scientific, Fairlawn, NJ, U.S.A.); glass double-distilled water was used in preparing the buffers and all the other aqueous solutions. All solutions used in the HPLC system were filtered through a membrane filter (average pore size 0.22 μm; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum immediately before use.

Drug

2-β-D-Ribofuranosylthiazole-4-carboxamide was obtained from Mrs. Ruth Davis, National Cancer Institute (Bethesda, MD, U.S.A.).

Buffer preparation

A 5 m*M* ammonium formate solution was freshly prepared in glass double-distilled water, filtered through a membrane filter and degassed under vacuum immediately before use in the HPLC system.

Sample collection and storage

Female B6DZF_1 mice (25—30 g) were used in all the experiments. The animals were housed under natural lighting and fed a standard laboratory chow (Wayne Lab. Animal Diets, Chicago, IL, U.S.A.) ad libitum. Each mouse was administered intraperitoneally (i.p.) with 0.25 ml of thiazole nucleoside in normal saline (20 mg/ml). Urine specimens (24-h) were collected in metabolic cages before and after administration of the drug without preservative and kept frozen. About 0.5 ml of blood was collected at different time intervals (0, 1, 2, 3, 4, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480, 600, 720, 1440 min) from the nasal sinus of the mice using heparinized micro-hematocrit capillary tubes, and immediately centrifuged for 2 min. Plasma was separated and stored in ice until the collection was completed and then kept frozen at −20°C.

Reversed-phase HPLC determination of thiazole nucleoside

The first 24-h urine specimen was diluted 1:100 with glass-distilled water whereas subsequent 24-h specimens were undiluted. All urine specimens were filtered through Millipore membrane filters. Plasma (5 μl) was placed in an Eppendorff microcentrifuge tube and 45 μl of 6% trichloroacetic acid were added to deproteinize the plasma, vortexed with 50 μl of water and centrifuged for 5 min. An aliquot of 20 μl was injected into the chromatograph. The drug was quantitated by its absorbance at 235 nm. The areas under the peaks were integrated with a Data Module, Model 730 and a system controller, Model 720 and the amount of the drug in the biological sample was calculated as follows.

$$\text{amount of drug per ml of sample} = \left[\frac{\text{Area test}}{\text{Area standard}}\right]_{\text{sample}} \times$$

$$\text{amount of standard} \times \frac{\text{ml sample}}{\text{volume of standard}}$$

The peak was identified on the basis of retention time and co-chromatography of the authentic compound with test materials.

Analytical recovery of standard thiazole nucleoside

An equal volume of thiazole nucleoside (2—20 m*M*) was added to urine or plasma and processed as described earlier and an aliquot of 20 μl of solution (10—100 μ*M*) was analyzed by reversed-phase HPLC for quantitation.

Animal experiment

Thiazole nucleoside was injected into three mice in each group. Urine specimens (24-h) of the same day from all three mice were pooled and analyzed for drug content. Plasma levels of drug were analyzed in a total number of 21 mice, which were divided into 7 groups with 3 mice in each group. Blood was drawn 3 times in each mouse, i.e., a total number of 63 times were drawn from 21 mice at various time intervals as described earlier.

RESULTS AND DISCUSSION

UV absorption spectra of standard thiazole nucleoside (200 μ*M*) were obtained at wavelengths ranging from 220—280 nm and the drug had an absorption maximum at 235 nm (Fig. 1).

Quantitation of thiazole nucleoside at different concentrations ranging from 20 pmol to 20 nmol (1 μ*M* to 1 m*M*) showed that linearity was observed

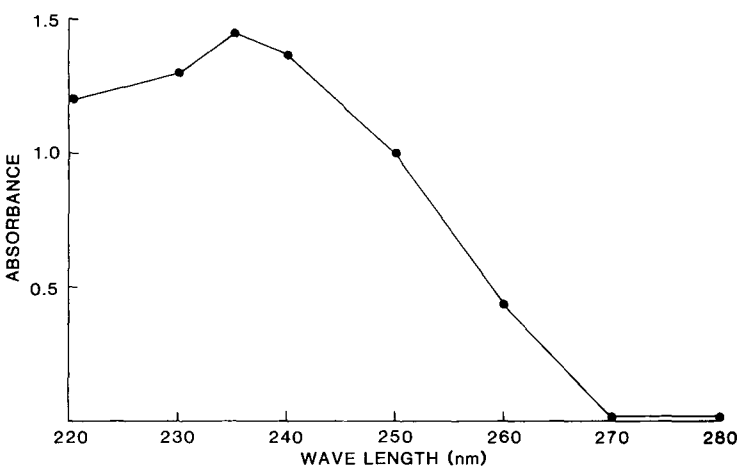

Fig. 1. UV absorption spectra of standard thiazole nucleoside (200 μ*M*) measured in a Gilford Spectrophotometer Model 250.

TABLE I

PRECISION OF HPLC ANALYSIS FOR THIAZOLE NUCLEOSIDE USING AUTHENTIC COMPOUND

Thiazole nucleoside standards (nmol/ml)		
Injected	Recovered*	Average correction value** (%)
100	96.71	3.29
50	49.47	1.06
20	19.77	1.15
10	9.82	1.8
5	4.95	1

*Each value is the mean of three or more determinations.
**Average of the difference between injected and recovered thiazole nucleoside standard of three or more determinations.

in the concentration range from 0.2 to 2 nmol (10—100 μM). The peak areas corresponding to the concentrations of 0.2, 0.4, 1.0, and 2.0 nmol of the authentic drug were $0.8 \cdot 10^6$, $1.6 \cdot 10^6$, $4 \cdot 10^6$ and $8 \cdot 10^6$, respectively, and the high efficiency of the HPLC separation allows an extremely low detection limit of 20 pmol.

The reversed-phase HPLC analytical recovery of standard drug from biological samples gave excellent precision at concentrations easily obtained from small samples of biological fluids (Table I). Repeated injection of drug (0.1—2 nmol) at each of five concentrations gave average correction values (1—3%).

When an aliquot of 20 μl of the filtrate of control urine or plasma was subjected to HPLC analysis, there was no non-specific peak corresponding to the thiazole nucleoside; a similar observation was also found in undiluted control urine. Retention times and peak areas of authentic drug and urine or plasma containing known amounts of drug were found to be the same at isocratic conditions (Fig. 2) and so no attempt was made to purify urine or plasma further for drug assay.

Analysis of the drug in 24-h urine specimens showed that more than 99% of the drug was excreted in the first 24 h and the remaining portion was completely excreted on the subsequent day (Table II). The drug could not be detected in the third 24-h urine specimen.

TABLE II

HPLC ANALYSIS FOR THIAZOLE NUCLEOSIDE IN URINE

Each value is the average of three independent runs. A total dose of 15 mg of drug is given to three mice and the 24-h urine collections of the same day were pooled.

Sample	Excretion of drug (mg)	
	Mean	S.D.
First 24-h urine specimen	14.97	± 0.29
Second 24-h urine specimen	0.023	± 0.004
Third 24-h urine specimen	0	0

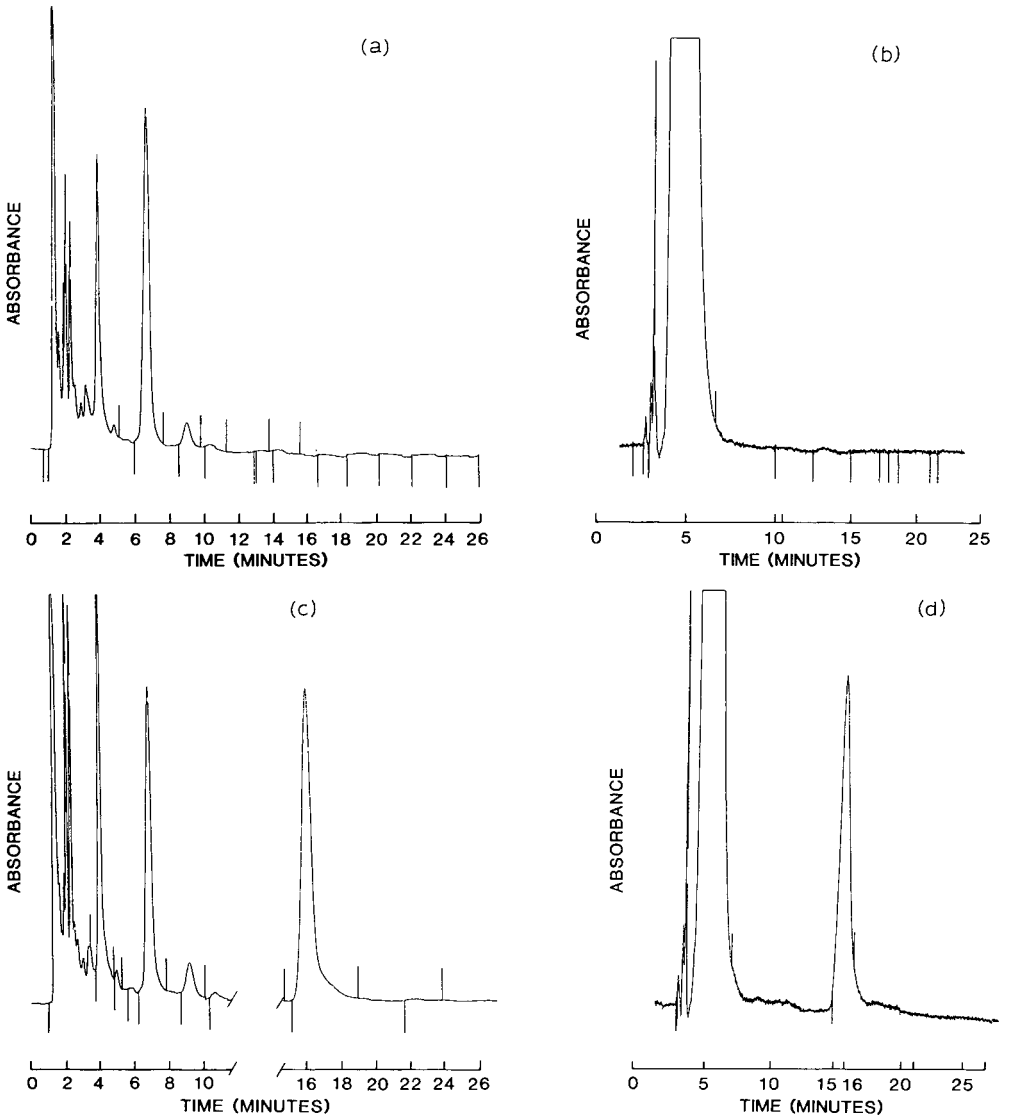

Fig. 2. High-performance liquid chromatograms obtained from mouse samples of (a) control urine; (b) control plasma; (c) urine containing drug; and (d) plasma containing drug. A total dose of 15 mg of drug was injected i.p. into 3 mice and 24-h urine collections of the same day were pooled. Sample, 20 μl equivalent to 0.2 μl or urine or plasma; column, μBondapak C_{18} (300 × 4 mm); buffer, 5 m*M* ammonium formate (native pH); flow-rate, 1 ml/min; detector, 235 nm; 0.01 absorbance; temperature, 24°C.

Analysis of the drug in plasma showed the presence of the drug within 1 min after administration, reaching a maximum by 10 min and steadily decreasing thereafter. The drug could be quantitated up to the 2-h specimen and it had disappeared completely from blood by 3 h (Fig. 3).

The reversed-phase HPLC method described for the separation and quantitation of thiazole nucleoside from biological samples with UV absorption detec-

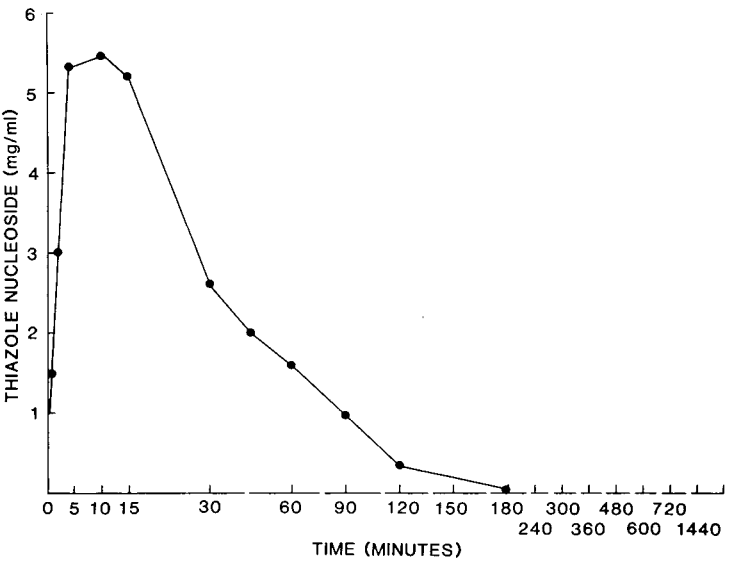

Fig. 3. Time course analysis of drug in plasma of mice using reversed-phase HPLC. A single dose of 0.25 ml of thiazole nucleoside (20 mg/ml) was injected i.p, into a total number of 21 mice, which were divided into 7 groups of 3 mice in each group. Blood was drawn 3 times in each mouse, i.e., a total number of 63 times were drawn from 21 mice at various time intervals as described in Experimental. All other conditions are the same as in Fig. 2.

tion is a rapid, efficient, selective, highly sensitive, non-destructive and quantitative method. There is no method available for the estimation of the drug in the literature. Thiazole is soon to enter Phase I study. The elucidation of this method will throw some light on pharmacology of the drug.

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Note

High-performance thin-layer chromatographic assay for the routine determination of piroxicam in plasma, urine and tissue

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For monitoring the non-steroidal anti-inflammatory agent piroxicam in plasma and serum after administration of therapeutic doses, assays have been described that use spectrophotometric [1, 2] and fluorimetric [1, 3] wet chemistry quantitation and high-performance liquid chromatography (HPLC) with UV detection [4, 5]. Whereas the spectrophotometric method is lacking in both selectivity and sensitivity, the improvement of the wet chemical fluorimetric assay resulted in a limit of detection of 0.2 μg/ml, which was sufficient for first-stage pharmacokinetic investigations. However, the latter method is based on the measurement of 2-aminopyridine, which is formed by the acidic cleavage of piroxicam [1]. As 2-aminopyridine may also be generated by the acidic hydrolysis of some metabolites of piroxicam [6], the selectivity of this assay is questionable. A further disadvantage from a practical point of view is the lack of internal standardization. This drawback also impairs the practical value of HPLC, which was used for pharmacokinetic investigations in animals and man [4].

None of the published methods was described as being applicable to biological materials other than plasma and serum. This paper describes the use of high-performance thin-layer chromatography (HPTLC) for the quantitation of piroxicam in plasma, urine and tissue after administration of therapeutic doses. The assay is based on fluorodensitometric quantitation with internal standardization.

EXPERIMENTAL

Standard and reagents

Piroxicam (Lot No. 503/2148) and six metabolites of piroxicam were

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supplied by Pfizer (Groton, CT, U.S.A.). As the internal standard, piretanide (HOE-118, OP.30) [4-phenoxy-3-(1-pyrrolidinyl)-5-sulphamoylbenzoic acid] was used (a gift from Hoechst, Frankfurt, G.F.R.).

All reagents were of analytical-reagent grade, obtained from Merck (Darmstadt, G.F.R.), and were used without further purification. Plasma was obtained from blood samples to which heparin (25 I.E. per 10 ml of blood) was added before centrifugation at 800 *g* for 10 min. Tissue was frozen with liquid nitrogen and mechanically homogenized with a dismembrator (Braun, Melsungen, G.F.R.).

Extraction procedure

In a 10-ml round-bottomed centrifuge tube, 0.5 ml of plasma was spiked with 0.5 μg of the internal standard (50 μl of a 10 μg/ml solution of piretanide in methanol), 100 μl of 1 *N* hydrochloric acid and 8 ml of dichloromethane were added and the mixture was agitated vigorously with a helix-shaped stirrer for 1.5 min. The tube was then centrifuged for 4 min at 2500 *g*, the aqueous phase was discarded and the organic layer was evaporated to dryness in a water-bath at 40°C under a stream of nitrogen. The dried extracts can be kept at 4°C for at least 2 days without degradation. In our laboratory, the described extraction was fully automated. We used a programmable ASA modular system (Ismatec, Zürich, Switzerland), which represents a sort of assembly line: by means of a central transport module with a capacity of 160 sample tubes the samples are moved along special modules which carry out the various necessary steps such as pipetting, stirring, centrifuging and separation and transfer of phases. The system allows the extraction of at least 320 samples during a working day. The last step of drying the extracts was achieved by simultaneous evaporation of 60 samples, so this did not diminish the total number of samples per day.

Extracts of urine were obtained by the same procedure as described for plasma.

For the extraction of piroxicam from tissue, 0.5 g of the homogenized sample was vortexed together with 0.5 ml of chloroform containing the internal standard and 1.75 ml of 1 *N* sodium hydroxide solution. The mixture was centrifuged for 15 min at 2000 *g* and to the separated aqueous layer 100 μl of 15% hydrochloric acid were added. This solution was transferred to an Extrelut column (Merck). Piroxicam and the standard were eluted with 6 ml of diethyl ether and the organic solution was evaporated to dryness under a stream of nitrogen.

Thin-layer chromatography

The extraction residues were taken up with 50 μl of dichloromethane and 500 nl of the solution were applied to a pre-washed (methanol—25% ammonia solution, 9:1), pre-coated silica gel 60 HPTLC plate (10 × 20 cm) using a Camag-Nanomat sample applicator (Camag, Muttenz, Switzerland). During routine analysis, 35 spots, of which 9 were calibration samples, were placed at opposite sides of the plate. Chromatography was performed in a Camag HPTLC linear developing chamber, in which both sides of the plate were developed simultaneously with chloroform—acetic acid—methanol (18.2:1.0:0.8)

after saturating the chamber for 15 min. The developed plate was dried in a desiccator under vacuum and then dipped into a 10% (v/v) solution of paraffin oil in pentane to enhance the intensity of the fluorescence signals [7, 8]. The air-dried plate was scanned fluorimetrically with a TLC Scanner (Camag) with a 400-nm edge-filter at an excitation wavelength of 366 nm (Hg lamp). The scanner was controlled by a computer (HP 9826) allowing an automatic, peak-maximum-adjusted scan of one plate within 75 min. Simultaneously, the chromatographic peaks were integrated by a computer program. The ratio of the piroxicam to piretanide peak areas was used to determine piroxicam.

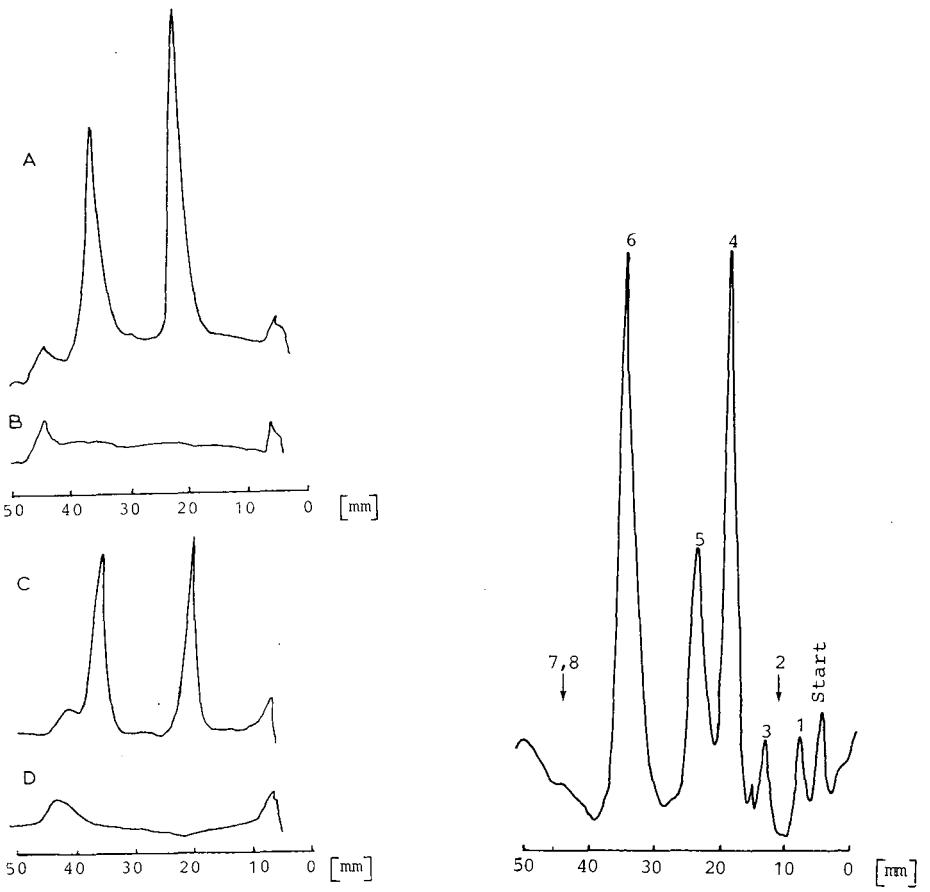

Fig. 1. Thin-layer chromatograms of processed plasma samples. A, Plasma spiked with equal amounts of piroxicam and piretanide; B, blank plasma; C, tissue from a patient under piroxicam medication; D, blank tissue.

Fig. 2. Thin-layer chromatogram of processed plasma samples spiked with piroxicam (6), piretanide (4) and the piroxicam metabolites 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylic acid 1,1-dioxide (1), 1,2-benzisothiazol-3(2H)-one 1,1-dioxide (2)*, 6-methyl-6H-7-oxopyrido[1,2-*a*]pyrimido[5,4-*c*]-1,2-benzothiazine 5,5-dioxide (3), N-(5′-hydroxy-2′-pyridyl)-4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide (5), 2H-1,2-benzothiazine-4(3H)-one 1,1-dioxide (7)* and 2-methyl-1,2-benzisothiazol-3(2H)-one 1,1-dioxide (8)* (* = no fluorescence signal observed).

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms of human plasma and tissue extracts. Piroxicam has an R_F value of 0.65 and piretanide appears at R_F 0.39. Neither the piroxicam nor the internal standard spots are subject to interference from co-extracted endogenous compounds, which is demonstrated by the corresponding blank chromatograms. The assay is specific for metabolites of piroxicam. The chromatogram of a plasma sample spiked with six metabolites before extraction (see Fig. 2) shows sufficient separation from piroxicam and internal standard.

As piroxicam is used in long-term therapy, other drugs might be taken simultaneously with piroxicam treatment by rheumatic patients. Therefore, we tested the interference of this assay with other frequently prescribed drugs. The results are shown in Table I. The piroxicam assay described here is specific for the listed drugs with the exceptions of tinidazole and sulphamethoxypyridazine.

TABLE I

THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR OF SOME COMMON DRUGS

Drug	R_F	Drug	R_F
Piroxicam	0.65	Tinidazole	0.39
Piretanide	0.39	Caffeine	0.45*
Cefoperazone	0.00*	Nordiazepam	0.55
Cimetidine	0.00*	Indomethacin	0.56
Codeine	0.00	Naproxen	0.56
Doxycycline	0.00	Diclofenac	0.57*
Prazosin	0.04	Tolbutamide	0.57*
Metoclopramide	0.05	Flufenaminic acid	0.58
Doxepin	0.11*	Prazepam	0.58
Propranolol	0.13	Diazepam	0.59
Sulphamethoxypyridazine	0.38		

*No fluorescence signal observed.

TABLE II

ANALYTICAL VALUES AND REGRESSION LINES OF FIVE DIFFERENT CALIBRATION GRAPHS

Amount of piroxicam added (μg/ml)	Parameter	Calibration graph No.				
		1	2	3	4	5
0.5	Ratio of peak areas	0.334	0.300	0.363	0.275	0.302
1	(piroxicam:piretanide)	0.723	0.592	0.775	0.622	0.703
2		1.543	1.222	1.538	1.639	2.018
4		2.754	2.439	3.110	2.876	3.086
	Slope	0.689	0.613	0.782	0.750	0.804
	Intercept	0.046	−0.011	−0.021	−0.054	0.020
	Correlation coefficient	0.997	0.999	0.999	0.994	0.979

Calibration graphs were calculated by linear regression of the ratio of the peak area of piroxicam to that of the internal standard as a function of piroxicam concentration. The calibration graphs were linear over the range 0.1–15 μg/ml with correlation coefficients > 0.98. Table II shows the measured values for five calibration graphs that were established on different plates and in different weeks. The variance of the calibration slope is attributed mainly to different concentrations of the internal standard solutions.

With the routine procedure described here, drug levels of 100 ng/ml in plasma or urine are easily quantified. The limit of sensitivity (signal-to-noise ratio = 3:1) can be improved to 50 ng/ml by taking up the dried residue of the extract in only 20 μl of dichloromethane before it is applied to the thin-layer plate.

The recovery after the extraction from plasma was calculated using ten samples each spiked with 10 μg/ml of piroxicam or piretanide. For piroxicam a mean extraction rate of 94.8 ± 2.4% (S.D.) was obtained. Piretanide was extracted from plasma with an average recovery of 85.1 ± 3.4% (S.D.).

In the assay of tissue samples, piroxicam and piretanide were almost completely partitioned into the aqueous sodium hydroxide phase. The recovery from the off-column extraction was 79.5 ± 5.9% (S.D.) for piroxicam and 74.3 ± 4.2% (S.D.) for piretanide.

To test the reproducibility of the assay, spiked plasma samples containing 1, 2 and 5 μg/ml were run in parallel on the same thin-layer plate. The reproducibilities of the measured concentrations for eight samples each were 4.9, 3.8 and 3.1%, respectively.

As a check of the reliability of the analytical results during routine application of the method, spiked piroxicam plasma samples with concentrations

TABLE III

RELIABILITY OF MEASURED PIROXICAM CONCENTRATIONS

The amounts added were unknown to the analyst.

Amount of piroxicam (μg/ml)		Deviation (%)
Added	Found	
0.26	0.25	−3.8
0.28	0.29	3.6
0.42	0.40	−4.8
0.62	0.53	−14.5
0.71	0.81	14.1
1.04	0.94	−9.6
1.32	1.27	−3.8
1.77	1.65	−6.8
1.89	1.89	0
2.50	2.76	10.4
3.08	2.86	−7.1
3.53	3.76	6.5
4.80	4.26	−11.3
9.01	8.95	−0.7

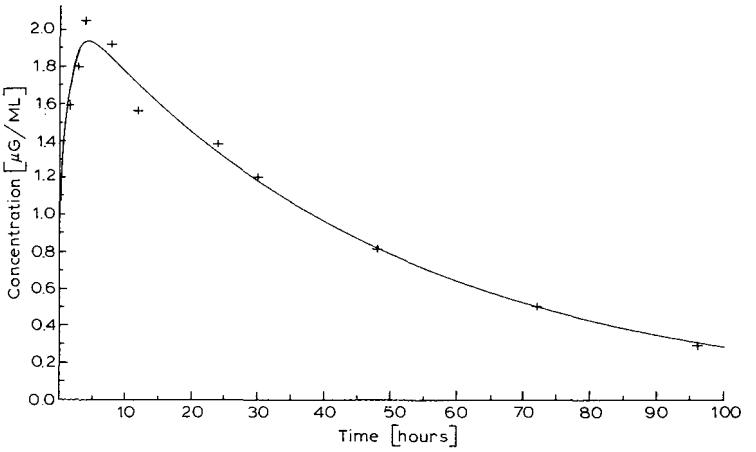

Fig. 3. Plasma concentrations in human subjects after oral administration of 20 mg of piroxicam.

unknown to the analyst were mixed with the original samples. Table III presents the results of assays performed on different days during a period of 6 months. The mean deviation of the measured values from the amounts of the piroxicam added to the test samples was 6.9%. The suitability of this assay procedure is demonstrated by the analysis of plasma samples from a human subject after a single oral dose of 20 mg of piroxicam (see Fig. 3). The half-life of elimination was 33.8 h.

The characteristics of our piroxicam assay make it well suited for routine work in clinical laboratories. Further, it proved to be compatible with extensive automation. With the equipment described, about 150 samples including standards can be handled in a working day, which corresponds to an average of 4.5 min per sample.

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Vol. 276, No. 1

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

WORKSHOP ON LOW DISPERSION LIQUID CHROMATOGRAPHY, AMSTERDAM, THE NETHERLANDS, JANUARY 19–20, 1984

"Microbore", "miniature" and "high speed" are words that are appearing more and more frequently in references to high-performance liquid chromatography. The trend that they reflect is a reduction in volume of all the components in a chromatographic system. The critical implication is that the dispersive contributions of all instrument factors external to the column have to be kept to a minimum. Therefore these techniques are grouped under the name: low dispersion liquid chromatography (LDLC). There are obvious benefits to be gained by applying LDLC: savings in stationary phase and solvent, reduced analysis time, improved absolute detection limit, enhanced separation efficiency, easier compatibility with hyphenated systems, etc.

The forthcoming LDLC workshop will address this topic in detail, with special emphasis on miniaturized columns and particle dimensions, high speed separations, systems for the generation of low flows, small volume injectors, miniaturization of system elements, detectors with small detection volumes and fast response and measurements of dispersion effects.

The workshop will be held at the Free University, in Amsterdam. The workshop language will be English (no simultaneous translation will be offered). The proceedings will be published by Elsevier Science Publishers B.V. as a special issue of the *Journal of Chromatography*, after the usual refereeing procedure. Besides regular lectures, instrument demonstrations and applications will be offered and discussed. A short course on "Low Dispersion LC" will be held at the Free University on January 17 and 18. The deadline for registration is December 15, 1983.

Further information may be obtained from: LDLC Workshop Office, Dept. of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

BIOCHEMISCHE ANALYTIK 84 – INTERNATIONAL CONFERENCE ON BIOCHEMICAL AND INSTRUMENTAL ANALYSIS, MUNICH, G.F.R., APRIL 10–13, 1984

The 9th Conference on Biochemical Analysis will be held in 1984 at the Münchener Messegelände. The breadth of the scientific themes will be considered in 16 half-day symposia. The themes selected by the Scientific Committee may be supplemented by poster demonstrations. Besides the provision of information during the course of the scientific programme, more time than is usual will be allowed for discussion in individual scientific meetings. The conference will be broadened in

the direction of the technical development and field of application by the "Analytica Forum München", at which novelties of industrial development by the technical exhibition will be presented.

Further information may be obtained from: Dr. Rosmarie Vogel, General Secretary, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München, Nussbaumstrasse 20, D-8000 München 2, G.F.R. Tel: (089) 15 30 32; telex 5 216 018 bird d.

THIRD WORKSHOP ON LC–MS AND MS–MS, MONTREUX, SWITZERLAND, OCTOBER 24–26, 1984

The above-mentioned workshop is being organized by the International Association of Environmental Analytical Chemistry and sponsored by instrumental companies and national bodies and will include an exhibition.

The topics will include technical developments in LC–MS and MS–MS with on-line and off-line aspects and applications of these techniques in environmental analysis, clinical analysis and other areas. Subtopics will be introduced by plenary lectures, followed by brief research presentations and posters, and by panel discussions on the state-of-the-art of LC–MS and MS–MS. The proceedings will be published in a special issue of the *Journal of Chromatography*.

For further information on attendance and submission of papers contact: Professor Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

CALENDAR OF FORTHCOMING EVENTS

Aug. 24–26, 1983 Breda, The Netherlands	**4th International Conference on Computing in Clinical Laboratories** Contact: The Secretary of the 4th Conference, Mr. R.C.J. Galle, Stichting Medische Laboratoria, Bergschot 69, 4817 PA Breda, The Netherlands. Tel: (076) 789000.
Aug. 29–Sept. 2, 1983 Bratislava, Czechoslovakia	**4th Danube Symposium on Chromatography and 7th International Symposium "Advances and Application of Chromatography in Industry"** Contact: Professor J. Garaj, Department of Analytical Chemistry, Faculty of Chemical Technology, Jánska 1, 81237 Bratislava, Czechoslovakia. (Further details published in Vol. 235, No. 1.)
Sept. 5–9, 1983 Rome, Italy	**1st Italo-Hungarian Symposium on Spectrochemistry: Environmental Protection and Spectrochemistry** Contact: S. Caroli, Laboratorio di Tossicologia Applicata, Istituto Superiore di Sanità, Viale Regina Elena, 299, I-00161 Rome, Italy.
Sept. 5–9, 1983 Montreux, Switzerland	**43rd International Congress on Pharmaceutical Sciences** Contact: Mr. L.G. Felix-Faure, Administrative Director, International Pharmaceutical Federation, 11 Alexanderstraat, 2514 JL The Hague, The Netherlands.
Sept. 6–9, 1983 Guildford, Great Britain	**5th International Bioanalytical Forum** Contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Surrey GU2 5UL, Great Britain. Tel.: 0483-65324. (Further details published in Vol. 275, No. 1.)
Sept. 6–9, 1983 Heidelberg, G.F.R.	**International Conference on Heavy Metals in the Environment** Contact: Heavy Metals Secretariat, CEP Consultants, Ltd., 26 Albany Street, Edinburgh EH1 3QH, Great Britain. Tel.: 031-557 2478.

Sept. 21–29, 1983 Amsterdam, The Netherlands	**het instrument** Contact: het instrument, Birkstraat 108, Postbus 152, 3760 AD Soest, The Netherlands. Tel. (02155) 18204.
Sept. 22–23, 1983 Cambridge, Great Britain	**Symposium: "Columns in High-Performance Liquid Chromatography"** Contact: Mrs. Annet Pullen, Hewlett-Packard Ltd., Analytical Instrumentation, Nine Mile Ride, Easthampstead, Wokingham, Berks. RG11 3LL, Great Britain. Tel.: (03446) 3100, ext. 3465.
Sept. 25–30, 1983 Philadelphia, PA, U.S.A.	**10th Annual Meeting of the Federation of Analytical and Spectroscopy Societies** Contact: FACSS X Program Chairman, John O. Lephardt, Philip Morris Research Center, P.O. Box 26583, Richmond, VA 23261, U.S.A. (Further details published in Vol. 264, No. 3.)
Sept. 28–30, 1983 Würzburg, G.F.R.	**International Workshop on Analysis of Volatiles: New Methods and Their Applications** Contact: Prof. Dr. P. Schreier, University of Würzburg, Food Chemistry, Am Hubland, D-8700 Würzburg, G.F.R.
Sept. 29–30, 1983 Schliersee, G.F.R.	**Symposium "Chiralität und Aktivität"** Contact: Gesellschaft Deutscher Chemiker, Geschäftsstelle, Postfach 90 04 40, D-6000 Frankfurt am Main 90, G.F.R. Tel.: 0611/7917-366.
Oct. 3–4, 1983 Bad Nauheim, G.F.R.	**Anwender-Kolloquium über die Gaschromatographische Dampfraumanalyse** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, D-6000 Frankfurt am Main 90, G.F.R. Tel.: 0611/7917-366.
Oct. 3–6, 1983 Amsterdam, The Netherlands	**20th Anniversary – International Symposium on Advances in Chromatography** Contact: Professor A. Zlatkis, Department of Chemistry, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 272, No. 2.)
Oct. 10–12, 1983 Tarrytown, NY, U.S.A.	**Capillary Chromatography – 2nd International Symposium** Contact: Professor A. Zlatkis, Department of Chemistry, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 275, No. 1.)
Oct. 17–21, 1983 Neubrandenburg, G.D.R.	**Analytiktreffen 1983: Fortschritte in der Gas- und Flüssigkeits-Chromatographie** Contact: Dr. sc. W. Engewald, Karl-Marx-Universität Leipzig, Sektion Chemie, Leibigstrasse 18, DDR-7010 Leipzig, G.D.R.
Oct. 18–19, 1983 Saarbrücken, G.F.R.	**"Dünnschichtchromatographie – Säulenflüssigkeitschromatographie: Partner oder Konkurrenten?"** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, D-6000 Frankfurt am Main 90, G.F.R. Tel.: (0611) 7917-366.
Nov. 10–16, 1983 Düsseldorf, G.F.R.	**9th International Congress and Exhibition for Instrumentation and Automation (INTERKAMA 83)** Contact: INTERKAMA 83, Düsseldorfer Messegesellschaft mbH, NOWEA, Postfach 32 02 03, D-4000 Düsseldorf 30, G.F.R.

Nov. 14–16, 1983 Monte Carlo, Monaco	**3rd International Symposium on HPLC of Proteins, Peptides and Polynucleotides** Contact: Shirley E. Schlessinger, 400 East Randolph, Chicago, IL 60601, U.S.A. Tel: (312) 527-2011. (Further details published in Vol. 275, No. 1.)
Nov. 16–18, 1983 New York, NY, U.S.A.	**22nd Eastern Analytical Symposium** Contact: Norman Gardner, 73 Ethel Street, Metuchen, NJ 08840, U.S.A. Tel.: (201) 548-7377.
Nov. 24–25, 1983 Lausanne, Switzerland	**Workshop on Handling of Environmental and Biological Samples in Chromatography** Contact: Prof. R.W. Frei, The Free University of Amsterdam, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 275, No. 1.)
Dec. 7–10, 1983 Singapore, Singapore	**Chem Asia '83 Conference** Contact: Singapore Exhibition Services, Ltd., 601 Cathay Building, Singapore 0922, Singapore.
Jan. 19–20, 1984 Amsterdam, The Netherlands	**Workshop on Low Dispersion Liquid Chromatography** Contact: LDLC Workshop Office, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.
March 5–9, 1984 Atlantic City, NJ, U.S.A.	**35th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A.
April 8–13, 1984 St. Louis, MO, U.S.A.	**187th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 10–13, 1984 Munich, G.F.R.	**9th Conference on Biochemical Analysis (BIOCHEMISCHE ANALYTIK 84) & ANALYTICA 84 Exhibition** Contact: Secretary General, Dr. Rosmarie Vogel, Abteilung für Klinische Chemi und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München, Nussbaumstrasse 20, D-8000 München 2, G.F.R. Tel.: (089) 15 30 32 Telex: 5 216 018 bird d.
April 16–19, 1984 New York, NY, U.S.A.	**20th International Symposium on Chromatography** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.
April 29–May 4, 1984 Rio de Janeiro, Brazil	**12th International Congress of Clinical Chemistry, 7th Latin American Congress of Clinical Biochemistry & 12th Brazilian Congress of Clinical Analysis** Contact: 12th International Congress of Clinical Chemistry, Rua Vicente Licinio 95, Tijuca, 20270 Rio de Janeiro, RJ, Brazil.
May 9–11, 1984 Dourdan, France	**4th Weurman Flavour Research Symposium** Contact: J. Adda, Laboratoire de Recherches sur les Arômes, 17 rue Sully, 21034 Dijon Cedex, France.

May 15–18, 1984 Ghent, Belgium	**5th International Symposium on Mass Spectrometry in Life Sciences** Contact: Prof. Dr. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: (091) 21.89.51.
May 20–26, 1984 New York, NY, U.S.A.	**8th International Symposium on Column Liquid Chromatography** Contact: Professor Cs. Horváth, Mason Laboratory, Yale University, P.O. Box 2159, Yale Station, New Haven, CT 06520, U.S.A. (Further details published in Vol. 272, No. 2.)
July 29–Aug. 3, 1984 Washington, DC, U.S.A.	**36th National Meeting of the American Association for Clinical Chemistry** Contact: American Association for Clinical Chemistry, 1725 "K" Street, NW, Washington, DC 20006, U.S.A.
Aug. 21–24, 1984 Colombo, Sri Lanka	**Analytical Chemistry in Development** Contact: Centre for Analytical Research and Development, Department of Chemistry, University of Colombo, Colombo, Sri Lanka; or, Trace Analysis Research Centre, Chemistry Department, Dalhousie University, Halifax, N.S. B3H 4J1, Canada.
Aug. 26–31, 1984 Philadelphia, PA, U.S.A.	**188th National Meeting of the American Chemical Society** Contact: A.T. Winstead, American Chemical Society, 1155 16th Street, NW, Washington, DC 20036, U.S.A.
Aug. 26–Sept. 1, 1984 Cracow, Poland	**EUROANALYSIS V – 5th European Conference on Analytical Chemistry** Contact: Professor Zygmunt Kowalski, Secretary-General, Euroanalysis V, Academy of Mining and Metallurgy, Mickiewicza 30, 30-059 Kraków, Poland. (Further details published in Vol. 261, No. 3.)
Sept. 2–6, 1984 Hradec Králové, Czechoslovakia	**4th International Symposium on Isotachophoresis – ITP 84** Contact: ITP 84, Dr. Z. Prusik, C.Sc., Institute of Organic Chemistry and Bic chemistry, Czechoslovak Academy of Sciences, Flemingovo nám. 2, CS-166 10 Praha 6, Czechoslovakia. (Further details published in Vol. 272, No. 2.)
Sept. 23–28, 1984 Philadelphia, PA, U.S.A.	**11th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies** Contact: R.F. Hirsch, Division of Analytical Chemistry, American Chemical Society, 304 Beach Wood, Orange, NJ 07050, U.S.A.
Oct. 1–5, 1984 Nürnberg, G.F.R.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.
Oct. 8–10, 1984 Tarrytown, NY, U.S.A.	**3rd International Symposium on Capillary Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.
Oct. 24–26, 1984 Montreux, Switzerland	**3rd Workshop on LC–MS and MS–MS** Contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.
Nov. 22–24, 1984 Barcelona, Spain	**14th Annual Symposium on Analytical Chemistry of Pollutants** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E.

Nov. 22–24, 1984 Barcelona, Spain	**3rd International Congress on Analytical Techniques in Environmental Chemistry** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; Telex: 50458 FOIMB-E.
Feb. 25–March 1, 1985 New Orleans, LA, U.S.A.	**36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Biggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A.
April 28–May 3, 1985 Miami Beach, FL, U.S.A.	**189th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
July 1–5, 1985 Edinburgh, Scotland, Great Britain	**9th International Symposium on Column Liquid Chromatography** Contact: J.H. Knox, Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, Scotland, Great Britain.
Sept. 8–13, 1985 Chicago, IL, U.S.A.	**190th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.

NEW BOOKS

Isoelectric focusing: theory, methodology and applications (Laboratory Techniques in Biochemistry and Molecular Biology, edited by T.S. Work and R.H. Burdon, Vol. 11), by P.G. Righetti, Elsevier Biomedical, Amsterdam, New York, 1983, XVI + 386pp., price US$ 80.75 (U.S.A. and Canada), Dfl. 190.00 (rest of world), ISBN 0-444-80498-6 (hardback); US$ 25.50 (U.S.A. and Canada), Dfl. 60.00 (rest of world), ISBN 0-444-80467-6 (paperback).

Morphinomimetic and antagonist drugs (Proc. XIIIth Int. Meeting of Anaesthesiology and Resuscitation), edited by C. Conseiller, M.-T. Cousin, J.-M. Desmonts, P. Duvaldestin, P. Glaser, A. Lienhart, J. Montagne, J.-C. Salamagne, K. Samii, J. Seebacker, Ph. Scherpereel, P. Viars and G. Vourc'h, Excerpta Medica, Amsterdam, New York, 1982, 289 pp., price US$ 63.75 (U.S.A. and Canada), Dfl. 150.00 (rest of world), ISBN 0-444-90327-5.

Side effects of drugs annual 7 – 1983, edited by M.N.G. Dukes, Excerpta Medica, Amsterdam, New York, 1983, 500 pp., price US$ 70.25 (U.S.A. and Canada), Dfl. 165.00 (rest of world), ISBN 0-444-90279-1.

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PUBLICATION SCHEDULE FOR 1983

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1982	J	F	M	A	M	J	J	A	
Journal of Chromatography	252 253/1 253/2	254 255 256/1	256/2 256/3 257/1	257/2 258 259/1	259/2 259/3 260/1	260/2 261/1 261/2	261/3 262 263	264/1 264/2 264/3 265/1	265/2 266	The publication schedule for further issues will be published later.
Chromatographic Reviews					271/1		271/2		271/3	
Biomedical Applications		272/1	272/2	273/1	273/2	274	275/1	275/2	276/1	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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