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CHROMBIO. 349

ESTIMATION OF CATECHOLAMINES IN HUMAN PLASMA BY ION-EXCHANGE CHROMATOGRAPHY COUPLED WITH FLUORIMETRY

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(Received February 2nd, 1979)

SUMMARY

Estimation of catecholamines in human plasma was made by ion-exchange chromatography coupled with fluorimetry.

Catecholamines in deproteinized plasma were adsorbed onto Amberlite CG-50 (pH 6.5, buffered with 0.4 *M* phosphate buffer) and selectively eluted by 0.66 *M* boric acid. The catecholamine fraction was separated further on a column of Amberlite IRC-50 which was coupled with a device for the automated performance of the trihydroxyindole method (epinephrine and norepinephrine) or the 4-aminobenzoic acid—oxidation method (dopamine). One sample could be analysed within 25 min with either method. The lower detection limits were 0.02 ng for epinephrine and dopamine, and 0.04 ng for norepinephrine.

Plasma catecholamine contents of healthy adults at rest were epinephrine 0.07 ± 0.01 ng/ml (n = 19), norepinephrine 0.27 ± 0.03 ng/ml (n = 19) and dopamine 0.22 ± 0.03 ng/ml (n = 26).

The procedure of adsorption and elution of the plasma catecholamines by ion-exchange resin was simple, the simplicity contributing to constant recovery. The catecholamine fraction could be analysed without evaporation of the eluate. The analytical column could be used for the analysis of more than 1000 samples before excessive back-pressure developed. Our method of continuous measurement of plasma catecholamine fulfils clinical requirements.

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INTRODUCTION

Estimation of catecholamines has been performed by, among others, the trihydroxyindole (THI) method [1—3] and the ethylenediamine condensation method [4]. Recently, several improved methods have been established: high-performance liquid chromatography coupled with fluorimetric detection [5—8] or coupled with electrochemical detection [9], high-performance liquid chromatography of fluorescent derivatives of catecholamines [10], gas—liquid chromatography with electron-capture detection [11], gas chromatography—mass spectrometry [12] and radioenzymatic methods [13—18]. These methods enable picogram amounts of catecholamines to be estimated.

With the exception of some of the radioenzymatic methods [15, 18], catecholamines of biological extracts have to be preseparated by adsorption onto an alumina [19], borate gel [20], or cation-exchange column [21—24]. Furthermore, the catecholamine fraction has to be evaporated and dissolved in a small volume for high-performance liquid chromatography or converted into volatile derivatives for gas—liquid chromatography.

However, it has been reported that catecholamines can be eluted from a column of Amberlite IRC-50, buffered at pH 6.0—6.5, by an aqueous solution of boric acid [25—28]. One of the authors reported that the eluates obtained from Amberlite CG-50 can be separated on a column of Amberlite IRC-50 using a borate-containing buffer as eluant simply by pH adjustment [29]. This method was used for the analysis of urinary catecholamines in combination with a modified ethylenediamine condensation method [30], but it was not sensitive enough for the estimation of catecholamines in plasma. In the present work, an automated THI method [31] and 4-aminobenzoic acid—hexacyanoferrate(III) [4-aminobenzoic acid (PABA)—oxidation] method [32] was used for the fluorimetric detection of catecholamines in eluates of Amberlite IRC-50 columns.

EXPERIMENTAL

Reagents

Epinephrine bitartrate, norepinephrine bitartrate and dopamine hydrochloride were purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.). 3-Mercaptopropionic acid was obtained from Aldrich Chemical Company (Milwaukee, Wis., U.S.A.). 4-Aminobenzoic acid and other chemicals were of reagent grade and obtained from Yashima Pharmaceutical Co. (Osaka, Japan). Stock solutions of catecholamines were prepared in 0.01 *N* HCl.

Equipment

A constant-flow pump (Jasco, Model TRI ROTAR) was used to pump buffer through the chromatographic columns. Peristaltic pumps (Atto Corporation, Models SJ-1211 H and L) were used to pump air and reagents into a gas-segmented flow reaction detector. A spectrofluorimeter (Jasco, FP-550) equipped with a flow-cell (square cross-section, inner width 4 mm) was used to measure fluorescence. Samples were injected by motor-driven injector (Kyowa Seimitsu, KUH-6000).

Preparation of Amberlite CG-50 columns

Amberlite CG-50 (type 2) was graded according to size to obtain particles of 90—120 μm. The resin was buffered as described previously [33]. The buffered resin was poured into a tube of 4 mm I.D. with 0.4 *M* phosphate buffer (pH 6.5) and allowed to settle under gravity to a height of 12 cm. The column was closed with a glass-wool plug at the bottom; it had a 10-ml reservoir on the top.

Preparation of the Amberlite IRC-50 column

Amberlite IRC-50 (45—60 μm, Na^+) was prepared and washed as described previously [33]. A suspension of the washed resin was equilibrated at pH 6.3 with a succinic acid solution (0.02 *M*). Then it was washed with eluant A (Table I) and poured into two serially connected chromatographic tubes. The tubes were fitted with column adjusters and then the same eluant was pumped through the columns at a flow-rate of 0.8 ml/min for several hours at 42°. The total height of the resin was 25 cm (10 cm in the shorter column and 15 cm in the longer column). Column B, for the separation of dopamine, was packed in the same way using eluant B (Table I). The resin height was 11 cm.

For the preparation of the eluants see Tables I and II.

Preparation of the catecholamine fraction from human plasma

Heparinized blood was drained into chilled tubes containing 5 m*M* reduced glutathione and centrifuged immediately at 4° at 1500 *g* for 15 min. The plasma was stored at —20°. Frozen plasma (1.0—2.0 ml) was mixed with 1.0 ml of 1 *N* HCl and thawed. Then it was deproteinized by addition of 1.0 ml of 2.0 *M* perchloric acid and centrifuged at 1500 *g* for 15 min. The supernatant

TABLE I

COMPOSITION OF THE ELUANTS

Eluant	pH*	Solutions to be mixed**
A	6.4	1 and 2
B	6.3	3 and 4

*pH was measured at 20° with a pH meter manufactured by TOA Electrics (Tokyo, Japan) using a Model GS-135 electrode.
**Succinate—boric acid—NaOH solutions listed in Table II.

TABLE II

COMPOSITION OF THE SOLUTIONS USED FOR THE PREPARATION OF THE ELUANTS

Solution	Composition (*M*)			
	Succinic acid	Boric acid	Disodium EDTA	NaOH
1	0.08	0.66	0.002	0.3
2	0.08	0.66	0.002	0.1
3	0.12	0.35	0.002	0.3
4	0.12	0.35	0.002	0.2

was transferred to a 20-ml beaker, and the protein precipitate was mixed with 2 ml of 0.6 *M* perchloric acid and centrifuged again. The supernatants were combined, chilled on ice and then 0.5 ml of a solution of 5% (w/v) disodium EDTA and 0.5% (w/v) ascorbic acid was added. The mixture was adjusted to pH 6.2 with 1.0 *M* potassium carbonate. The supernatant was applied to the Amberlite CG-50 column. The potassium perchlorate precipitate was mixed with 2 ml of disodium EDTA solution (pH 6.2, 0.005 *M*) and chilled on ice for several minutes. The washings were also added to the column.

The column was washed with 4 ml of deionized water and with 1.0 ml of 0.66 *M* boric acid; 2.0 ml of 0.66 *M* boric acid solution were used for the elution of the catecholamines. The eluate was collected in a tube with 0.07 ml of 1.0 *N* HCl which contained disodium EDTA (0.005 *M*) and sodium dihydrogen phosphate (0.05 *M*). It was diluted with succinate buffer (0.08 *M* succinic acid, 0.002 *M* disodium EDTA, pH 6.3) to 4.0 ml and stored in a refrigerator until analysis ("sample solution").

Chromatographic separation of samples

One millilitre of the sample solution was applied to column A for the detection of epinephrine and norepinephrine, and 1 ml to column B for the detection of dopamine. Elution of each column was carried out with the corresponding eluant at a flow-rate of 0.7 ml/min. When the back-pressure exceeded 30 kg/cm^2 (column A) or 5—10 kg/cm^2 (column B), the columns were repacked.

Fluorimetric determination

THI method. A gas-segmented flow reaction detector was assembled from commercial parts (Technicon Instruments Co., Tokyo, Japan) and Pyrex coils, as shown in Fig. 1. Pyrex coils were made by winding Pyrex tubes of 4 mm O.D. The eluate from column A was segmented by air, mixed with 0.1% (w/v) hexacyanoferrate(III) and heated at 42° (mixing coil: 15 turns). Finally, it was mixed with a solution of 4 *M* sodium hydroxide and stabilizing agents: 2% (w/v) 3-mercaptopropionic acid, 7.5% (w/v) sodium sulfite and 0.3% (w/v)

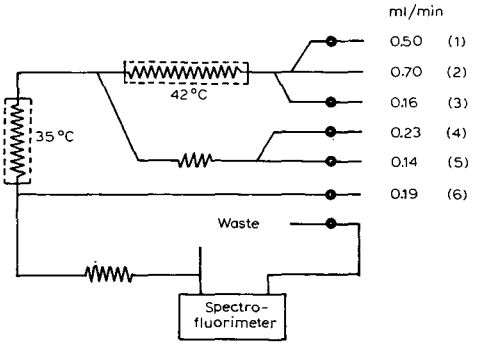

Fig. 1. Schematic diagram of the gas-segmented flow reaction detector using the trihydroxyindole method. Flow-lines: 1, air; 2, column eluate; 3, 0.1% (w/v) hexacyanoferrate(III); 4, 4 *M* sodium hydroxide; 5, stabilizing agents; 6, 6 *M* acetic acid. The pH of the waste was 5.2.

ascorbic acid. After equilibration at 35° (mixing coil: 16 turns), 6 *M* acetic acid was added to the stream and mixed (mixing coil: 8 turns) and debubbled. Fluorescence was measured at 500 nm (excitation at 405 nm). The slit width was 20 nm for excitation and 40 nm for emission.

PABA—oxidation method. We reported previously [32] that dopamine was converted into fluorescent product(s) by oxidation with hexacyanoferrate(III) in the presence of 4-aminobenzoic acid in an alkaline solution. Picogram amounts of dopamine can be measured by this reaction. The fluorimetric detector was assembled as shown in Fig. 2. The eluate from column B was seg-

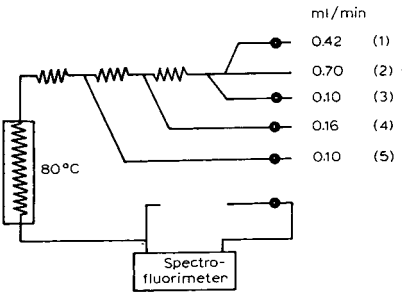

Fig. 2. Schematic diagram of the gas-segmented flow reaction detector using the 4-aminobenzoic acid (PABA)—oxidation method. Flow-lines: 1, air; 2, column eluate; 3, 1% (w/v) solution of 4-aminobenzoic acid in 0.1 *M* disodium hydrogen phosphate; 4, 0.8 *M* NaOH—0.1% (w/v) Brij-35; 5, 0.3% (w/v) hexacyanoferrate(III). The pH of the waste was 9.0.

mented by air and mixed with 1% (w/v) 4-aminobenzoic acid in 0.1 *M* disodium hydrogen phosphate (mixing coil: 7 turns), 0.8 *M* sodium hydroxide containing 0.1% (w/v) Brij-35 (mixing coil: 7 turns), 0.3% (w/v) hexacyanoferrate(III) (mixing coil: 7 turns) and heated at 80° (heating coil: 30 turns). The bubbles were removed from the stream and fluorescence was measured at 520 nm (excitation at 465 nm). The slit width was 20 nm for both excitation and emission.

RESULTS AND DISCUSSION

Chromatographic separation

Pretreatment. Catecholamines in biological extracts were adsorbed onto a weakly acidic ion-exchange resin and selectively eluted by a small volume of boric acid with constant recovery in agreement with previous results [26]. The addition of ascorbic acid and disodium EDTA prevented the oxidation of catecholamines during the procedure. The catecholamines in the eluate were stable below 10° for at least one week with less than 5% decomposition. It is convenient that the catecholamine fraction can be directly applied to the analytical column without evaporation.

Ion-exchange chromatography. As shown in Fig. 3, epinephrine and norepinephrine could be separated within 40 min with eluant A using column A. Eluant A, containing a higher concentration of borate, was used in order to accelerate the elution, but the peak of isoproterenol overlapped that of epinephrine, and the separation of epinephrine from norepinephrine was not

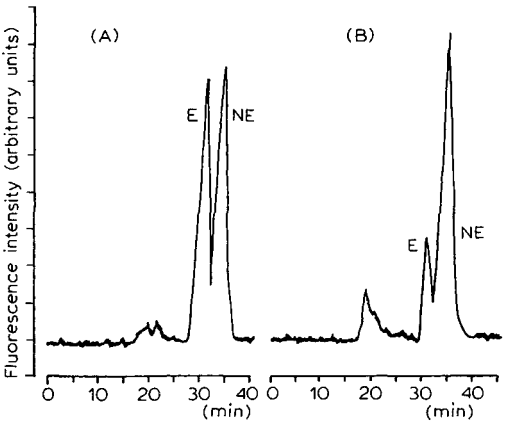

Fig. 3. Elution pattern and fluorimetric determination of epinephrine (E) and norepinephrine (NE). A, Standard sample of epinephrine (0.5 ng) and norepinephrine (1.0 ng). B, Plasma sample.

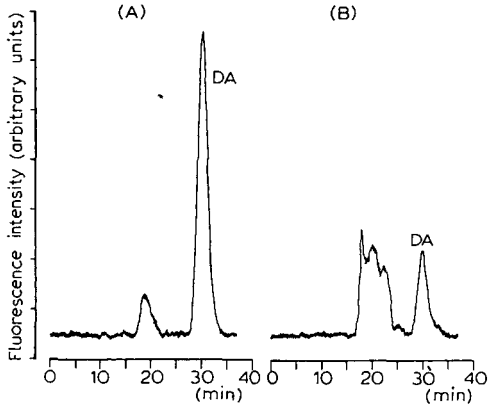

Fig. 4. Elution pattern and fluorimetric determination of dopamine (DA). A, Standard sample of dopamine (1.0 ng). B, Plasma sample.

complete. In a sample containing ten times more norepinephrine than epinephrine, the epinephrine peak was 5% higher than it was expected to be. In a sample with an epinephrine/norepinephrine ratio of 10, the norepinephrine peak was even 20% higher due to tailing. In these cases, repetition using an eluant with lower boric acid concentration is advisable [29].

We used a shorter chromatographic column (column B) to reduce analysis time and to improve sensitivity for dopamine (Fig. 4). The analytical columns (A or B) can be used for the separation of more than 1000 samples.

Fluorimetric determination

Epinephrine and norepinephrine were determined by the THI method and dopamine by the PABA—oxidation method. An automated reaction system for the THI method was assembled according to the description of Martin and Harrison [31] with minor modifications. The reaction was complete within 8 min. Lowering the pH with acetic acid has a favorable effect on stability and

sensitivity of fluorescence [8]. The elution pattern was quite reproducible. Intra-assay variation was 1.6% for epinephrine (1 ng) and 1.5% for norepinephrine (1 ng) (n = 6). Inter-assay variation was 1.5% (1 ng) and 3.7% (0.3 ng) for epinephrine and 2.6% (1 ng) and 6.5% (0.3 ng) for norepinephrine (n = 10). Intra-assay variation for plasma samples was 6.2% for epinephrine and 2.6% for norepinephrine (n = 6). Intra-assay variation for dopamine standard (1 ng) was 1.2% (n = 7) and for plasma samples 2.1% (n = 7). During storage at −20° for three months, the decrease of plasma catecholamine contents was less than 10%.

A linear relationship between peak height and amount of the amines added to the column was obtained over the range of 0.05—500 ng for epinephrine and norepinephrine, and 0.05—100 ng for dopamine. One nanogram of epinephrine and norepinephrine were detected at a signal-to-noise ratio of 100 and 50, respectively. Thus, the lower detection limits were about 20 pg of epinephrine and 40 pg of norepinephrine. One nanogram of dopamine was also detected at a signal-to-noise ratio of 100; so 20 pg of dopamine could be detected. The sensitivity was sufficient for the measurement of catecholamines in 1—2 ml of human plasma.

Over-all recoveries for epinephrine, norepinephrine and dopamine added to plasma are shown in Table III. These recoveries are superior to those obtained by the alumina adsorption method [3, 19]. Probably our method has the following advantages: (1) no troublesome handling of plasma samples; (2) isolation of catecholamines by chromatography on Amberlite CG-50 is simple; and (3) regeneration of Amberlite IRC-50 is not necessary. By continuous measurement of fluorescence, samples could be injected every 25 min before the emergence of epinephrine peak (column A) or dopamine peak (column B).

Normal values are shown in Table IV. Peripheral venous samples of healthy adults at rest were obtained painlessly from an antecubital vein through an indwelling catheter. The mean values of epinephrine (0.07 ± 0.01 ng/ml) and norepinephrine (0.27 ± 0.03 ng/ml) were close to the values obtained by a previous fluorimetric method [3], radioenzymatic methods [13—18] or gas

TABLE III

RECOVERY OF CATECHOLAMINES ADDED TO PLASMA

	Amount added (ng)	Recovery (%, mean ± S.E.)
Epinephrine	0.30	86.7 ± 3.5
Norepinephrine	0.50	95.4 ± 4.0
Dopamine	1.00	86.9 ± 1.5

TABLE IV

VALUES OF PLASMA CATECHOLAMINES OF HEALTHY INDIVIDUALS

	Concentration (ng/ml)	
	mean ± S.E.	Range
Epinephrine	0.07 ± 0.01	0.18—0.03
Norepinephrine	0.27 ± 0.03	0.63—0.06
Dopamine	0.22 ± 0.03	0.60—0.04

chromatography—mass spectrometry [12]. However, the mean value of dopamine (0.22 ± 0.03 ng/ml) was 2—5 times higher than the values obtained by other methods [12, 14, 15]. Even higher than our values were those reported by Pederson and Christensen [17]. Comparison with other fluorimetric estimations of plasma dopamine levels was not possible because no other fluorimetric method was sufficiently sensitive for the measurement of plasma dopamine.

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SELECTED ION MONITORING ASSAY FOR BIOGENIC AMINE METABOLITES AND PROBENECID IN HUMAN LUMBAR CEREBROSPINAL FLUID

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SUMMARY

Details are presented of an improved selected ion monitoring assay for the major biogenic amine metabolites and probenecid in human lumbar cerebrospinal fluid (CSF). The metabolites and probenecid are simultaneously extracted with ethyl acetate from an acidified aqueous phase, and are simultaneously converted to pentafluoropropionyl esters by reaction with pentafluoropropionic anhydride and pentafluoropropanol. The esters of the metabolites are analyzed following a single injection of the derivatized sample onto the gas chromatographic column, while the ester of probenecid is analyzed following a separate injection onto the gas chromatographic column. Quantitation is achieved using for internal standards deuterated analogues of the metabolites and a chemical analogue of probenecid. Data are presented on the concentration of free and conjugated forms of the metabolites in lumbar CSF taken from healthy volunteers.

INTRODUCTION

A primary goal of recent studies on affective disorders in humans has been to compare the concentrations of neurotransmitters and neurotransmitter metabolites in the body fluids of normal and affected subjects. In particular, the concentrations of 5-hydroxyindole-3-acetic acid (5-HIAA), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), the major central nervous system metabolites of serotonin, dopamine, and norepinephrine, respectively, have been measured in urine and lumbar cerebrospinal fluid (CSF) in an attempt to describe the neurochemical abnormalities which may occur during mania, depression, and schizophrenia [1, 2]. In this report we describe details of an integrated selective ion monitoring (SIM) method for measuring the concentrations of 5-HIAA, HVA,

MHPG, 3,4-dihydroxyphenylacetic acid (DOPAC), and *p*-(di-*n*-propylsulfamyl)-benzoic acid (probenecid), in small volumes of human lumbar CSF. This methodology is of note because by simultaneously extracting and derivatizing these compounds the quantification is achieved with the same aliquot of CSF, thus providing a valuable economy of time and materials. We also report, for the first time, the concentration of free and conjugated forms of the metabolites in human lumbar CSF taken from healthy volunteers.

METHODS

Reagents

Pentafluoropropionic anhydride (PFPA) was obtained from Pierce Chemical Co. (Rockford, Ill., U.S.A.); 2,2,3,3,3-pentafluoro-1-propanol was from ICN Pharmaceuticals (Plainview, N.Y., U.S.A.); HVA, MHPG (piperazine salt), and DOPAC were from Calbiochem (Los Angeles, Calif., U.S.A.); 5-HIAA and 5-methoxyindole-3-acetic acid (5-CH_3OIAA) were from Regis Chemical Co. (Chicago, Ill., U.S.A.); sulfatase (arylsulfatase containing some β-glucuronidase, type H-1) was from Sigma; probenecid was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); 5-hydroxyindole-3-acetic-2,2-d_2 acid (d_2-5-HIAA, 98 atom % ^{2}H), ^2H_2O (99.7 atom % ^{2}H), $C^2H_3COO^2H$ (99.5 atom % ^{2}H) and ^{2}HCl (99 atom % ^{2}H) were from Merck (Rahway, N.J., U.S.A.); borane methylsulphide complex was from Aldrich; MHPG sulfate was kindly donated by Hoffman La Roche (Nutley, N.J., U.S.A.) (Ro 4-6028); 3% (w/w) OV-17, 80—100 mesh Gas-Chrom Q, was from Applied Science Labs (State College, Pa., U.S.A.). All other reagents and compounds were of the highest purity available.

Collection of CSF

The subjects were normal males, none of whom had received psychotropic drugs for at least three weeks prior to the study. Having given informed consent, the subjects received two lumbar punctures in the lateral decubitus position, after an overnight fast, before arising from bed. The lumbar punctures were performed at 8.00 a.m. on consecutive days, and prior to the second lumbar puncture the subjects were administered probenecid in six oral doses of 12.5 mg/kg and a final oral dose of 25 mg/kg for a total of 100 mg/kg, given 18, 16, 14, 12, 10, 8 and 3 h before the lumbar puncture. A total of 24 ml of CSF was collected in 6-ml aliquots, from each lumbar puncture, and the second 6-ml aliquot was used for the analyses described here. No CSF specimens were used which contained blood; immediately after collection ascorbic acid was added (1 μmole/ml) and the samples were frozen and stored at $-70°$ until they were analyzed.

Preparation of deuterated internal standards

Deuterated internal standards were prepared by exchange reactions [3, 4] in ^2H_2O—^{2}HCl. Assessment of the isotopic composition and purity of the products was made by combined gas chromatography—mass spectrometry (GC—MS) of the pentafluoropropionyl (PFP) esters (prepared as described below) after a correction was made for the natural isotope satellite ion in the authentic non-deuterated compound. In the case of vanillylmandelic acid (VMA) and MHPG

it was not possible to weigh the deuterated products accurately. In this situation SIM analysis, using the non-deuterated compound as a standard, was employed to determine the concentration of d_3-VMA and d_3-MHPG in solution.

DOPAC. A solution of HVA (300 mg) in ^2H_2O (2 ml) and ^2HCl (2 ml, 38% in ^2H_2O) was heated in a sealed glass tube at 130° for 16 h. After cooling, the mixture was extracted three times with 5 ml of ethyl acetate. The pooled organic phase was shaken with activated charcoal, filtered, and dried under vacuum (< 40°). On standing, the resulting oil crystallized. Recrystallization from an ether—benzene mixture yielded needles which were collected by filtration. The final product contained d_5-DOPAC with less than 1% d_0-DOPAC, and no detectable amount of HVA.

HVA. A solution of HVA (30.5 mg) in ^2H_2O (2 ml), ^2HCl (2 ml, 38% in ^2H_2O) and $C^2H_3COO^2H$ (0.5 ml), was gently refluxed in an open-ended test-tube on a heating block at 190° for 30 min. After cooling, the mixture was extracted three times with 5 ml of ethyl acetate, and the cooled organic phase was taken to dryness under vacuum (< 40°). The resulting oil crystallized on standing, and recrystallization from ether—benzene yielded needles containing d_3-HVA with less than 1% d_0-HVA.

VMA. A solution of VMA (400 mg) in ^2H_2O (6 ml), ^2HCl (10 ml, 20% in ^2H_2O) and $C^2H_3COO^2H$ (2.5 ml) was heated gently under reflux in an open-ended test-tube (15 cm × 2.5 cm) on a heating block at 170° for 15 min. The mixture was then quickly cooled on ice, filtered under vacuum, and extracted three times with 3 ml of ethyl acetate. The pooled organic phase was shaken with activated charcoal and filtered again. The yellow-red filtrate was dried under vacuum (< 40°), and the resulting oil was dissolved in H_2O (1 ml) and loaded onto a Sephadex G-10 column (11.9 × 1.0 cm) suspended in H_2O (Pharmacia, Uppsala, Sweden). As the column was eluted with H_2O, 5-ml fractions were collected. Aliquots (100 μl) of each fraction were removed, dried under a stream of nitrogen, and derivatized (as described below) for examination by GC—MS. Fractions 2, 3, and 4 contained VMA and were pooled and lyophilized to dryness, yielding a yellow powder (weighing approximately 10 mg) which contained d_3-VMA with less than 3% d_0-VMA.

MHPG. To a solution of d_3-VMA (5 mg of the powder containing d_3-VMA) in tetrahydrofuran (1 ml) was added borane methyl sulphide complex (1 ml, containing approximately 5% methyl sulphide). The mixture was left to stand at room temperature with occasional manual agitation for 1 h, after which anhydrous methanol (2.5 ml) was added slowly. After drying under a stream of nitrogen, the resulting clear oil was dissolved in ^2H_2O (2.5 ml). The final solution contained d_3-MHPG with less than 1% d_0-MHPG and a trace of d_3-VMA but no detectable amount of d_0-VMA.

Synthesis of m-(di-isobutylsulfamyl)benzoic acid (DBSB)

DBSB was synthesized from *m*-(chlorosulfonyl)benzoic acid and di-isobutylamine as already described [5].

Sample preparation for unconjugated monoamine metabolites and probenecid

To a 1.0-ml aliquot of CSF are added 100 μl of an aqueous solution containing d_5-DOPAC (10 pmoles), d_3-HVA (400 pmoles), d_3-MHPG (15

pmoles), d_2-5-HIAA (400 pmoles), 5-CH_3OIAA (500 pmoles) and ascorbic acid (100 nmoles). A solution of DBSB (44 nmoles) in 20 μl of 20 m*M* NaOH is then added, and the sample, which is kept on ice (0—4°) during the solvent extraction process, is acidified by the addition of formic acid (4 *N*, 50 μl) and extracted twice with freshly redistilled ethyl acetate (4 ml each time) using centrifugation to separate the phases (3000 *g* for 5 min). The organic phases are pooled in a screw-capped test-tube and taken to dryness under a stream of nitrogen. The dried contents are washed to the bottom of the tube by addition of anhydrous methanol (100 μl), which is evaporated under a stream of nitrogen. The drying process is completed by addition of benzene (50 μl), which is also evaporated under a stream of nitrogen.

Hydrolysis of conjugated forms of the monoamine metabolites

To a 1.0-ml aliquot of CSF containing the internal standards, aryl sulfatase (type H-1, 100 units in 100 μl of 1 *M* sodium acetate buffer. pH 6.2) is added. The solution is incubated on a gently shaking water-bath at 37° for 1 h, after which it is extracted in the same manner as described for the free monoamine metabolites.

Preparation of standard curves

With each set of samples (free and hydrolyzed), a standard curve is prepared for each compound by addition of the internal standards and known amounts of each compound to samples of artificial CSF (containing m*M* concentrations of NaCl (140), KCl (3.4), $CaCl_2$ (1.3), $MgCl_2 \cdot 7H_2O$ (0.54), urea (0.22), NaH_2PO_4 (0.25), Na_2HPO_4 (0.25), glucose (3.33), and $NaHCO_3$ (3.57). These standard samples are then treated in the manner already described for the free and conjugated metabolites.

Derivatization of monoamine metabolites and probenecid [6]

Pentafluoropropionic anhydride (40 μl) and 2,2,3,3,3-pentafluoropropanol (10 μl) are added to the dried ethyl acetate extracts, and the mixture is heated to 75° for 15 min. The tubes are then cooled and dried under a stream of nitrogen. Additional pentafluoropropionic anhydride (50 μl) is added, and the mixture heated to 75° for a further 5 min. Excess reagent is again removed in a stream of nitrogen, and the dried residue is stored at —70° until analyzed.

Gas chromatography

The derivatives of the monoamine metabolites are separated on a silanized glass column (5 ft. × 2 mm I.D.) packed with 3% OV-17 (80—100 mesh) with helium as the carrier gas (25 ml/min) and the injector port at 200°. The column temperature is 115° and is increased at a rate of 6°/min from the time of sample injection. The derivatives of probenecid and DBSB are chromatographed on the same column but with the injector port at 250° and the column isothermal at 200°.

Typically, for analysis of the monoamine metabolites the derivatized sample, prepared from an extract of 1 ml of lumbar CSF, is dissolved in 20 μl of ethyl acetate of which 2—5 μl are injected onto the GC column. For analysis of probenecid and DBSB the remainder of the derivatized extract is dissolved in a

further 100 μl of ethyl acetate of which 1—2 μl are injected onto the GC column.

The gas chromatograph was provided with a toggle switch to divert the solvent from the mass spectrometer; the diverter was switched off 0.5 min after sample injection.

Mass spectrometry

Mass spectrometry was carried out with a Finnigan 3200 GC—MS system integrated with a Finnigan 6000 data system. The parameters of the source are manipulated to maximize the signal at *m/e* 464 (obtained from standard calibration gas: perfluoro-tri-*n*-butylamine). The system was provided with a baffle; ethanol was used as the cooling agent and a cryocool unit (Neslabs Instruments) afforded the refrigeration.

RESULTS

Monoamine metabolites

Under the conditions used, the PFP derivatives of DOPAC, VMA, MHPG, HVA, 5-HIAA, and 5-CH_3OIAA separated from one another on the OV-17 column (Table I). The mass spectra of these derivatives (Table I) are dominated by the presence of relatively intense molecular ions with characteristic losses of 177 and 163 mass units due respectively to losses of the esterified carboxyl function ($OCOCH_2C_2F_5$) and an esterified hydroxyl function ($OCOC_2F_5$). Other characteristic fragments correspond to the loss of 147 and 150 mass units, due presumably to loss of C_2F_5CO and $C_2F_5CH_2OH$, respectively, from the molecular ions.

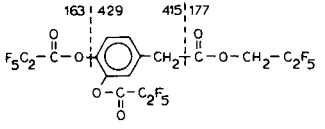
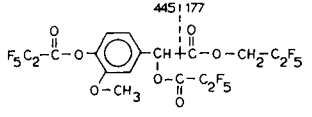
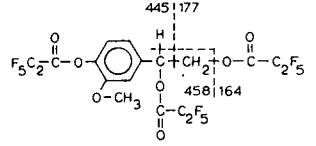
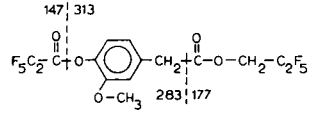
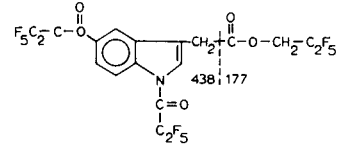
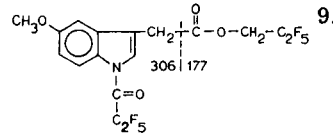
Using the technique of multiple-ion SIM, we have been able to identify DOPAC, MHPG, HVA, and 5-HIAA in extracts of human CSF (Table II). There is also a small peak in the *m/e* 445 ion trace at the retention time of VMA, but we have been unable to confirm the identity of VMA with peaks in other ion traces characteristic of this compound.

The parameters used for quantitative SIM data collection of the PFP derivatives of the monoamine metabolites can be chosen so that DOPAC, VMA, MHPG, HVA and 5-HIAA are analyzed during the same GC run (Table III). In this case, the ion at *m/e* 445 is used for quantitative measurements of both d_0-VMA and d_0-MHPG, and the ion at *m/e* 448 can be used for quantitative measurements of both d_3-VMA and d_3-MHPG. Otherwise, the close proximity of the DOPAC, VMA, and MHPG peaks would not permit the analysis of all compounds in the same GC run. We have not routinely measured VMA concentrations in human CSF samples, however, because the amount present is usually below the sensitivity limit of our mass spectrometer. Therefore, in the 2.5—3.3-min time frame, we monitor the ions at *m/e* 458 and 622 (d_0-MHPG), and *m/e* 461 and 625 (d_3-MHPG), and thereby obtain a stronger signal for the MHPG peak. In this case, the ratio of peak heights in the ion traces at *m/e* 458 and 461 is used in the quantitative measurements.

The degree of completion of hydrolysis of conjugated forms of the metabolites was checked with time-course experiments using 230-μl aliquots of artificial CSF samples obtained from rat brain perfusion experiments [7].

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIMES AND MASS SPECTRAL CHARACTERISTICS OF THE PFP DERIVATIVES OF THE MONOAMINE METABOLITES

Compound	Structure of derivative and probable fragmentation pattern	Retention time (min)*	Major mass spectral ions (*m/e*) and their relative intensities (%)
DOPAC		2.35	592 (44%, M^+), 429 (19%, $M^+ - 163$), 415 (100%, $M^+ - 177$), 387 (28%)
VMA		2.63	622 (33%, M^+), 472 (28%, $M^+ - 150$), 445 (100%, $M^+ - 177$), 417 (28%)
MHPG		3.04	622 (43%, M^+), 458 (100%, $M^+ - 164$), 445 (52%, $M^+ - 177$), 417 (26%)
HVA		3.90	460 (100%, M^+), 313 (13%, $M^+ - 147$), 283 (6%, $M^+ - 177$)
5-HIAA		6.37	615 (30%, M^+), 438 (100%, $M^+ - 177$)
5-CH_3OIAA		9.21	483 (100%, M^+), 306 (88%, $M^+ - 177$)

*Column: 3% OV-17 initially 115° and increased linearly at the time of sample injection at a rate of 6°/min.

TABLE II

SIM IDENTIFICATION OF THE DERIVATIVES OF DOPAC, MHPG, HVA, and 5-HIAA IN EXTRACTS OF HUMAN CSF

Compound	Source	Retention time (min)	Ions monitored (*m/e*) and ratio of peak intensities (%)
DOPAC	Authentic standard	2.34	592 (35%), 415 (100%), 387 (40%)
	Hydrolyzed CSF	2.17	592 (57%), 415 (100%), 387 (35%)
MHPG	Authentic standard	3.04	622 (41%), 458 (100%), 445 (53%), 417 (25%)
	Unhydrolyzed CSF	3.00	622 (51%), 458 (100%), 445 (58%), 417 (27%)
	Hydrolyzed CSF	2.90	622 (51%), 458 (100%), 445 (59%), 417 (27%)
HVA	Authentic standard	3.90	460 (100%), 313 (25%), 283 (29%)
	Unhydrolyzed CSF	3.93	460 (100%), 313 (26%), 283 (31%)
	Hydrolyzed CSF	3.82	460 (100%), 313 (27%), 283 (33%)
5-HIAA	Authentic standard	6.37	615 (27%), 438 (100%)
	Unhydrolyzed CSF	6.29	615 (27%), 438 (100%)

TABLE III

PARAMETERS USED DURING QUANTITATIVE SIM DATA COLLECTION FOR DOPAC, VMA, MHPG, HVA, AND 5-HIAA

Time after sample injection (min)	Ions monitored (*m/e*)	Compound	Ratio of peak heights used in the quantitation
1.5—2.5	415, 592	DOPAC	592/597
	420, 597	d_5-DOPAC	
2.5—3.3	445, 622	VMA	445/448
	448, 625	d_3-VMA	
	445, 622	MHPG	445/448
	448, 625	d_3-MHPG	
3.3—5.3	460	HVA	460/463
	463	d_3-HVA	
5.3—7.3	438, 615	5-HIAA	438/440
	440, 617	d_2-5-HIAA	

Using the Sigma H-1 enzyme preparation (which is a mixture of aryl sulfatase and β-glucuronidase) we found that the concentration of the free forms of DOPAC, MHPG, and HVA in the samples increased during the first hour of the incubation and thereafter remained constant.

Deuterated internal standards which were carried through the extraction and derivatization procedure were found to contain the same distribution of deuterium as the standards which had been converted directly to the PFP derivatives. This result indicates no loss of deuterium occurred by back-exchange with hydrogen during the extraction process.

Complete disappearance of DOPAC was found to occur when solutions of artificial CSF (250 μl) containing DOPAC (24 and 48 pmoles) were incubated for 2 h at 37° prior to extraction and derivatization. No detectable amount of breakdown of DOPAC occurred, however, when ascorbic acid was added to the CSF (1 μmole/ml of CSF) and the solutions were kept at 0° for 2 h.

The recovery of 5-HIAA (500 pmoles) during ethyl acetate extraction from solutions of artificial CSF (1.0 ml) in the absence of added 5-CH_3OIAA, was found to be 5%, but the recovery was increased to 37% by the addition of 100 μg of 5-CH_3OIAA to the artificial CSF. Use of ethyl acetate that had not been freshly redistilled immediately before the extractions resulted in variations between replicate samples in the intensity of the signals obtained for DOPAC and 5-HIAA. This variation was avoided by redistilling the ethyl acetate immediately before use. Variability between replicate samples in the intensity of the peaks for DOPAC, MHPG, HVA and 5-HIAA was also obtained when no care was taken to dry the ethyl acetate extracts completely prior to derivatization. This source of variability was overcome when, prior to derivatization, the residue remaining after the ethyl acetate was blown off in the nitrogen stream was first washed to the bottom of the tubes with methanol then dried in a nitrogen stream, and then dried again in a stream of nitrogen after addition of benzene. Using optimized conditions, the recovery of the monoamine metabolites during extraction was checked by adding DOPAC (540 pmoles), VMA (40 pmoles), MHPG (220 pmoles), HVA (440 pmoles), and 5-HIAA (500 pmoles) to 1.0 ml of artificial CSF. Percentage recoveries, calculated by

comparison with the peak intensities obtained from the same amount of compound which was derivatized directly, were 31%, 75%, 79%, 80%, and 40% for DOPAC, VMA, MHPG, HVA, and 5-HIAA, respectively.

In the concentration ranges for DOPAC, VMA, MHPG, HVA, and 5-HIAA of 2—200, 1—100, 5—140, 6—1400, and 5—1300 pmoles, respectively, the standard curves are linear. Inverse linear regression analysis has been used to determine the concentrations of DOPAC, MHPG, HVA, 5-HIAA, and probenecid in human lumber CSF taken before and after probenecid administration (Table IV).

Probenecid

Details of the procedure used in the SIM analysis of probenecid have been presented elsewhere [5], so only a brief synopsis will be given here. The PFP derivatives of probenecid and DBSB elute from the GC column with similar retention times (Table V). The mass spectra of these derivatives are characterized by weak molecular ions (< 1%) with base peaks at *m/e* 388 and 402 for probenecid and DBSB, respectively, corresponding to losses of C_2H_5 and C_3H_7 from the molecular ions (Table V). The quantitative assay is based on recording, in the SIM mode, the peaks in the *m/e* 388 and 402 ion traces. In the concentration range of 4—100 nmoles, the standard curve is linear, and inverse regression analysis has been used to determine the concentration of probenecid in lumbar CSF of patients given an oral load of 351 nmoles/kg of the drug (Table IV).

DISCUSSION

We have described a sensitive SIM assay for the quantitation of DOPAC, VMA, MHPG, HVA, 5-HIAA, and probenecid in 1.0-ml aliquots of human lumbar CSF. The method, which employs a chemical analogue as an internal standard for probenecid and deuterated analogues as internal standards for all other compounds, utilizes the fact that the chemical properties of DOPAC, VMA, MHPG, HVA, 5-HIAA, and probenecid permit their simultaneous extraction and derivatization in adequate yield for SIM analysis.

The mass spectral data presented (Table II) verify the occurrence of MHPG, HVA, and 5-HIAA in human lumbar CSF. The absence of significant amounts of other compounds co-eluting with these compounds validates the SIM procedures used for their quantitation in extracts of human lumbar CSF. In derivatized extracts of hydrolyzed and unhydrolyzed human CSF DOPAC eluted in close proximity to a compound which contributed a significant signal to the *m/e* 415 and 387 ion traces. This made it difficult to measure accurately the intensity of the DOPAC peak in these two ion traces and accounts for the differences obtained (Table II) in the relative intensities of the peaks in the 592, 415, and 387 ion traces between authentic DOPAC and DOPAC in human CSF samples. The *m/e* 592 (d_0-DOPAC) and 597 (d_5-DOPAC) ion traces, however, were not confused with extraneous peaks and the intensities of the peaks in these ion traces have been used in the quantitative measurements.

Sensitive and reliable SIM assays for measuring the concentrations of several catecholamine metabolites but no serotonin metabolites in the same sample of CSF, using different extraction procedures and different

TABLE IV

MEAN CONCENTRATIONS (± S.E.) OF COMPOUNDS IN HUMAN LUMBAR CSF BEFORE AND AFTER PROBENECID ADMINISTRATION

Subjects were 23 normal males: age range 22—69 years; mean age 43 years; mode 20—30-year age bracket.

Compound	Form of metabolite			Conjugated (percentage total)
	Free	Total	Conjugated	
Before probenecid administration				
DOPAC (pmoles/ml)	2.38 ± 0.22 (21)*	3.80 ± 0.31 (18)	1.41 ± 0.54 (17)***b	37
MHPG (pmoles/ml)	48.40 ± 2.70 (23)	53.10 ± 3.30 (22)	2.40 ± 0.80 (22)***c	5
HVA (pmoles/ml)	239.50 ± 20.10 (23)	241.10 ± 20.20 (22)	1.40 ± 3.20 (22)	0.6
5-HIAA (pmoles/ml)	153.90 ± 13.40 (23)	Not done		
After probencid administration**				
DOPAC (pmoles/ml)	21.00 ± 4.41 (10)	22.00 ± 2.85 (12)	0.60 ± 0.84 (9)	2.7
MHPG (pmoles/ml)	59.51 ± 4.46 (13)	62.83 ± 4.57 (13)	3.32 ± 1.47 (13)***a	5.3
HVA (pmoles/ml)	957.64 ± 75.44 (14)	961.04 ± 75.93 (14)	3.00 ± 11.10 (44)	0.3
5-HIAA (pmoles/ml)	608.95 ± 53.66 (14)	Not done		
Probenecid** (nmoles/ml)	47.80 ± 5.4 (12)	Not done		

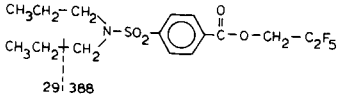
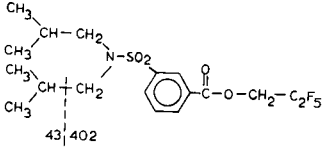
*Figure in parentheses refers to the number of subjects whose CSF concentrations were used in the calculation of each mean and standard error.

**Subjects were administered probenecid at 100 mg/kg (351 μmoles/kg) in six divided oral doses of 12.5 mg/kg and a final oral dose of 25 mg/kg, taken 18, 16, 14, 13, 10, 8 and 3 h, respectively, prior to the lumbar puncture.

***Significantly greater than zero using a one-tailed Student's t-test. $^{a}p < 0.025$; $^{b}p < 0.01$; $^{c}p < 0.005$.

TABLE V

GAS CHROMATOGRAPHIC RETENTION TIMES AND MASS SPECTRAL CHARACTERISTICS OF THE PFP DERIVATIVES OF PROBENECID AND DBSB

Compound	Structure of derivative and probable fragmentation pattern	Retention time (min)*	Major mass spectral ions (*m/e*) and their relative intensities (%)
Probenecid		1.89	417 (<1%, M^+), 388 (100%, $M^+ - 29$)
DBSB		2.00	445 (<1%, M^+), 402 (100%, $M^+ - 43$)

*Column: 3% OV-17 isothermal at 210°.

derivatives than those used in this report, have been previously described 6 by Karoum and others [4, 8, 9]. Swahn et al. [10] have described a SIM method for the simultaneous extraction and derivatization of the catecholamine metabolites MHPG and HVA and the serotonin metabolite 5-HIAA from human CSF. We have improved the sensitivity of this method for 5-HIAA and have extended the assay to include the additional catecholamine metabolites DOPAC and VMA and the drug probenecid. By adopting some relatively simple procedural changes we have also been able to improve the reliability of the assay for DOPAC, MHPG, HVA and 5-HIAA.

In order to obtain high recoveries of DOPAC it was found essential to maintain an ascorbic acid concentration of 1—2 m*M* in the aqueous phase during the extraction process. Also, to avoid large losses of DOPAC, we found it necessary to reduce the time of hydrolysis of the conjugated metabolite. In contrast to described procedures [4, 10—12], 1 h at 37° with the aryl sulfatase—β-glucuronidase enzyme preparation proved to be sufficient to hydrolyze completely the conjugates in samples of rat brain perfusate. The concentrations of conjugated forms of DOPAC, MHPG, and HVA in rat brain perfusion fluid is much greater than the concentration of these compounds in human CSF [7]. Therefore, if the conjugates in human CSF are hydrolyzed with equal efficiency with the H-1 enzyme preparation, 1 h at 37° is sufficient to complete hydrolysis of conjugated forms of these compounds in human lumbar CSF.

In quantitating the conjugated metabolites, the approach we have taken has been to measure free and total metabolite concentrations in separate aliquots of CSF, and thus to determine the concentration of the conjugated metabolite by difference. An alternative approach used by others [4, 11, 12] has been to measure conjugated metabolite concentrations directly in samples of CSF. This has been done by first removing the free metabolites by extraction, and then hydrolyzing the remaining conjugated metabolite. This approach suffers from the disadvantage of measuring either artifactually high conjugated metabolite concentrations, if the free metabolite is not completely removed by extraction, or artifactually low conjugated metabolite concentrations, if a significant proportion of the conjugated metabolite is removed during extraction.

Bertilsson et al. [13] found that the addition of 11 nmoles of 4-hydroxyindole-3-acetic acid to a 2-ml aliquot of CSF improved the recovery of 5-HIAA. In the work reported here the recovery of 5-HIAA was found to be significantly improved by the addition of 500 pmoles of 5-CH_3OIAA to the samples prior to extraction, and by using freshly redistilled ethyl acetate for the extractions. The use of 5-CH_3OIAA benefits from the advantage that the PFP derivative elutes after the 5-HIAA derivative and thus does not confound measurement of the 5-HIAA peak intensity.

In addition to their satisfactory GC and mass spectral characteristics and ease of preparation, the PFP derivatives of the metabolites and probenecid were all found to be relatively stable. When stored dry, they are stable for at least two weeks at −70°.

Takahashi et al. [9] have previously reported the concentrations of VMA, HVA and isohomovanillic acid in human lumbar CSF taken from healthy controls, but neither the concentrations of DOPAC, MHPG, and 5-HIAA in

lumbar CSF taken from healthy control subjects before probenecid administration, nor the concentration of DOPAC, MHPG, HVA, 5-HIAA and probenecid in lumbar CSF from healthy control subjects taken after probenecid administration have been previously reported. We report concentrations of the free forms of DOPAC, MHPG, HVA, 5-HIAA and probenecid in lumbar CSF taken from healthy control subjects, before and after probenecid administration, which are within the concentration ranges reported for these compounds in lumbar CSF taken from non-psychotic hospitalized patients [4, 10, 11, 13]. We have also demonstrated the presence of small but significant amounts of conjugated forms of DOPAC and MHPG in lumbar CSF, which is consistent with previous reports [11, 12, 14, 15].

ACKNOWLEDGEMENTS

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CHROMBIO. 355

URINARY EXCRETION OF METHYLATED PURINES IN MAN AND IN THE RAT AFTER THE ADMINISTRATION OF THEOPHYLLINE

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SUMMARY

Chromatographic characteristics of urinary metabolites of theophylline were studied by two-dimensional thin-layer chromatography, high-performance liquid chromatography and gas chromatography—mass spectrometry. Quantitative data for the urinary metabolites of theophylline in asthmatic children are given. It was shown that 1,3-dimethyluric acid is the predominant excretory product. In addition, smaller amounts of 1-methyluric acid, 3-methylxanthine and unchanged theophylline were found.

Excretory patterns after theophylline ingestion before and during the administration of allopurinol in asthma patients and in rats suggest the existence of three metabolic pathways of theophylline. The administration of this drug to a patient with xanthine oxidase deficiency resulted in the excretion of 1-methyluric acid in addition to 1,3-dimethyluric acid, 3-methylxanthine, 1-methylxanthine and unchanged theophylline. It was concluded that in man the oxidation of theophylline is not catalysed by xanthine oxidase.

INTRODUCTION

Theophylline is used extensively for the treatment of bronchial and cardiac asthma, cardiac and coronary insufficiency, and obstructive lung disease. Moreover, it has been applied recently for the management of apnea and of bradycardic spells in premature infants.

In man and rat theophylline is converted into 1,3-dimethyluric acid (1,3-diMeU), 1-methyluric acid (1-MeU) and 3-methylxanthine (3-MeX), which are excreted in the urine [1–3]. When present in human urine which is screened for inborn errors of purine and pyrimidine metabolism, these compounds can be expected to interfere. This screening is indicated especially in patients showing the characteristics of severe immune deficiency disease as a result of adeno-

sine deaminase (ADA) deficiency [4, 5] and purine nucleoside phosphorylase (NP) deficiency [6, 7].

We felt there was a need to study the chromatographic parameters of these compounds in detail. The methods employed include two-dimensional thin-layer chromatography (TLC) as described previously [8] and high-performance liquid chromatography (HPLC). As far as we know gas chromatography—mass spectrometry of the trimethylsilyl (TMS) derivatives of the methylated uric acids has not yet been described.

As little is known about the quantitative aspects of theophylline metabolism, we also investigated urinary concentrations in patients under treatment. The metabolic pattern does suggest that xanthine oxidase (XO) is a catalyst for the in vivo oxidation of theophylline. However, milk XO does not have this activity in vitro [1]. We had the opportunity to study the effect of allopurinol on the metabolism of this drug in asthma patients under treatment and in rats. Also the oxidation of theophylline in a patient with XO deficiency could be investigated. In this paper we present the data obtained from these studies.

METHODS

Screening for urinary metabolites of theophylline

Screening was performed by two-dimensional TLC, after isolation of these substances from the urine by anion-exchange column chromatography as described previously [8]. Elution was performed with 5 ml of 0.1 *M* ammonia (fraction I), 40 ml of water (fraction II), 150 ml of 0.01 *M* formic acid (fraction III) and 150 ml of 4 *M* formic acid (fraction IV).

Preparative TLC

The same stationary and mobile phases as described for the screening procedure were used. Unknown compounds were scraped off and extracted from the cellulose with 0.1 *M* ammonia. The extracts were centrifuged and investigated by UV spectrometry at various pH values.

High-performance liquid chromatography

HPLC was performed as described previously [8]. For identification of the peaks a Perkin-Elmer Model LC-55 UV—Vis spectrophotometric detector with scanning accessory was used. Quantitative HPLC of urinary 1,3-dimethylxanthine (1,3-diMeX), 3-MeX and 1-methylxanthine (1-MeX) was performed on fraction III, and HPLC of 1-MeU and 1,3-diMeU on fractions III and IV of the isolation procedure using as the mobile phase acetonitrile (UV grade)—sodium acetate/acetic acid buffer (10 mmol/l, pH 4.0) (7:93, v/v) (see ref. 9). A solvent flow-rate of 2.0 ml/min was used and continuous monitoring of the eluent was performed at 280 nm. At the top of the peaks UV spectra were scanned for confirmation.

Calibration curves were prepared and a linear relationship between extinction peak area and concentration was found. For determination of the recoveries the synthetic compounds were added to a preanalyzed urine.

1-Methyluric acid

1-Methyluric acid was synthesized by the oxidation of 0.2 m*M* 1-MeX in oxygen-saturated water, adjusted to pH 7.4 with ammonia, and an excess of xanthine oxidase (from milk, Boehringer, Mannheim, G.F.R.). The course of the reaction was monitored by UV spectrometry. Isolation of 1-MeU from the reaction mixture was performed by anion-exchange column chromatography as described for urine [8].

Gas—liquid chromatography—mass spectrometry

The 70 eV mass spectra of the trimethylsilyl (TMS) derivatives of 1,3-diMeU, 1-MeU and 3-MeU were recorded on a Jeol JGC-20 KP/JMS-D 100/W-JMA system combination at an ion source temperature of 150°, an accelerating voltage of 3 kV and an ionizing current of 300 μA.

For this purpose the extracts obtained from preparative TLC were evaporated to dryness under reduced pressure at 40°. The extracted or synthesized compounds were converted to TMS derivatives with a mixture of N,O-bis(trimethylsilyl)acetamide (BSA) and pyridine (1 : 3) plus 1% trimethylchlorosilane (TMCS) [10, 11] in a tightly closed glass tube at 70° for 30 min. Gas chromatographic conditions were as follows: dual glass columns (8 ft. × 1/8 in. I.D.) packed with 5% GE SE-52 on Chromosorb W AW DMCS, 100—120 mesh (HP); carrier gas, helium, 30 ml/min; oven temperature 250°

Uric acid

Uric acid was determined with uricase.

RESULTS

Identification of 1,3-dimethyluric acid and 1-methyluric acid

During the isolation of the purines from the urine by anion-exchange chromatography, 1,3-diMeU was mainly eluted with fraction III, to a lesser extent with fraction IV. Theophylline (1,3-diMeX) was eluted completely in fraction III. Fig. 1 represents the chromatogram of fraction III from the urine of a six-month-old boy with the characteristics of severe combined immune deficiency disease (SCID) under treatment with theophylline. A marked spot (48) in the position of 1,3-diMeU is present. Only a small spot of 1,3-diMeX can be seen. Both substances gave a blue to pink colour with mercuric acetate—diphenylcarbazone. The UV absorption spectrum of compound 48, isolated from the two-dimensional chromatogram, was identical with that of authentic 1,3-diMeU. Characteristic UV absorption data of the methylated uric acids are given in Table I. In HPLC the isolated compound eluted in the position of 1,3-diMeU. The

TABLE I

UV ABSORPTION MAXIMA (nm) IN 0.1 *M* HYDROCHLORIC ACID (I), 0.05 *M* SODIUM PHOSPHATE, pH 7.4 (II) AND 0.1 *M* AMMONIA (III)

	I	II	III
1,3-diMeU	286	294	295
1-MeU	283	288	292
3-MeU	286	292	292

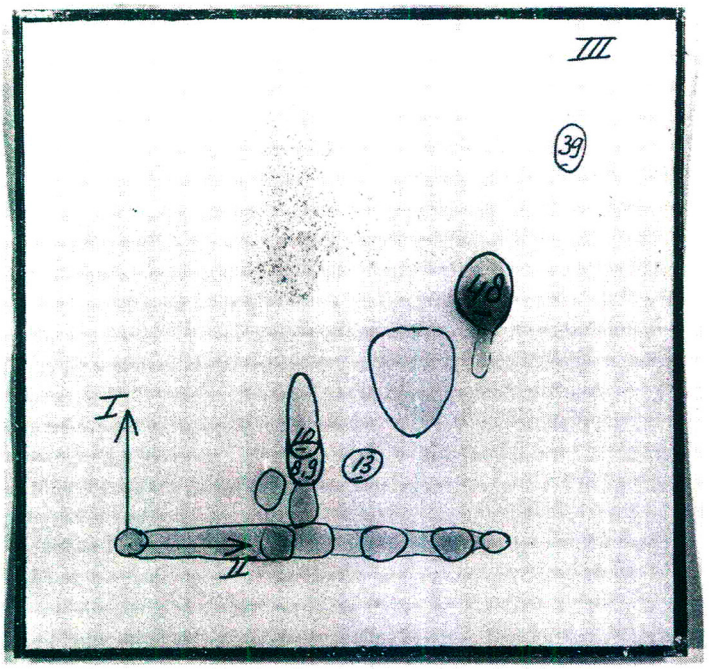

Fig. 1. Two-dimensional chromatogram of fraction III from the urine of the patient with severe combined immune deficiency disease. Sorbent: cellulose 0.1 mm on DC-Alufolien (Merck, Darmstadt, G.F.R., No. 5552). Solvent 1 = isopropanol—5% ammonia (8:2, v/v). Solvent 2 = butanol—acetic acid—water (8:2:2, v/v/v). Chromatogram is developed twice by the ascending technique in both solvents. Large spot 48 represents 1,3-di-MeU; spot 39 = 1,3-diMeX. See also ref. 8.

UV spectrum of the eluate corresponding to the peak of the isolated compound matched that of 1,3-diMeU. The mass spectrum obtained after GLC of the TMS derivative (see Fig. 2a) was identical with that of 1,3-diMeU obtained from Fluka (Buchs, Switzerland).

1-Methyluric acid was isolated from the urine of an asthma patient under treatment with theophylline. Isolation and identification of this compound was performed using the same procedures as for 1,3-diMeU. The mass spectra of 1-MeU and 3-MeU are given in Fig. 2b and c.

Quantitative analysis

Fig. 3 shows representative chromatograms on the μBondapak C_{18} column of fractions III and IV from a patient on theophylline therapy. 3-MeU, 1-MeU, 3-MeX, 1-MeX, 1,3-diMeU and 1,3-diMeX are eluted after 131, 166, 183, 205, 237 and 400 sec and their overall recoveries are 93, 88, 92, 92, 89 and 92% respectively (n=5).

Allopurinol and its metabolites oxipurinol, allopurinol riboside and oxipurinol-7-riboside are eluted after 143, 134, 145 and 174 sec, respectively. Hypoxanthine and xanthine are eluted after 128 and 133 sec, respectively, and uric acid is eluted after 102 sec. So these compounds do not interfere in the analysis of theophylline metabolites.

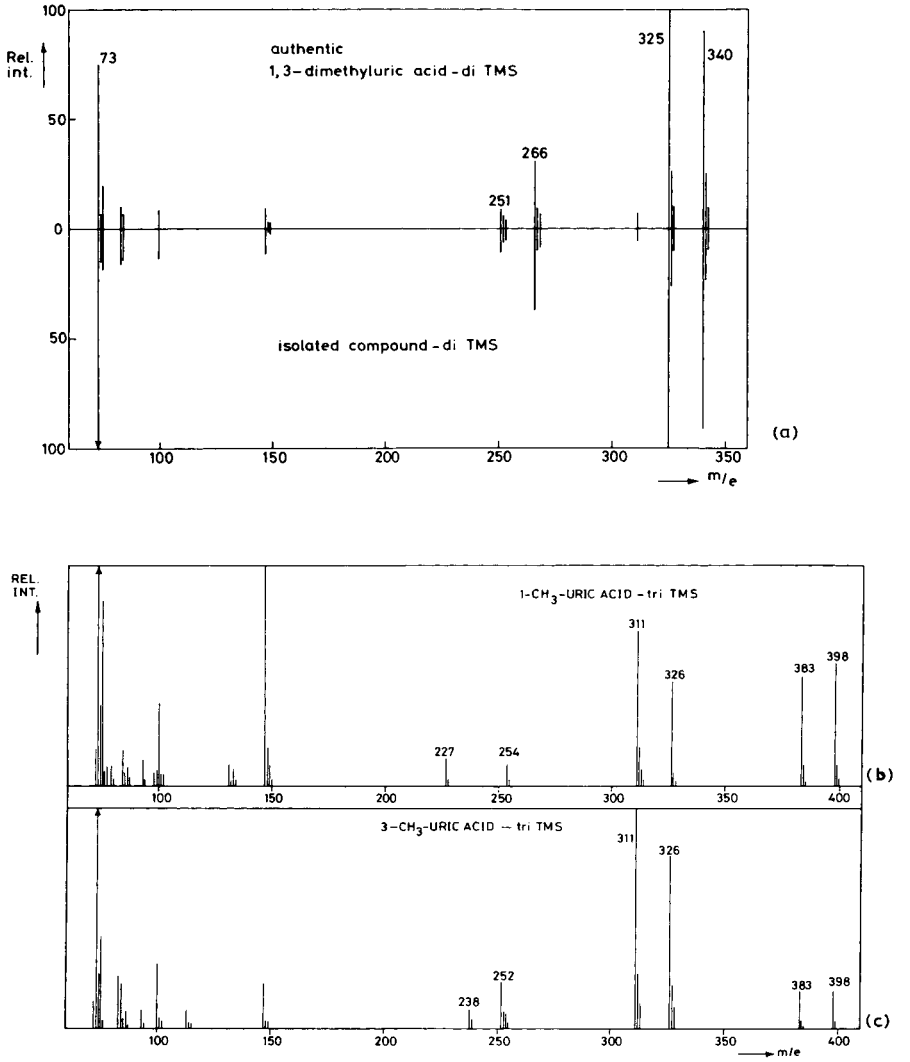

Fig. 2. Mass spectra of the trimethylsilyl derivatives of 1,3-diMeU (a), 1-MeU (b) and 3-MeU (c) using a GLC inlet system. 1,3-diMeU was isolated from the urine by preparative TLC. Chromatographic and mass spectrometric conditions as described in Methods.

Comparison of the HPLC elution profiles of fractions III and IV from the urine of patients and rats before and during therapy with theophylline revealed that, in the urine without theophylline, some UV absorbing compounds were eluted in front of 1-MeU. However, the area of the chromatogram occupied by theophylline and its metabolites was practically empty and as confirmed by "peak top UV spectrometry" there was no interference by other substances.

Excretion values

Excretion data for the SCID patient and five asthma patients under treatment with theophylline are summarized in Table II. In four of them 24-h urine

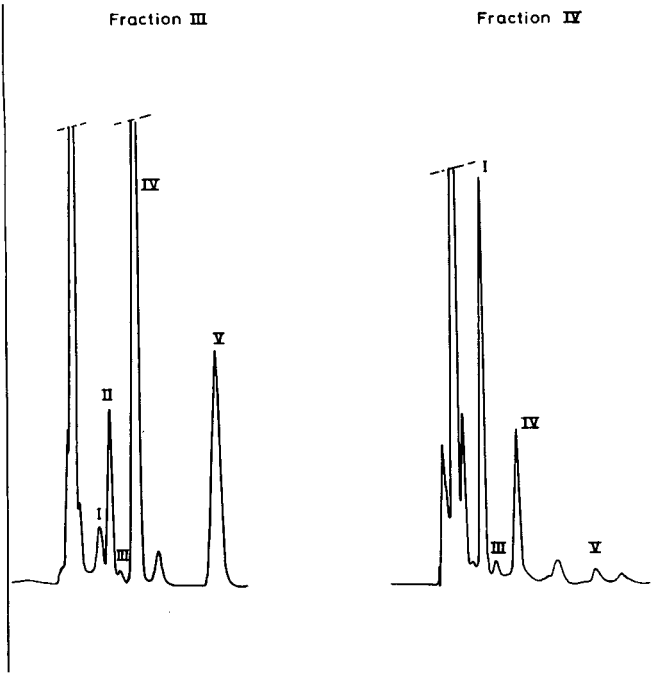

Fig. 3. HPLC of urinary N-methylpurines in fractions III and IV from a patient on theophylline therapy. Column: μBondapak C_{18}, 30 cm × 4 mm I.D. Eluent: acetonitrile (UV grade)—sodium acetate/acetic acid buffer (10 mmol/l, pH 4.0)(7:93, v/v). Flow-rate: 2.0 ml/min. UV detection at 280 nm. Peaks: I = 1-MeU; II = 3-MeX; III = 1-MeX; IV = 1,3-diMeU; V = 1,3-diMeX.

samples were collected and excretion values were related to the intake (see Table III). The results demonstrate that 1,3-diMeU is the main excretion product, followed by 1-MeU, 3-MeX and unchanged 1,3-diMeX. Only traces of 1-MeX were seen. However, in the SCID patient only small amounts of 1-MeU and trace amounts of 3-MeX were present; 1-MeX was absent.

Excretion patterns in rats following oral administration of theophylline before and during loading with allopurinol

Five male WAG rats each weighing approximately 300 g were maintained on a fixed diet during the course of the experiment. A 24-h control urine from each rat was collected and stored in a freezer (−20°) until analyzed. Subsequently 10 mg of theophylline in 2 ml of water were administered to each of four rats by stomach tube three times a day on two successive days. The remaining rat served for comparison. On the three following days two rats of the group received 15 mg of theophylline two times a day, the remaining two rats 15 mg of theophylline two times a day as well as 60 mg and 30 mg allopurinol, respectively, in one dose. From each rat 24-h urine samples were collected daily and analyzed for theophylline metabolites by two-dimensional TLC and HPLC. The quantitative results are summarized in Fig. 4. After the ingestion of theophylline a large amount of 1,3-diMeU, moderate amounts of 1-MeU and theophylline and small amounts of 3-MeX were excreted. In all samples trace

TABLE II

URINARY EXCRETION OF THEOPHYLLINE, 1,3-DIMETHYLURIC ACID, 1-METHYLURIC ACID, 1-METHYLXANTHINE AND 3-METHYLXANTHINE IN PATIENTS RECEIVING THEOPHYLLINE

In H.O. (SCID patient) and K.T. (asthma patient) random urine samples, and in the others 24-h samples, were analyzed. H.O. received theophylline 4 × 154 μmol per day, and K.T. 2 × 528 μmol per day (as suppositories). For the other patients see Table III.

Subject	Creatinine (mmol/l)	1,3-diMeX (μmol/l)	1,3-DiMeU (μmol/l)	1-MeU (μmol/l)	1-MeX (μmol/l)	3-MeX (μmol/l)
H.O. (1)	1.9	174	684	56	n.d.*	19
H.O. (2)	1.9	246	872	52	n.d.	20
H.O. (3)	2.3	169	1684	46	n.d.	28
K.T. (1)	3.7	169	571	466	n.d.	173
K.T. (2)	3.1	76	361	407	23	163
K.T. (3)	6.3	140	811	709	52	257
R.C.	11.4	79	786	772	n.d.	218
R.H.	4.1	89	628	604	8	335
D.A.	4.9	212	401	204	6	71
H.P.	8.3	151	2306	346	15	675

*n.d. = Not detected.

amounts of 1-MeX were present before, as well as during, the loading with the drugs. After administration of allopurinol there was a decrease in the excretion of 1,3-diMeU. However, the excretion of 1-MeU was reduced to zero and a sharp increase in the excretion of 1-MeX was noted; 3-MeX was unaltered. Uric acid became negligible, demonstrating that XO was strongly inhibited.

TLC patterns of the urinary purines before and after the administration of allopurinol to rats receiving theophylline were in agreement with the HPLC data shown in Fig. 4.

Excretion patterns in asthma patients on treatment with theophylline before and during loading with allopurinol

Two boys aged 10.5 and 6.8 years were treated for their asthma with aminophylline 3 × 200 mg per day orally and 3 × 150 mg per day as suppositories, respectively. During this treatment 24-h urine samples were collected and stored in a freezer (−20°) until analyzed. Subsequently, on the three following days, the patients received allopurinol 200 mg per day orally in addition to their theophylline and 24-h urine samples were collected on the third day. Theophylline metabolites were analyzed by both two-dimensional TLC and HPLC. Quantitative excretion values are given in Table IV. After the administration of allopurinol in addition to theophylline an obvious increase in urinary 1-MeX was observed in both patients. But the excretion of 1-MeU was decreased while 1,3-diMeU remained almost unchanged. A reduction in urinary 3-MeX was found in one patient only.

TABLE III

EXCRETION VALUES OF THEOPHYLLINE METABOLITES IN RELATION TO THE AMOUNT OF THEOPHYLLINE ADMINISTERED

Subject	1,3-diMeX administered* (μmol)	Excretion (%)					
		1,3-diMeX	1,3-diMeU	1-MeU	1-MeX	3-MeX	Total
R.C.	1972 (s)	2.5	25.3	24.8	0	7.0	59.6
R.H.	4557 (i)	2.2	15.7	15.1	0.2	8.4	41.6
D.A.	5583 (i)	9.7	18.3	9.3	0.3	3.2	40.8
H.P.	594 (s)	3.4	52.4	7.9	0.3	15.3	79.3

*s = As suppositories; i = infusion.

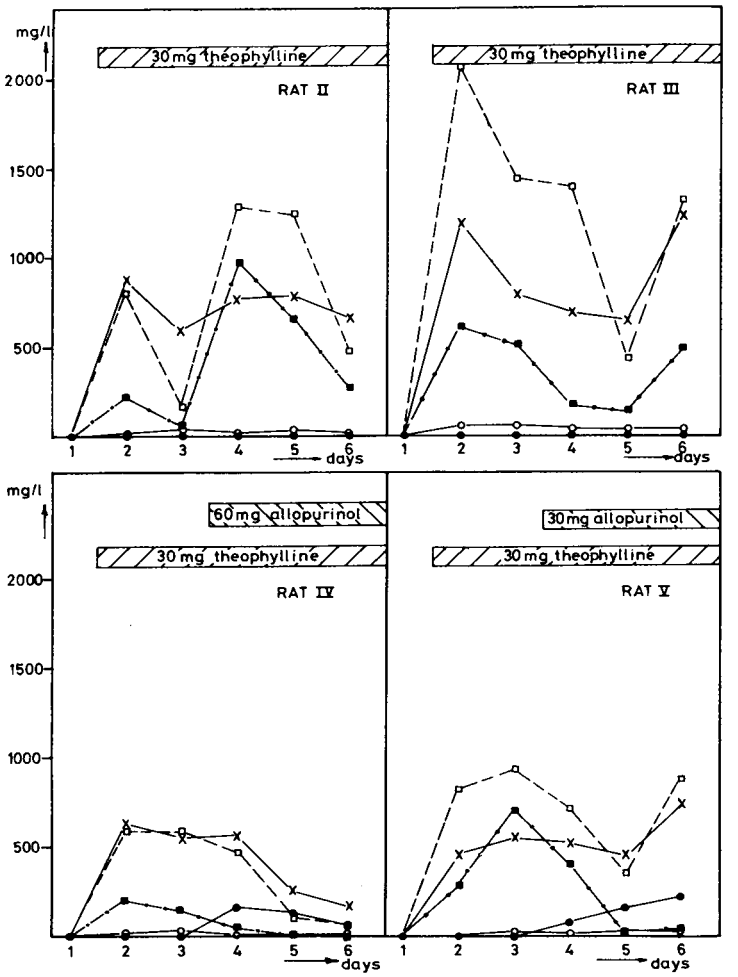

Fig. 4. Urinary theophylline metabolites in rats following oral administration of theophylline before and during loading with allopurinol. x——x, Theophylline; □– – –□, 1,3-diMeU; ■ —·—·—■, 1-MeU; ○———○, 3-MeX; ●———●, 1-MeX.

Excretion pattern in a patient with XO deficiency after ingestion of theophylline

In XO deficiency xanthine is not oxidized to uric acid. XO is also necessary for the oxidation of hypoxanthine to xanthine. A xanthinuric girl, aged 15 months (described elsewhere [12]), was given a single dose of 25 mg euphyllin (3 mg per kg body weight) as a suppository and a 24-h urine sample was collected in order to investigate the metabolic fate of theophylline.

1,3-diMeU was found to be the most prominent metabolite of theophylline in the urine. Also moderate amounts of 1-MeU and 1-MeX were present. This was confirmed by the UV spectra scanned at the tops of the peaks during HPLC. Quantitative data are given in Table V.

TABLE IV

URINARY EXCRETION OF N-METHYLATED PURINES AND URIC ACID IN TWO ASTHMA PATIENTS UNDER TREATMENT WITH THEOPHYLLINE BEFORE AND DURING LOADING WITH ALLOPURINOL

For doses see text.

Medication		Theophylline			Theophylline + allopurinol		
Patient	Compound	μmol/l	μmol/g creatinine	μmol per 24 h	μmol/l	μmol/g creatinine	μmol per 24 h
M.S.	1,3-diMeX	42	39	38	169	226	220
	1,3-diMeU	1260	1162	1123	944	1266	1227
	1-MeU	518	478	461	209	280	271
	1-MeX	n.d.*	n.d.	n.d.	320	429	416
	3-MeX	580	534	516	383	513	497
	Uric acid	3100	2858	2759	1300	1743	1690
J.R.	1,3-diMeX	57	41	16	31	43	23
	1,3-diMeU	1398	990	391	404	567	303
	1-MeU	769	545	215	78	110	59
	1-MeX	n.d.	n.d.	n.d.	126	177	94
	3-MeX	1229	870	344	214	300	160
	Uric acid	4000	2832	1120	1800	2528	1350

*n.d. = Not detected.

TABLE V

URINARY EXCRETION OF N-METHYLPURINES IN A PATIENT WITH XO DEFICIENCY BEFORE AND AFTER THE ADMINISTRATION OF THEOPHYLLINE (110 μmol)

Excretion product	Without theophylline			Theophylline administered		
	μmol/l	μmol/g creatinine	μmol per 24 h	μmol/l	μmol/g creatinine	μmol per 24 h
Theophylline	n.d.*	n.d.	n.d.	36.1	118	9.4
1,3-diMeU	n.d.	n.d.	n.d.	268	879	69.7
1-MeU	n.d.	n.d.	n.d.	214	702	55.6
3-MeX	4.8	9.3	1.1	45.2	148	11.8
1-MeX	26.5	86.9	6.2	78.3	257	20.4

*n.d. = Not detected.

DISCUSSION

By two-dimensional TLC screening of urinary purines and pyrimidines in a patient with SCID, an abnormal high excretion of an unknown compound, later identified as 1,3-diMeU, was observed. It is known from the literature [1—3] that 1,3-diMeU is the main metabolite of theophylline and indeed the SCID patient appeared to have been treated with theophylline. In addition, 1-MeU [2, 3] and 3-MeX [3, 13] are reported to be theophylline metabolites. Both

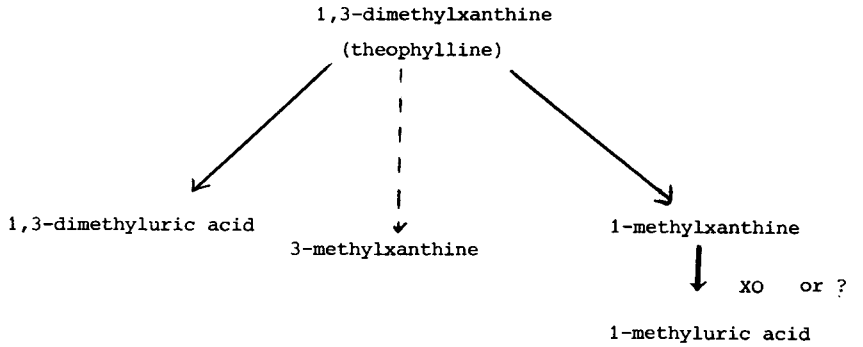

Fig. 5. Metabolic pathways of theophylline in man and in the rat.

compounds are excreted in moderate amounts in the urine of adults receiving theophylline. However, our patient excreted only small amounts of 1-MeU and traces of 3-MeX. This raised the question of whether theophylline in children and adults could follow different metabolic pathways. Such a hypothesis was supported by the fact that in children the plasma theophylline clearance is approximately 40% greater than that in adults [14]. Investigation of the urines of five asthmatic children treated with theophylline revealed the excretion of considerable amounts of 1,3-diMeU, moderate amounts of 1-MeU and unchanged theophylline and small amounts of 3-MeX and 1-MeX. These findings argue against the theory of different pathways for theophylline in children and adults. The higher plasma clearance in children could be explained by a greater capacity of the liver in children to metabolize theophylline. The very low urinary concentrations of 1-MeU, 3-MeX and 1-MeX in the terminal phase of the patient with SCID, may be caused by a decreased metabolic activity of his liver resulting in a reduced N-demethylation of theophylline.

The results of the experiments with theophylline and allopurinol in rats and in asthma patients point to probable metabolic pathways of theophylline in rats and in man being those shown in Fig. 5. The oxidation of theophylline without demethylation appears to be the major route. For a small part theophylline is demethylated at position 1 giving 3-MeX, which does not seem to be metabolized further because urinary 3-MeU was not found. On the other hand, theophylline is demethylated at position 3 to give 1-MeX, which is excreted in small amounts.

Moreover, the moderate excretion of 1-MeU indicates that 1-MeX may be an intermediate in the formation of 1-MeU. This is supported by the significant increase of 1-MeX and the decrease of 1-MeU after the administration of allopurinol in the theophylline-treated patients and rats. These findings also indicate that 1-MeU is formed by the oxidation of 1-MeX, catalyzed by XO or another enzyme which is inhibited by allopurinol and not by demethylation of 1,3-diMeU, as was suggested in the literature [3]. It can be concluded from in vitro experiments [1], our experiments in rats and the results from our patient with XOD, that the XO-catalyzed oxidation of theophylline cannot be the sole pathway of 1,3-diMeU formation. With XO from milk and from human liver 3-MeX cannot be converted into 3-MeU either, but 1-MeU and 7-MeU do arise from 1-MeX and 7-MeX, respectively [15, 16]. Apparently, a methyl substitution on

position 3 prevents oxidation by XO. This is supported by the fact that allopurinol did not suppress the excretion of 1,3-diMeU to zero level in the rats.

In our XO-deficient patient a considerable amount of theophylline is oxidized to 1,3-diMeU and to 1-MeU. It is not known which enzyme activity is responsible for this oxidation, but the results from our patient suggest that it is not XO. Demethylation of 1,3-diMeU at position 3 might explain the excretion of 1-MeU, but in this case it is difficult to understand why it should not happen in the asthma patients and the rats on theophylline and allopurinol.

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CHROMBIO. 351

RAPID ASSAY FOR THEOPHYLLINE IN CLINICAL SAMPLES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A fast, sensitive and highly specific method for the determination of theophylline in human serum is reported. Using a C_{18}-bonded reversed-phase column with an acetonitrile—acetate buffer mobile phase theophylline is completely resolved not only from other dietary xanthines and their metabolites but also from co-administered drugs such as paracetamol and phenobarbitone. Use of β-hydroxyethyltheophylline as internal standard allows a within batch precision of 2.0% and a between batch variation of 3.0%. Factors involved in the development of the method and its performance are discussed.

INTRODUCTION

The therapeutic effects of many drugs are dependent on their concentrations in biological fluids rather than their administered dosages. As the rate of metabolism of such drugs may differ widely between individuals the monitoring of drug levels in body fluids can be very important. Theophylline, a bronchodilator used extensively in the treatment of asthma operates over a narrow therapeutic range (10—20 μg ml^{-1} [1—3]) below which it is ineffective and above which toxic side-effects may occur [4, 5].

Plasma rather than saliva or urine has usually been chosen as the biological fluid for analysis of theophylline for three main practical reasons. Firstly, the levels of theophylline in plasma are approximately twice those present in saliva [6]. Secondly, apart from 3-methylxanthine [6, 7], plasma contains negligible

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quantities of the theophylline metabolites that are present in urine and therefore the possibility of interference is reduced. Thirdly, no simple correlation exists between the levels of theophylline in urine and plasma. Urine theophylline levels therefore do not provide a reliable indication of the concentration of the drug in the body.

Before the estimation of theophylline in plasma can be performed the plasma proteins, which account for about 7% by mass [8], must be removed. This may be achieved by simple denaturation with trichloroacetic acid [9] or organic solvents such as acetonitrile [10] or by ultrafiltration using a high-molecular-weight filter [6, 11]. Difficulties may occur, however, because the volume of sample available for analysis is often limited, particularly for neonates, where a heel-prick will provide approximately 100 μl of sample.

Methods which have been used to determine theophylline include ultraviolet spectrophotometry [12], radioimmunoassay [13], thin-layer chromatography [14], together with gas [15] and liquid chromatographic techniques [10, 16—23]. The high-performance liquid chromatographic (HPLC) determination of theophylline has usually been performed with a C_{18} reversed-phase bonded to an irregular microparticulate support. Typical column efficiencies ranged from 3000 to 8500 plates metre^{-1} [10, 18—23]. However, many of these methods suffer from one or more serious disadvantages such as lengthy sample preparation [19], poor chromatography (bad peak shape, low column efficiency, incomplete resolution of theophylline from plasma peaks [23]), high retention time [18] or applicability only to macrosamples [11].

The present work describes a rapid, specific method for the determination of theophylline in microsamples of human plasma. Studies of some seventy-five patients over a three-month period [24] suggest that this procedure is not only superior in performance to other gas and liquid chromatographic methods but also is sufficiently reliable to be used routinely in clinical laboratories.

EXPERIMENTAL

Liquid chromatography

The liquid chromatograph consisted of a Waters Assoc. Model 6000 pumping system and a Pye Unicam LC3 variable wavelength UV detector fitted with an 8-μl flow-cell. The detection wavelength was 273 nm and the detector sensitivity was 0.02 a.u.f.s. The 10 cm × 5 mm I.D. column (Shandon Southern Products, Runcorn, Great Britain, integrated column and septum injector system) was slurry packed in methanol containing a trace of acetate buffer, with 5-μm ODS-Hypersil (Shandon Southern Products), a C_{18}-bonded spherical 5-μm packing material. The solvent used to compress the slurry was hexane. The packing pump was of the pneumatic amplifier type (H.S.C.P., Bourne End, Great Britain) and the packing pressure was 6000 p.s.i. The detector output was to a suitable recorder with 10 mV f.s.d. All chromatograms were obtained at ambient temperature. A Hamilton 10-μl syringe with 75 mm length needle was used for injection of sample volumes. Quantitation was by comparison of peak heights with an internal standard. Operating pressure was 1200—1300 p.s.i. with a flow-rate of 1.5 ml min^{-1}.

Reagents and chemicals

The mobile phase was acetate buffer—acetonitrile (92:8). The buffer was prepared by adjusting the pH of a 20 mmol l^{-1} solution of sodium acetate in singly distilled water to 4 with reagent-grade glacial acetic acid. The buffer was prepared weekly and stored at 4° until required. Due to a negative volume of mixing, volumes of acetate buffer and acetonitrile were measured separately and subsequently mixed together. Degassing of the mobile phase was achieved by warming to ca. 40° under reduced pressure (ca. 15 mm Hg) for 10 min. During use solvents were maintained in the degassed state with a slow stream of helium. Care must be taken to ensure that the helium flow does not alter the composition of the acetonitrile—buffer mixture as a small reduction in the acetonitrile content leads to a large increase in retention time (Fig. 1). All mixed solvents were stirred continuously whilst the chromatograph was running.

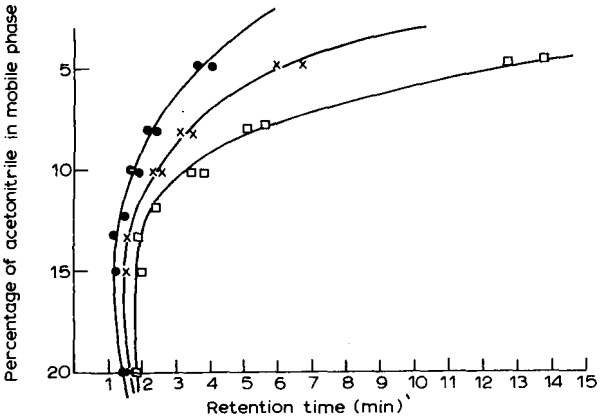

Fig. 1. Variation of retention time with percentage of acetonitrile in the mobile phase. •, Theobromine; x, theophylline; □, caffeine.

Sample collection and storage

Samples were collected from out-patients attending an asthma clinic. Upon receipt in the laboratory the samples were centrifuged and the plasma stored at - 20° until required for analysis.

Sample preparation

A stock solution of 237.5 ml chloroform and 12.5 ml isopropanol (95:5 mixture) together with 1.4 mg of β-hydroxyethyltheophylline as internal standard was prepared and stored at 4° until used. An aliquot of 25 μl of plasma was transferred via a 100-μl syringe into a 10-ml tapered centrifuge tube and 250 μl of the organic extracting solution (see above) were added from a 500-μl syringe. The tube was sealed with a rubber septum, the contents vortexed for one minute to give thorough mixing and centrifuged for one minute. A 200-μl amount of the chloroform layer was removed and evaporated to dryness. The residue was dissolved in 50 μl of mobile phase and vortexed for one minute prior to injection. For a 25-μl sample of plasma in 250 μl of extracting solution recovery of theophylline was 71%.

RESULTS AND DISCUSSION

Chromatographic aspects

The ratio of acetonitrile to acetate buffer was found to be critical to the separation. Slight alteration of the ratio brought about significant variations in retention time (Fig. 1). An acetate buffer—acetonitrile (92:8) mixture was selected for use, this being a compromise between maximum resolution of peaks and minimum analysis time per chromatogram.

Variation in the pH of the mobile phase was also found to be significant to the determination. The maximum difference in retention time between theophylline and dietary xanthines occurred at pH 4.0 (Fig. 2).

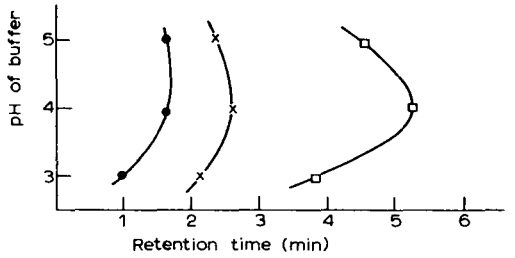

Fig. 2. Variation of retention time with the pH of the acetate buffer in the mobile phase. •, Theobromine; x, theophylline; □, caffeine.

The optimum flow-rate was found to be 1.5 ml min^{-1} which allowed an operating pressure of 1200—1300 p.s.i. At this working pressure regular changes of septum every fifteen to twenty injections were necessary.

The eluate was monitored at 273 nm corresponding to λ_{max} for theophylline (ϵ = 96,700) i.e. the optimum detection wavelength. Detection at 254 nm or 280 nm resulted in greatly reduced sensitivity. As these two wavelengths are commonly available in "fixed-wavelength detectors" it should be recognised that the use of such detectors seriously degrades the performance of the method.

The use of a spherical 5-μm particle packing material gave considerable improvement in performance when compared with other methods [10, 18—23]. Typical column efficiency with this material was 30,000 plates per metre. Whilst this was more than adequate for the resolution of theophylline, theobromine and caffeine (Fig. 3A) such performance is desirable. For example one of the most likely, and often neglected, of possible interferents in the determination of theophylline, is paracetamol, a common analgesic. Indeed paracetamol was found to be present in several of the seventy-five patients studied during this work. The resolution of paracetamol from theophylline is illustrated in Fig. 3B. Other possible interferents include the metabolites of theophylline, 1,3-dimethyluric acid, 1-methyluric acid and 3-methylxanthine.

The retention times of these and other related compounds are given in Table I. The only interfering compound was 1,7-dimethylxanthine, a metabolite of caffeine [25]. As this compound has not been detected in plasma in significant concentrations, interference is unlikely in practice. A second advantage, result-

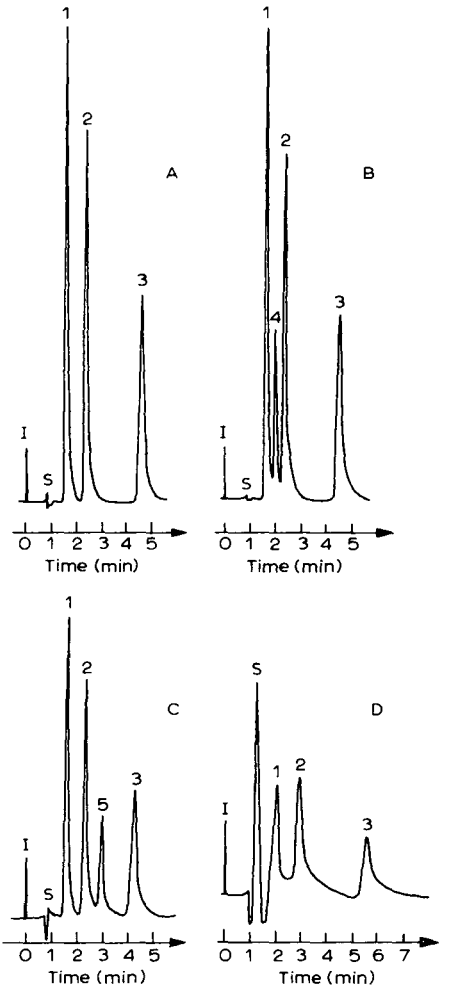

Fig. 3. (A) Optimised separation of theobromine (1), theophylline (2), and caffeine (3). S = solvent front. (B) The resolution of paracetamol (4) from theophylline (2). (C) The position of the internal standard (5) relative to theophylline (2) and caffeine (3). (D) The effect of injecting the sample in 100% acetonitrile. 1 = Theobromine; 2 = theophylline; 3 = caffeine.

ing from the high efficiency of the column was that considerable deterioration in the condition, and thus performance, of the column could be tolerated before column repair became essential. Acceptable results were still obtained when column efficiency had decreased to half of its original value. A daily wash of the column with methanol was found to extend its working lifetime thus reducing routine column maintenance still further.

Internal standard

Substituted xanthines which are not used as drugs, do not occur in dietary items, and are not metabolic products, were obvious choices for the internal standard. Three possible compounds were investigated, β-hydroxyethyltheo-

TABLE I

RETENTION TIMES OF THE VARIOUS COMPOUNDS STUDIED

Compound	Retention time (min)
Theophylline	2.50
Theobromine	1.65
Caffeine	4.65
Hypoxanthine	0.95
3-Methyluric acid	0.95
Xanthine	1.00
1-Methyluric acid	1.15
3-Methylxanthine	1.35
1-Methylxanthine	1.40
1,3-Dimethyluric acid	1.65
1,7-Dimethylxanthine	2.50
Paracetamol	2.15
(Phenobarbitone	29.0)*
β-Hydroxyethyltheophylline (I.S.)	3.20

*Phenobarbitone was only detected as a very small peak at high concentrations (due to a very low absorbance at 273 nm), and thus it can be ignored.

phylline, 8-chlorotheophylline and 7-β-hydroxypropyltheophylline. The retention times of these were 3.20, 5.45 and 5.50 min, respectively. All three showed similar absorbance at 273 nm and chromatographed well giving resolved symmetrical peaks. 8-Chlorotheophylline was rejected because of low solubility in the mobile phase at the 40 $\mu g\ ml^{-1}$ level. β-Hydroxyethyltheophylline, rather than 7-β-hydroxypropyltheophylline, was selected as the best internal standard because it eluted between theophylline and caffeine (Fig. 3C) thus adding no extra time to a chromatographic run. Chromatographic analysis time is thus reduced to 5 min.

Linearity of the assay

Using the extraction procedure detailed above, standard plasma samples with theophylline concentrations of 0, 0.5, 1, 2, 5, 10, 20, 40, 60, 80 and 100 $\mu g\ ml^{-1}$ were analysed. The results obtained over the range 0—40 $\mu g\ ml^{-1}$ were linear (index of determination 0.9977) but a slight upward curve was displayed at higher concentrations. The index of determination was 0.9927 for the range 0—100 $\mu g\ ml^{-1}$. As the therapeutic range (10—20 $\mu g\ ml^{-1}$) is contained within the linear portion of the calibration graph, the departure from linearity at high concentrations is not significant.

Analysis of plasma samples

A set of seventy-five samples from subjects attending out-patient asthma clinics was analysed. All samples had previously been analysed by the gas chromatographic method currently in use [15] at the Bristol Royal Infirmary. Batches of sixteen samples were analysed, consisting of thirteen prepared plasma samples together with two standards of concentrations 10 $\mu g\ ml^{-1}$ and 20 $\mu g\ ml^{-1}$ respectively and one control sample with a concentration near the middle of the therapeutic range. Duplicate injections of each sample were made.

The results obtained by HPLC are compared directly with those produced by gas chromatography in Fig. 4. The within batch coefficient of variation was determined by ten replicate assays of the control sample to be 2.0% (mean value 14.9 $\mu g\ ml^{-1}$). The between batch coefficient of variation was 3.0% (thirteen determinations, mean value 15.1 $\mu g\ ml^{-1}$). The figures may be directly compared with values for the gas chromatographic method [15] of 3.5% within batch and 5.6% between batch.

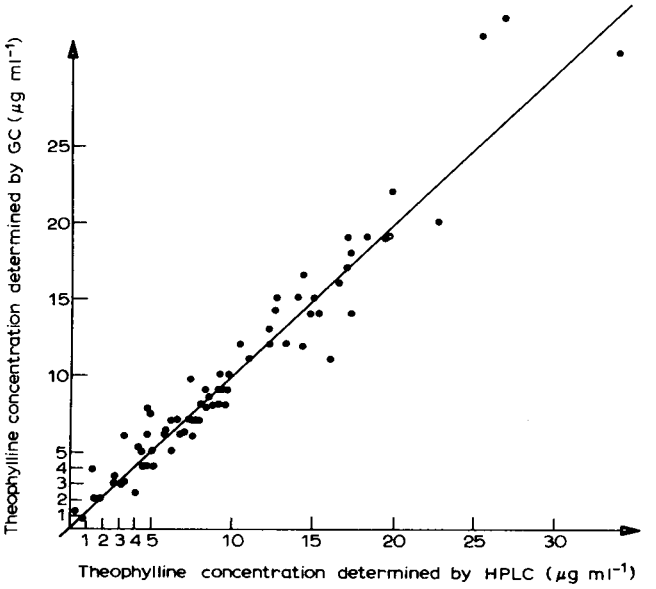

Fig. 4. Correlation of data from HPLC and gas chromatographic analyses. Index of determination, 0.964; slope, 1.011; intercept, + 0.089.

Due to the nature of the assay, many of the normal sources of variation have been eliminated. The only accurate volume measurements required are those of the sample (25 μl) and the 250 μl of extracting solution. The internal standard removed any errors caused by subsequent volume measurements, including injection. Furthermore any reduction in peak height, caused by variable increases in retention time or by band broadening as the column deteriorated, resulted only in a loss of sensitivity. Without an internal standard, this would have drastically affected the peak height response with respect to concentration.

Daily calibration of the method, by using two standards at 10 μg/ml and 20 μg/ml concentrations, eliminated errors due to slight changes in the response of the assay. These can be caused by such factors as small changes in the extracting solution (chloroform readily evaporates for example).

The speed of the method can be most important for clinical usage, especially in emergency cases, and therefore this procedure was designed to give accurate analyses in the minimum time. The fastest procedure developed was to initially prepare two samples of the batch while the liquid chromatograph stabilised. This preparation took 12 min. These samples were then run with duplicate injections. Since the possible elution of caffeine had to be allowed for, the chro-

matography time per injection was 5 min and thus 10 min per sample. The time per injection could be reduced to 3 min by injection of samples immediately after elution of the internal standard peak. The caffeine peak would then elute near the solvent front of the next injection, without interference with the theophylline peak. Other samples were then prepared during the chromatographic runs (each pair taking 12 min to prepare, while the previous pair takes 20 min to chromatograph). Computation of results was then performed during chromatography of the final pair of samples.

Potential chromatographic difficulties

As the developed method is very simple to operate it is likely that routine clinical determinations will be performed by chromatographically inexperienced personnel. We therefore report three areas where particularly careful observation is necessary.

Contrary to published results [26], the solvent in which the theophylline samples are injected was shown to be critical. Injection in pure acetonitrile produced poor peak shapes (Fig. 3D, compared with 3A). The decrease in peak height resulted in significantly reduced sensitivity. This effect is probably due to the theophylline being present in an un-ionised form when dissolved in pure acetonitrile, but existing in a protonated form when the recommended mobile phase (i.e. pH 4) is used. The step in the method involving evaporation of the chloroform extract to dryness and dissolution in the mobile phase is thus essential.

Injection of the sample at any height above the top of the column packing reduced the column efficiency. The sample should be injected into a layer of glass beads packed on top of the column as close as possible to the packing material without disturbing it. Disturbance of the column causes decreased column efficiencies and tends to block the syringe. For example, injection of the sample at a point ca. 5 mm above the column bed i.e. towards the top of the layer of glass beads was observed to reduce column efficiency from 25,000 to 7,700 plates m^{-1}. The injection of samples as close to the top of the packing as possible is therefore essential.

During use the packing material of the column shrinks slightly producing a void at the top of the column. The reason for this is uncertain, but may be due to further polymerisation of the bonded phase via cross-linking of the silane groups. The consequence, however, is the doubling (Fig. 5B), and in severe cases tripling, of peaks. The initial observation is the development of shoulders on each peak (Fig. 5A). The effect is readily cured by removal of the top few millimetres of dirty packing followed by repacking with fresh material using the chromatographic pump. We have restored the efficiency of the column used in the development of this work to about 30,000 plates m^{-1} five times. No permanent deterioration of the column has occurred.

In an attempt to prevent void development the top of the column was modified (Fig. 6). A gauze, permeable to 5-μm particles was placed on the top of the column packing. The column insert was positioned to hold this gauze in place and was then half filled with packing material as in normal column repair. A second gauze, that retained 5-μm particles, was cut to fit easily inside the column insert on top of the packing material. The remaining space was filled

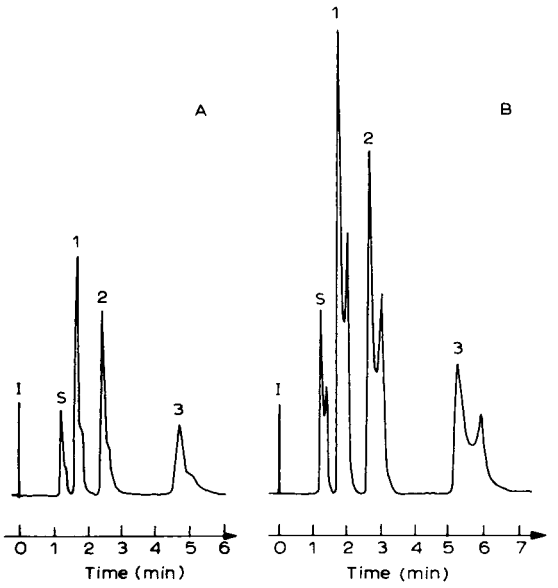

Fig. 5. Degradation of chromatography caused by void formation at the top of the column. For details, see text. 1 = Theobromine; 2 = theophylline; 3 = caffeine; I = point of injection; S = solvent front.

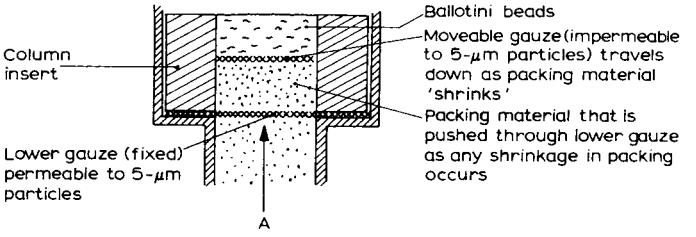

Fig. 6. Moving gauze modification to head of column. Void formation at point A is prevented.

with small glass beads (ballotini). As bedding down occurred the top gauze was forced down by the high pressure preventing the formation of voids at the top of the packing material. Instead the void was generated at the top of the bed of glass beads. Some success has been achieved using this modification as the problem of double peaks has not been observed during the last two hundred injections.

CONCLUSION

An HPLC method has been developed that is entirely suitable for the routine clinical monitoring of theophylline. The high specificity, high sensitivity, applicability to microsamples, good accuracy, precision and speed of analysis make it an attractive procedure when compared with other HPLC methods, gas chromatographic methods and the more conventional techniques of thin-layer chromatography and UV spectrophotometry.

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EVALUATION OF A MODIFIED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR ACEBUTOLOL AND ITS MAJOR METABOLITE

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SUMMARY

Extensive modification of an existing high-performance liquid chromatography assay for acebutolol and its major metabolite has markedly improved chromatographic stability eliminating the previous need for frequent adjustment of the eluent composition to accommodate continuous loss of column retention. The eluents now used and avoidance of the requirement for elevated column temperature may be significant factors in the ability to maintain column life over 8 months of continuous use with little decrease in retention. As a result of the improved chromatographic stability full advantage can now be taken of automatic injection devices for the unattended processing of large numbers of samples. A significant modification of the work-up of blood samples has improved precision of the assay in whole blood. Nevertheless, it is recommended that plasma samples rather than whole blood be analyzed, since the plasma assay is faster and still more precise.

INTRODUCTION

Acebutolol (Fig. 1) is a new beta-adrenergic receptor antagonist [1, 2]. In contrast to propranolol, its cardioselectivity allows treatment of cardiac arrhythmias in asthmatics [3]. A major metabolite of the drug, (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane (metabolite I; Fig. 1), has been isolated and identified [4]. This metabolite may exceed the plasma concentrations of the parent drug several-fold, but its contribution to the antiarrhythmic effect of acebutolol in man is unclear. Several specific assays which allow a comparison of activity of acebutolol with other β-blockers and a test for possible contribution of metabolites to the pharmacodynamic response have been developed. Meffin et al. [4] reported on a gas chromatographic procedure suitable for simultaneous but separate quantitation of the

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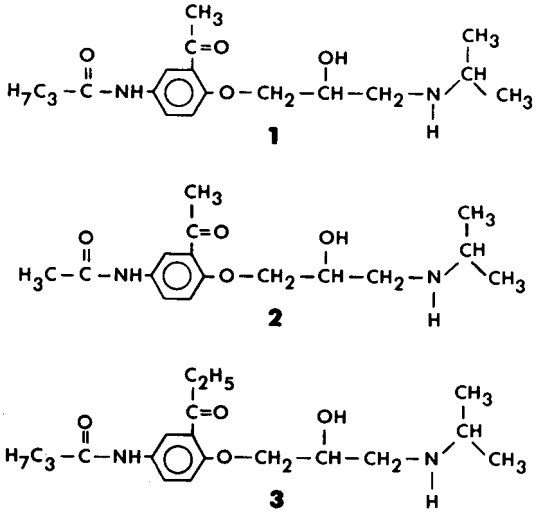

Fig. 1. Molecular structures of (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (acebutolol; 1), (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane (metabolite I; 2) and (±)-1-(2-propionyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (internal standard; 3).

drug and its major metabolite. The sample work-up procedure in this assay is, however, tedious. Three extraction and two derivatization steps are necessary to provide a sample suitable for injection. The thin-layer chromatographic procedure described by Steyn [5] uses quinidine, another antiarrhythmic drug, as internal standard and will give inaccurate results in patients where this drug is co-administered. The high-performance liquid chromatographic (HPLC) assay reported by Meffin et al. [6] uses an ion-pair reversed-phase system at slightly elevated column temperature (30°). When applying this method we noted periodic changes in retention times of the compounds necessitating frequent readjustment of the solvent composition. Rapid deterioration and a short life of the column were observed. This seemed to be due to the ion-pairing reagent used (sodium dodecyl sulfate) and its high surfactant activity. Changing the counter-ion in the system resulted in insufficient retention and/or resolution of the drug, its metabolite and the internal standard used. With the possibility of processing large numbers of samples with the aid of an automatic injector we have developed a reversed-phase chromatographic system not using ion-pairs.

EXPERIMENTAL

Materials

Acetonitrile and methanol were obtained from Fisher (Pittsburgh, Pa., U.S.A.) (HPLC grade) and ethyl acetate from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Sodium hydroxide pellets (USP), phosphoric acid (N.F. 85% wt/wt), sulfuric acid (95—98%) and potassium phosphate monobasic, all of analytical reagent grade, were supplied by Mallinckrodt (St. Louis Mo., U.S.A.)

Water was demineralized, distilled in glass and filtered before use. The reference substances, (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (acebutolol), (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane (metabolite I) and (±)-1-(2-propionyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (internal standard) (Fig. 1) were kindly supplied by May & Baker (Dagenham, Great Britain).

Instrumentation and chromatographic conditions

A Varian (Palo Alto, Calif., U.S.A.) Model 8500 high-performance liquid chromatograph was used equipped with a Varichrom (Varian) UV detector set at λ = 240 nm. The Altex (Berkeley, Calif., U.S.A.) Spherisorb ODS 5-μm HPLC column (25 cm × 3.9 mm I.D.) was eluted with acetonitrile—0.1 *M* phosphate buffer (pH 3.3)—water (55:6:39) at a flow-rate of 60 ml/h.

Injections were made with a Varian automated injector (Model 8000) through a Valco sweepflow injector valve CV-6-UHPa-N60 with a 50-μl loop. A dual pen recorder (Linear, Irvine, Calif., U.S.A.) enabled an eighty-fold range to be covered.

Assay procedure

Plasma. A 1.0-ml volume of methanolic internal standard solution containing 2.0 μg/ml of the internal standard is measured into a test-tube (18 × 150 mm with PTFE-lined screw cap) and evaporated to dryness at 35° under a stream of nitrogen. A 1.0-ml quantity of the plasma to be assayed is added and vortexed for a few seconds. After addition of 1.0 ml of distilled water and 200 μl of 2 *N* aqueous sodium hydroxide solution, the sample is vortexed for 90 sec with 10 ml of ethyl acetate. After centrifugation for 5 min at 600—1200 *g* the organic phase is transferred with a disposable pipette into an evaporation tube*, 150 μl of 0.01 *N* aqueous sulfuric acid added and the mixture vortexed for 90 sec. The tube is then placed into a dry-ice—acetone bath for 1 min to cause the aqueous component to separate and is then centrifuged for 3 min. The aqueous phase (150—250 μl) is sampled with a syringe through the ethyl acetate layer, transferred into vials and injected onto the column with an automatic sampler.

Blood. A 1.0-ml volume of the blood to be assayed is spiked in a disposable test-tube with 2.0 μg of internal standard as described in the plasma assay above. To precipitate proteins 2 ml of acetonitrile are added. After vortexing the sample for 1 min and centrifugation for 3—5 min at 600—1200 *g* the supernatant is decanted into another test-tube (18 × 150 mm with PTFE-lined screw cap), the acetonitrile evaporated at 35° under a stream of nitrogen and the sample further processed as described for plasma samples.

Calibration and interpretation of chromatograms

Quantitation of acebutolol and its metabolite was achieved on the basis of a standard curve. To accommodate small variations in the chromatographic system which may occur from one day to another, standard curves were prepared daily by spiking 1.0 ml of blank, drug-free plasma or blood with 2.0

*Centrifuge tube with a glass capillary tube (capacity ≈ 150 μl) fused to the base.

μg internal standard and 0, 50, 100, 200, 500, 1000, 2000 or 3000 ng of both acebutolol and metabolite I and assaying them together with the patients' samples. An unweighted least squares regression relationship between concentration and the peak height ratio of each compound to the internal standard, was calculated.

RESULTS AND DISCUSSION

Using the conditions described, the chromatographic system could be maintained for months provided the column was washed periodically with acetonitrile—water or pure acetonitrile and column frits were replaced as soon as significant increase in back-pressure was observed. Adjustment of neither the solvent composition nor the flow-rate was necessary, nor did the column show any sign of deterioration in spite of the large sample volumes injected. After an eight-month period of use of the column the retention times of acebutolol, metabolite I and internal standard had decreased slightly but the column was still usable.

Typical chromatograms from patient samples are shown in Figs. 2 and 3 for plasma and blood, respectively. Retention times for acebutolol, metabolite I

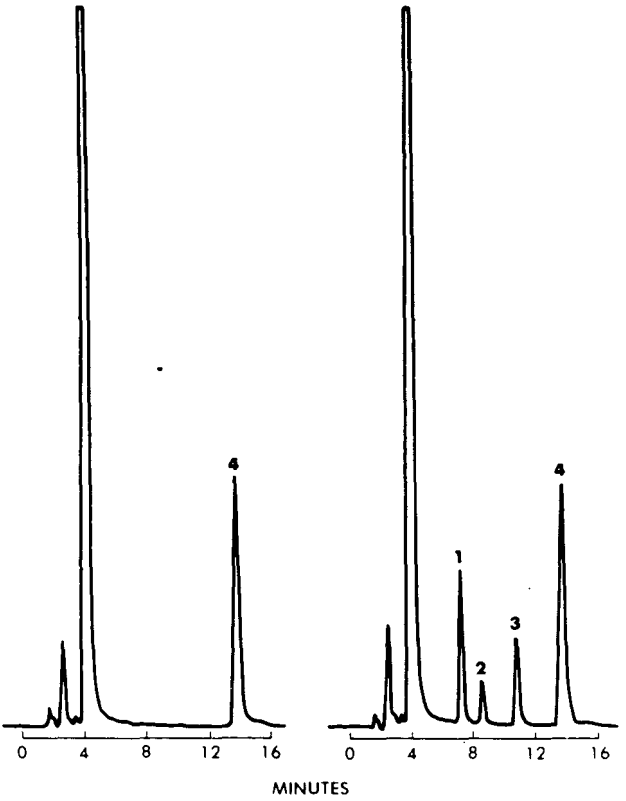

Fig. 2. Chromatograms of plasma extract prior to (left) and after (right) administration of acebutolol. Peaks: 1, metabolite I; 2, unknown substance; 3, acebutolol; 4, internal standard.

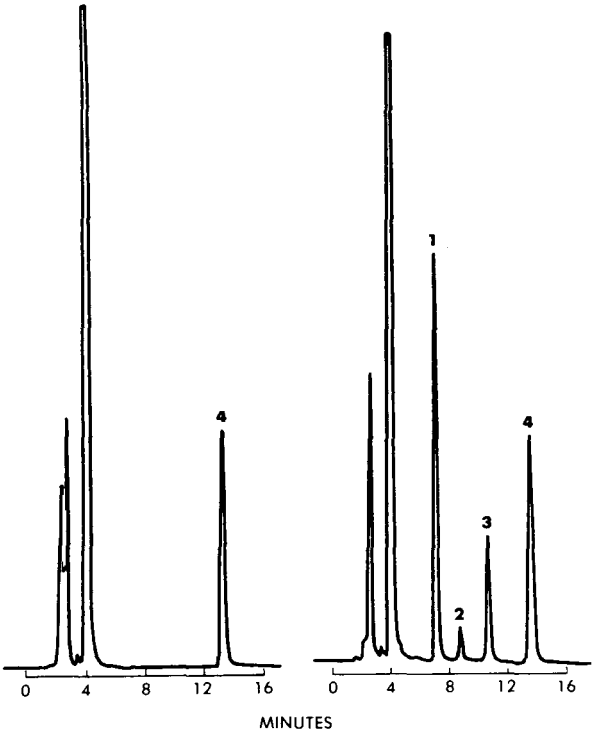

Fig. 3. Chromatograms of blood extract prior to (left) and after (right) administration of acebutolol. Peaks: 1, metabolite I; 2, unknown substance; 3, acebutolol; 4, internal standard.

and internal standard are 10.6, 7.0, 13.4 min, respectively. As can be seen, neither plasma nor blood obtained from the subject prior to administration of the drug gave rise to interfering peaks. Quinidine and oxprenolol, also antiarrhythmic drugs, did not interfere with the assay. Although propranolol did not itself interfere, one of its metabolites (N-desisopropyl-propranolol) had the same retention time as acebutolol. In some patients taking acebutolol, a small unidentified peak with a retention time of 8.7 min could be found (cf. Figs. 2 and 3). Since these patients took no other drugs and since the additional peak was not present in the sample taken prior to administration of acebutolol, it is likely that the additional substance found in our assay is a previously unknown minor metabolite. It could be quantitated with no changes in the procedure if a reference sample were available.

The assay procedures include extraction of the drug and its major metabolite from the biological sample into ethyl acetate and back-extraction into sulfuric acid. The acid is then injected directly onto the column. Since the column material is the most variable factor in a chromatographic system and may deteriorate under strongly acidic conditions, we investigated the minimum acid strength required to reproducibly extract the bases from the organic phase. It was shown that 0.01 *N* sulfuric acid was sufficient for extraction of the basic compounds and no increased peak height could be obtained by extracting identical samples with higher acidic strengths. Addition of 150 μl of acid

yielded, after chilling, sufficient aqueous phase for two injections with the automatic injector.

Since with automatic sampling the drug and its metabolite were kept in the sulfuric acid for up to 12 h prior to chromatography, the stability of the compounds in 0.01 *N* sulfuric acid was tested. In 0.01 *N* and even in 0.1 *N* sulfuric acid no degradation of either compound could be detected even after 24 h.

Using the same sample work-up procedure for both plasma and whole blood as suggested by Meffin et al. [6], there was distinct nonlinearity in the standard curves obtained for blood. We therefore altered the sample work-up for blood to precipitate proteins by addition of acetonitrile. After centrifugation of the precipitated proteins acetonitrile had to be evaporated from the supernatant to ensure complete extraction of the drug from the aqueous phase with ethyl acetate.

The sensitivity of the method described is dependent not only on the sample size taken for assay but also on the injection mode, since the automatic injector used requires excess sample to rinse away residue from the preceding sample. Taking 1 ml of biological sample and using the automated injector device the limit is about 50 ng/ml. Increasing the sample size to 2 ml, using only 50 μl of sulfuric acid for back-extraction of the bases from the ethyl acetate and injecting the sample manually with a Hamilton syringe onto the column, a sensitivity of 10 ng/ml can easily be achieved. The variability of the assay over the whole concentration range (50—3000 ng/ml) as reflected in the coefficient of variation for normalized peak height ratios in typical standard curves was 4.6% and 6.0% for acebutolol and metabolite, respectively, in plasma and 6.1% and 9.7% for these compounds in the blood.

Results from precision studies in patient samples with the plasma assay are shown in Tables I and II. Assaying the same plasma sample six times on one day, a coefficient of variation of 0.9% and 2.3% for acebutolol and metabolite determinations respectively was found. Repeating the determination of five plasma samples on different days yielded coefficients of variation from 2.3% to 6.8% (mean: 4.3%) for acebutolol and from 2.1% to 5.5% (mean: 3.8%) for the metabolite. Tables III and IV show the corresponding precision values for the blood assay. The slightly higher coefficients of variation for the blood

TABLE I

INTRADAY PRECISION OF PLASMA ASSAY (PATIENT SAMPLE)

Replicate No.	Concentration (ng/ml)	
	Acebutolol	Metabolite I
1	1425	3023
2	1389	2862
3	1400	2875
4	1389	2887
5	1397	2875
6	1397	2975
Mean	1400	2916
C.V.	0.9%	2.3%

TABLE II

INTERDAY PRECISION OF PLASMA ASSAY (PATIENT SAMPLES)

Subject	Concentration of acebutolol/metabolite (ng/ml)				C.V. (%)	
	1	2	3	4	Acebutolol	Metabolite
A	319/937	312/917	300/898		3.1	2.1
B	439/1696	440/1744	388/1587	394/1672	6.8	3.9
C	779/1908	853/2057	783/1883	764/1807	5.0	5.5
D	273/523	294/554	286/508	267/528	4.4	3.6
E	1147/2782	1130/2702	1153/2626	1193/2872	2.3	3.8
				Mean	4.3	3.8

assay, both within-day (4.0%, 4.5%) and between-day (7.1%, 5.4%), are probably due to the additional sample clean-up step necessary for blood. An additional possible source for the increased variability is the pipetting of the aliquot of sample (increased viscosity of blood) taken for assay.

The assay procedure was checked not only for precision but also for a consistent trend (bias) by evaluation of replicate determinations of plasma or blood samples spiked with a quantity of compound unknown to the analyst. As can be seen from Table V the coefficient of variation in repeated measurements in the low and high range of parent drug and metabolite concentrations was less than 4% and there appears to be no significant bias introduced in the assay. Precision and bias in spiked blood samples is shown in Table VI. Although the variability is higher than for the plasma assay the accuracy of the analytical procedure is still very acceptable.

To determine whether there are differences in plasma and whole blood concentrations of acebutolol and metabolite, whole blood samples and the plasma derived from them were both assayed. As can be seen from Table VII the blood: plasma ratio for acebutolol was very close to 1.0 for all the seven samples tested while metabolite levels determined in blood tended to be higher than in plasma (mean: +11%). This might be due to binding of the metabolite to red

TABLE III

INTRADAY PRECISION OF BLOOD ASSAY (PATIENT SAMPLE)

Replicate No.	Concentration (ng/ml)	
	Acebutolol	Metabolite I
1	517	1188
2	568	1333
3	525	1235
4	533	1242
5	529	1268
6	564	1341
Mean	539	1268
C.V.	4.0%	4.5%

TABLE IV

INTERDAY PRECISION OF BLOOD ASSAY (PATIENT SAMPLES)

Subject	Concentration of acebutolol/metabolite (ng/ml)						C.V. (%)	
	1	2	3	4	5	6	Acebutolol	Metabolite
F	(0)* /377	(28) /380	(26) /369	(5) /410			—	4.7
G	181/682	185/698	210/678	186/632	185/641	152/700	10.1	4.3
H	283/881	279/852	244/750	281/777	262/893		6.2	7.7
I	778/1215	721/1185	812/1274				6.0	3.7
K	1606/2959	1551/2975	1430/2671	1419/2615			6.1	6.7
						Mean	7.1	5.4

*Figures in parentheses denote estimates below assay sensitivity.

TABLE V

PRECISION AND BIAS IN PLASMA ASSAY (SPIKED SAMPLES)

Replicate No.	Measured concentration of acebutolol/metabolite (ng/ml)	
	Sample 1	Sample 2
1	284/276	1735/1720
2	269/290	1730/1689
3	268/284	1716/1695
4	263/277	1699/1696
5	280/279	1746/1718
Mean	273/281	1725/1704
C.V.	3.2%/2.1%	1.0%/0.84%
Added amount (ng) per ml	290/290	1690/1693
Bias	—5.8%/—3.1%	+2.1%/+0.7%

TABLE VI

PRECISION AND BIAS IN BLOOD ASSAY (SPIKED SAMPLES)

Replicate No.	Measured concentration of acebutolol/metabolite (ng/ml)	
	Sample 1	Sample 2
1	469/103	1681/341
2	488/101	1657/344
3	457/108	1646/323
4	517/117	1651/343
5	511/ 95	
Mean	488/105	1659/338
C.V.	5.3%/7.9%	0.9%/2.9%
Added amount (ng) per ml	507/102	1775/356
Bias	—3.7%/+2.7%	—6.5/—5.1%

TABLE VII

COMPARISON OF BLOOD AND PLASMA LEVELS OBTAINED IN THE SAME SAMPLE

Subject No.	Concentration of acebutolol/metabolite (ng/ml)		Blood:Plasma ratio for acebutolol/metabolite
	in blood	in plasma	
1	698/1223	706/1042	0.99/1.17
2	1859/1230	1836/1006	1.01/1.22
3	1100/1798	1067/1817	1.03/0.99
4	515/684	489/620	1.05/1.10
5	546/1285	517/1288	1.05/1.0
6	171/402	168/312	1.02/1.29
7	2218/3526	2295/3650	0.97/0.97
Mean			1.02/1.11

blood cells. The significance of such a finding is, however, unclear since there are no definitive studies correlating plasma or blood levels of acebutolol and metabolite to the pharmacodynamic response. Since the plasma assay, however, is faster and more accurate, analysis of plasma, where possible, appears to be preferable to determinations of whole blood.

ACKNOWLEDGEMENT

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Biomedical Applications

CHROMBIO. 357

TRACE ANALYSIS OF THE MIF* ANALOGUE PAREPTIDE IN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SHORT-WAVELENGTH EXCITATION FLUOROMETRY

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SUMMARY

A high-performance liquid chromatographic procedure was developed and applied to analysis of the pharmacologically active MIF* analogue pareptide in human plasma. The procedure involves formation of a fluorescent 7-chloro-4-nitrobenzyl-2-oxa-1,3-diazole (NBD-Cl) pareptide derivative followed by separation of the NBD derivative from plasma components on a 30-cm microparticle octadecylsilane bonded column. The separated derivative was quantitated using a short-wavelength excitation fluorometric detector. The detection limit of pareptide in plasma samples was 5 ng or 17 pmoles per ml of plasma. In the absence of plasma, the corresponding on-column detection limit was 0.5 pmoles.

INTRODUCTION

The analysis of a pharmacologically active peptide compound in body tissues presents a challenge to the analytical chemist; the relatively low concentrations involved and the presence of other peptides, proteins and amino acids complicate the problem. Furthermore, the peptide of interest in this study has no natural fluorescence or strong UV absorption and the only readily derivatized functional group is the secondary amine of the proline moiety. The peptide investigated was L-prolyl-N-methyl-D-leucyl-glycinamide, a synthetic analogue of MIF* [1—5]. The USAN*** name of this MIF analogue tripeptide is pareptide.

In view of the above complications, high-performance liquid chromatography(HPLC) of the fluorescent pareptide derivative was investigated. The reagent used for this purpose was 7-chloro-4-nitrobenzyl-2-oxa-1,3-oxadiazole (NBD-Cl) [6], which reacts with the prolyl secondary amino group. The

*MIF = melanocyte stimulating hormone release-inhibiting factor.
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***USAN = United States Adopted Name.

advantages of this reagent over the frequently used dansyl chloride [7] are lower fluorescent excess reagent background and higher solubility and stability of the derivative in aqueous solution. Although the NBD derivative, unlike the dansyl derivative, can be excited in the visible range, previously reported [8] short-wavelength excitation was used since it yielded lower detection limits.

There are numerous published applications of NBD derivatization to the analysis of thiols, phenols, and primary and secondary amines [9–14]; however, a study of optimum conditions for NBD derivatization was not reported. In the present study, the yield of NBD derivatization of the prolyl-tripeptide secondary amine was investigated as a function of pH, time, and reagent excess. The optimized conditions were applied to analysis of spiked human plasma samples. A calibration curve was prepared and detection limits were determined.

EXPERIMENTAL

Materials and reagents

The MIF analogue pareptide was obtained from Ayerst Research Labs. (Montreal, Canada). NBD-Cl reagent was purchased from Regis (Morton Grove, Ill., U.S.A.). Glass-distilled acetonitrile, ethyl acetate, methanol, and pentane were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). ACS analytical reagent grade diethyl ether, ammonium carbonate, dibasic potassium phosphate, and trichloroacetic acid were purchased from Mallinckrodt (Jersey City, N.J., U.S.A.).

Spherisorb-ODS chromatographic support (particle size 5 μm) was purchased from Spectra-Physics (Santa Clara, Calif., U.S.A.), while SAS-Hypersil was purchased from Shandon Southern Instruments (Sewickley, Pa., U.S.A.). Empty stainless-steel columns (4.1 mm I.D.) and zero-dead-volume fittings were purchased from Alltech (Arlington Heights, Ill., U.S.A.). The columns were packed in-house at 8,000 p.s.i., in an acetone slurry, with a Haskel Engineering and Supply Company (Burbank, Calif., U.S.A.) slurry packing apparatus.

Chromatographic equipment

Samples to be chromatographed were injected on column with a Valco CV-6-UHP 50-μl loop valve (Valco Instruments, Houston, Texas, U.S.A.). Chromatographic elution was monitored by a Schoeffel FS-970 fluorescence detector (Schoeffel, Westwood, N.J., U.S.A.). A Schott-Optical Glass (Duryea, Pa., U.S.A.) KV 370 cut-off filter was used to filter the fluorescence emission. Detector output was recorded with a Linear Model 385 recorder (Rainin Instruments, Brighton, Mass., U.S.A.).

Treatment of plasma prior to derivatization

Human blood plasma samples were spiked with an appropriate amount of MIF analogue pareptide to yield final concentrations ranging from 10 to 100 ng/ml. Aliquots (2 ml) of plasma samples were shaken briefly and allowed to stand for approximately 5 min. Trichloroacetic acid (100 μl of a 500 mg/ml solution) was added to each tube to precipitate the proteins. The tubes were capped, shaken for 5 min, and centrifuged at 2000 *g* for 25 min in an Inter-

national Equipment (Needham Heights, Mass., U.S.A.) Model UV centrifuge. The supernatant was decanted into a 10-ml screw-cap vial and extracted with two 1-ml portions of diethyl ether to remove fats and lipids. Residual diethyl ether was removed by a 2-min nitrogen flush of the solution.

Derivatization

The method used for derivatization of MIF analogue pareptide in plasma with NBD-Cl reagent is a modification of previously published methods [7, 11—14]. In general, previously published procedures [10—13] specified pH control with sodium bicarbonate or acetate buffers and derivatization temperatures ranging from 55° to 80°. In this study, saturated dibasic potassium phosphate solution, the pH of which was adjusted to 11.5 with 50% potassium hydroxide, was added to the deproteinized plasma until pH 9.5 was reached. (The volume of phosphate buffer necessary to reach pH 9.5 can be predetermined, thus simplifying the procedure.) One volume of pH 9.5 plasma was then mixed with two volumes of an acetonitrile solution of NBD-Cl reagent (0.5 mg/ml).

Due to high concentration of buffer in the aqueous phase, the water—acetonitrile mixture yields a two-phase system. Since most of pareptide is in the water phase while the NBD reagent is in the acetonitrile phase, derivatization was facilitated by continuous tumbling of capped vials in a 50° water bath for 40 min. A rotating wheel device was used for this purpose.

The vials were then removed from the bath and acetonitrile was evaporated by a stream of nitrogen. To reduce the overload of the reversed-phase column and prolong its life, the aqueous phase of the derivatized plasma solution was extracted with one 2-ml aliquot of pentane—diethyl ether (1:1) solution, which removed some of the NBD derivatives less polar than the NBD derivative of MIF analogue pareptide. The remaining partially purified aqueous phase was extracted with three 2-ml aliquots of ethyl acetate and the organic layers were combined. This procedure was carried out in a centrifuge tube so that the samples could be spun (1 min at 1000 *g*) to yield better separation of the aqueous and organic phases. The ethyl acetate fraction was then blown to dryness with a stream of nitrogen and the residue was dissolved in the chromatographic solvent (1 or 2 ml, depending on the amount of pareptide NBD derivative).

The above procedure was simplified when pareptide was in water rather than plasma solution. The acetonitrile solution of the NBD reagent was added to pH 9.5 aqueous solution of pareptide and the pareptide was derivatized as described. After the reaction, acetonitrile was blown off and the remaining aqueous phase was appropriately diluted with the chromatographic solvent.

Chromatography

The analytical system employed to generate the calibration curve was a combination of two 15 cm × 4.1 mm I.D. columns of Spherisorb-ODS and a 5 cm × 4.1 mm I.D. pre-column of Hypersil-SAS connected in series. The injection loop size was 50 μl. The chromatographic solvent system was a mixture of aqueous ammonium carbonate (200 mg/l)—methanol—acetonitrile (60:25:15, v/v) and was pumped at a flow-rate of 1.0 ml/min. Samples were

injected with the 50-μl loop valve, which was flushed with chromatographic solvent between each sample injection. Fluorometric detector settings were as follows: excitation at 220 nm, KV 380 nm emission filter, 0.02 μa full-scale expansion fluorometric response range, sensitivity 4.3, and a time constant of 8.0 sec. The NBD derivative of pareptide had a retention time of ca. 23 min.

RESULTS AND DISCUSSION

Although the NBD-Cl reaction and its derivatization conditions have been previously reported [7, 10—14], analysis of pareptide in blood plasma necessitated further refinement of the reaction variables. Thus it was ascertained that pH 9.5 and a large (5000-fold) excess of the NBD-Cl reagent are necessary for optimum pareptide derivative yield in the presence of blood plasma constituents. It was also established that at 50° the optimum reaction time is ca. 50 min.

However, even under optimum reaction conditions, the apparent NBD-Cl reaction yield in blood plasma was 50—60% of the yield obtained in the absence of blood plasma constituents. To correct for the incomplete reaction yield, quantitation of the pareptide in blood plasma was based on a calibration curve obtained by spiking blank plasma with known amounts of pareptide.

Fig. 1 depicts the calibration curve observed in the 20—100 ng of pareptide per ml of plasma concentration range. The observed linearity of the curve and zero intercept indicate that the blank response is negligible and the percentage yield is constant in the effective concentration range. The linear relationship between the fluorometric detector response and concentration of pareptide in the spiked blood plasma also indicates that the NBD-Cl reagent is present in a sufficient excess. Negligible blank plasma response may be attributed to the selectivity of the chromatographic system.

The chromatographic system selected for the separation and quantitation of pareptide NBD derivative involved reversed-phase chromatography on octadecylsilane (ODS) bonded support and detection with a fluorometric detector at a short excitation wavelength. The choice of the chromatographic system was based on the reproducibility of retention times, capacity of the support, and compatibility of the derivatized sample solution with the mixed aqueous—organic chromatographic solvent. Although an adsorptive support and organic chromatographic solvent system could also be used, adsorptive column supports usually have more limited column life and thus are subject to changing retention times and decreasing column efficiency. In view of the relative complexity of plasma extracts and presence of many relatively polar compounds, chromatography on adsorptive column supports was not investigated.

Selection of fluorometric detection was dictated by two considerations: inherent sensitivity of short excitation wavelength fluorescence [8] and the large excess of NBD reagent. Since only NBD derivatized tripeptide fluoresces, partial overlap between excess NBD reagent and NBD derivative of pareptide does not impair fluorometric quantitation.

Fig. 2 illustrates chromatograms of representative blank and spiked extracts of NBD derivatized human plasma extracts. Since the spiked sample contained 20 ng of pareptide per ml of plasma, the apparent detection limit is about 5 ng

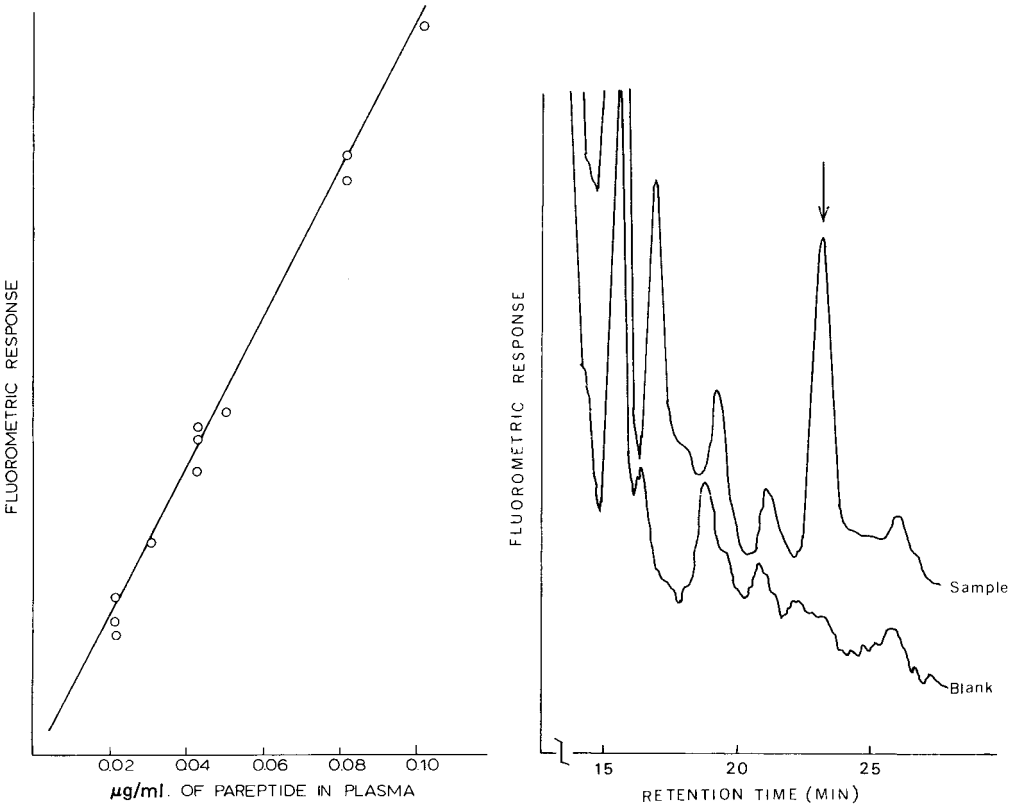

Fig. 1. Calibration curve obtained with human plasma containing variable amounts of pareptide.

Fig. 2. HPLC chromatograms of a blank plasma and a plasma sample containing pareptide at 20 ng/ml concentration; arrow identifies pareptide peak in the spiked plasma sample.

per ml of plasma. This limit involves an on-column injection of about 0.5 ng, which is equivalent to about 1.7 pmoles of tripeptide. Additional enhancement of sensitivity is possible if the chromatographic solvent is modified to yield a shorter retention time and consequently a sharper peak for the NBD pareptide derivative. However, relatively high background at short retention times requires chromatographic clean-up of the derivatized plasma extract before the analytical column solvent system can be modified. Since chromatographic clean-up of the derivatized plasma extract would significantly lengthen the procedure, it was not used in this study.

Fig. 3 illustrates chromatograms of pareptide derivatized in the absence of plasma and chromatographed using a solvent system which yielded a retention time of 8 instead of 22 min. The apparent detection limit under these conditions is about 0.5 pmoles which is almost 10 times more sensitive than the detection limit reported in the literature [41] for an NBD derivative of proline. The observed discrepancy may be attributed to the hemi-spherical mirror incorporated in the detector flow-cell and short excitation wavelength used in this

study. The latter contribution may be explained in terms of a relatively high electronic absorption band intensity of the singlet–singlet $\pi \rightarrow \pi^*$ transition which is promoted by the short-wavelength excitation [15]. The previously reported study [14] involved longer excitation wavelength and subsequently lower band intensity.

Although the observed 0.5-pmole detection limit for the NBD pareptide derivative in the absence of plasma is about five times less sensitive than the detection limit of previously reported [8] dansyl pareptide derivative, NBD derivatization was found to be more suitable for analysis of pareptide in the presence of plasma. Relative instability and high background of plasma derivatized with dansyl reagent offset its higher sensitivity advantage.

Since it is conceivable that some patient plasma samples could contain significant amounts of closely related tripeptides (e.g., natural MIF tripeptide) which could interfere significantly with the detection and quantitation of MIF analogue tripeptide, a mixture of pareptide and natural MIF tripeptide-NBD derivatives was chromatographed. Although the two tripeptide compounds have similar molecular structure, a complete separation of the two NBD-tripeptide derivatives was observed (Fig. 4). As expected, natural MIF tripeptide has a shorter retention time since it is a more polar compound than pareptide.

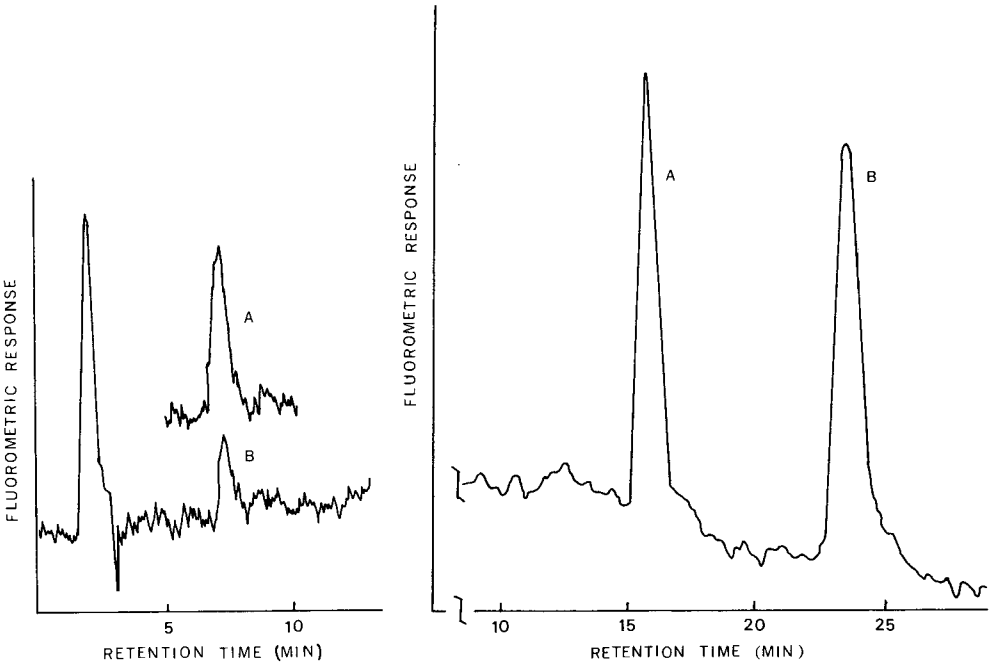

Fig. 3. HPLC chromatograms of NBD pareptide derivatives formed and chromatographed in the absence of plasma; scans A and B represent 0.4-ng and 0.2-ng on-column injection peaks of pareptide, respectively.

Fig. 4. HPLC chromatogram of the natural MIF tripeptide (A) and pareptide (B) NBD derivatives.

Subsequently, the natural MIF tripeptide cannot be used as an internal standard in our procedure since its peak is in an area where large excipient peaks are usually observed. However, an internal standard is not necessary in this procedure since no bias and a linear calibration curve (Fig. 1) were observed.

CONCLUSIONS

In conclusion, an NBD derivative of the MIF analogue pareptide was separated from plasma constituents by a HPLC reversed-phase system. As was reported in a previous publication [8], the fluorometric detection limit was enhanced by the use of short-wavelength excitation. In the presence of plasma excipients the apparent detection limit of pareptide is 5 ng per ml of plasma. Further enhancement of method sensitivity is possible with additional plasma extract clean-up which could allow shorter retention time for the pareptide NBD derivative and consequently enhanced peak height. Another method refinement which could increase the accuracy of the procedure involves use of an internal standard. However, the calibration curve obtained with spiked human plasma samples indicates that the present procedure can quantitate pareptide in human plasma samples in the low nanogram (or picomole) concentration range without the aid of an internal standard.

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Note

Determination of dextromethorphan in serum by gas chromatography

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Dextromethorphan (I) [(+)-3-methoxy-17-methylmorphinan] is a widely used antitussive agent of synthetic origin. It is sometimes rated the equal of codeine for this utility [1], though this is not universally true [2]. Very little is known about the pharmacokinetic behavior of I. One difficulty has been the lack of a sensitive, specific method for its determination in biological fluids. Most existing methods do not have the sensitivity for analyses in the low nanogram range [3—5]. The reported gas chromatographic (GC) methods [4, 5] are capable of detecting I in biological materials at levels in the low microgram range. However, it was not established that this sensitivity was adequate for the analysis of actual samples. I was reportedly found in human blood using a fluorometric method [3]. The specificity of this method was not established, and the interference, if any, of metabolites was not reported. Due to the lack of a sensitive determination for I, it was suggested that bioavailability studies be conducted using plasma levels of (+)-17-methylmorphinan-3-ol (dextrorphan), an active metabolite [6]. Although it was assumed that I would not interfere because of the extremely small amounts present, the interference of other metabolites was not determined. (+)-Morphinan-3-ol, for example, has a similar fluorescence spectrum [7]. Recently a sensitive, specific radioimmunoassay was described [8] which was capable of detecting approximately 1 ng/ml of I in plasma. No I was detected in the plasma of one human subject given a single 0.5-mg/kg dose. In a study involving seven subjects [2], 43% of a 60-mg dose of [N-methyl-^{14}C]I · HBr was excreted in the urine in 24 h and only 0.12% was recovered in feces. The maximum amount of radioactivity in plasma corresponded to about 20 ng/ml of I. These figures would have included any metabolite containing an N-methyl group, such as dextrorphan.

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This report describes a sensitive GC method for the determination of I in serum, and the application of this method to sera from dogs and humans after single doses of I · HBr.

EXPERIMENTAL

Reagents

All chemicals were analytical-reagent grade. Benzene, isopropyl alcohol, methylene chloride, and isooctane were a glass-distilled chromatographic grade obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Dextromethorphan · HBr was obtained from Hoffmann-LaRoche (Nutley, N.J., U.S.A.) and diphenylpyraline · HCl was purchased from K & K Labs. (Plainview, N.Y., U.S.A.). (+)-3-Methoxymorphinan was prepared from I and cyanogen bromide (Eastman-Kodak, Rochester, N.Y., U.S.A.) using the procedure of Clark [9]. The tartrate salt was recrystallized twice from ethanol—water, m.p. 209—211°. The structure was verified by elemental analysis and mass spectrometry. Dextrorphan tartrate and (+)-morphinan-3-ol were obtained from Hoffmann-LaRoche and Boehringer (Ingelheim am Rhein, G.F.R.), respectively. Pentafluorobenzyl chloroformate and trichloroethyl chloroformate were obtained from Pierce (Rockford, Ill., U.S.A.) and Aldrich (Milwaukee, Wis., U.S.A.), respectively.

Apparatus

The samples were analyzed using a gas chromatograph (Model 7610A, Hewlett-Packard, Avondale, Pa., U.S.A.) equipped with a ^{63}Ni electron-capture detector. A silanized glass column (120 cm × 4 mm I.D.) packed with 3% OV-25 on 80—100 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) was used at 250°. The injection port and detector were operated at 265° and 300°, respectively, with a 150-μsec pulse interval for the detector. The flow-rate of the carrier gas (5% methane in argon) was 50—55 ml/min. Under these conditions, the retention times of the pentafluorobenzyl carbamates of I and diphenylpyraline were 7.5 and 8.9 min, respectively, and the respective trichloroethyl carbamates eluted at 8.2 and 9.8 min.

Procedure

Two-milliliter aliquots or less of serum were diluted with 2 ml of distilled water in a 13-ml glass-stoppered centrifuge tube and a quantity of the internal standard, diphenylpyraline · HCl, roughly equivalent to the mid-point of the standard curve was added to all samples and standards. The mixture was made alkaline with 0.5 ml of 2 *M* potassium hydroxide and extracted vigorously with 4.0 ml of benzene—isopropyl alcohol (9:1, v/v) for 5 min. The upper layer was transferred to a 13 × 100-mm test-tube after centrifugation and one drop of acetic anhydride was added and mixed. After 5 min, 2.0 ml of 0.05 *M* sulfuric acid was added and the mixture was agitated for 1 min on a rotary tube mixer. After initially discarding most of the benzene, the sample was centrifuged and all remaining benzene was carefully removed. The remaining aqueous sample was made alkaline with 0.3 ml of 2 *M* potassium hydroxide and extracted with 2.0 ml of benzene—isopropyl alcohol (9:1, v/v) for 1 min on a rotary mixer.

After centrifugation, the benzene layer was transferred to a 2-ml glass ampoule and evaporated under a stream of nitrogen in a warm sand bath. A 0.25-ml aliquot of 0.5% (v/v) pentafluorobenzyl chloroformate (trichloroethyl chloroformate was used for the human samples) in methylene chloride and 0.15 ml of 5% (w/v) aqueous sodium bicarbonate were added to the sample, and the ampoule was sealed and heated at 80° in a sand bath for 2 h.

After cooling, the ampoule was scored, broken about 1 cm above the stricture, and the contents were evaporated to dryness under a stream of nitrogen in a warm sand bath. One milliliter of isooctane and 0.5 ml of 0.5 *M* potassium hydroxide in methanol—water (3:1, v/v) were added and mixed for 1 min on a rotary mixer. After centrifugation, the upper layer was transferred to a vial with PTFE-lined screw cap and concentrated before analysis if necessary. A standard curve was prepared daily (peak height ratio vs. concentration) by analyzing in duplicate at least three standards in serum. Linearity was observed through 150 ng/ml, the maximum level usually required.

In vivo studies

Three fasted male mongrel dogs received 1.0-mg/kg doses of I · HBr in aqueous solution by three routes of administration — intravenous (i.v.), intraperitoneal (i.p.), and per os (p.o.), with at least one week between doses. Blood samples were collected from the jugular vein 0.5, 1, 2, 3, 4, and 6 h after drug administration.

Twelve fasted male human volunteers, 21—30 years and 60—100 kg, were given single 20-mg doses of I · HBr p.o. The drug was administered as part of an experimental liquid formulation also containing 60 mg of pseudoephedrine · HCl and 200 mg of guaifenesin. Venous blood samples were taken 0.5, 1, 1.5, 2.5, and 5 h after drug administration.

Serum was prepared from all blood samples and stored at —15°.

RESULTS AND DISCUSSION

GC analysis of I

The absence of reactive groups on I makes the use of common acylating agents impossible. The tertiary nitrogen is susceptible to derivatization with chloroformates [10], with the loss of a methyl group, and this approach has been used in the development of a technique for the determination of a number of tertiary amines [11—13]. We have found this general method, with some modification, applicable to the determination of I in serum, with detection limits of < 1 ng/ml. Pentafluorobenzyl chloroformate is preferred for greater sensitivity, but trichloroethyl chloroformate has been used with similar results. Solvent extractions combined with a back-extraction with dilute aqueous acid provided adequate purification prior to derivatization. Sodium bicarbonate was superior to sodium carbonate as a catalyst for the derivatization reaction, and an aqueous bicarbonate solution, resulting in a two-phase system, gave higher yields than the solid reagent. The utility of alcoholic alkali for the reduction of interference resulting from the derivatizing reagent [13] was confirmed.

The derivatizing reagent also reacts with (+)-3-methoxymorphinan (II) to

form the same derivative, necessitating the removal of II with acetic anhydride. When 1.0 μg of II was added to control serum, less than 0.5% was recovered using this procedure. The interference of dextrorphan and (+)-morphinan-3-ol was likewise negligible (< 0.5%). The total amount of I + II can be estimated by omitting the acetic anhydride. This has not resulted in substantially larger values than for I alone, and it is known that II is not a major metabolite [14]. The analysis of ten 1-ml control serum samples spiked with 50 ng/ml I resulted in a mean of 50.7 ± 2.4 (S.D.).

Canine serum I

Serum I was determined in dogs after i.v., i.p., or p.o. administration of 1-mg/kg doses of I · HBr (Table I). The mean concentration of I in serum 0.5 h after i.v. administration was 78 ng/ml. The mean terminal $t_{1/2}$ after i.v. administration was 2.3 h. Values of 2.5 and 3.9 h were reported for two dogs treated with 2 mg/kg I i.v. [8]. After i.p. administration, there was a marked reduction in the amount of I present in serum with a further decrease to only about 2 ng/ml after oral administration. This contrasts with the results obtained with hydrocodone, where serum drug levels after i.v. and p.o. administration were similar [15]. Poor oral availability could be associated with low absorption, but the fact that i.p. availability was also low suggests a first-pass metabolic effect [16]. The rapid and extensive metabolism of I has been described

TABLE I

SERUM CONCENTRATIONS OF I AFTER INTRAVENOUS, INTRAPERITONEAL, AND ORAL ADMINISTRATION OF 1 mg/kg OF I · HBr IN DOGS

Hours	Serum I (ng/ml as free base)*
Intravenous administration	
0.5	78 ± 2
1.0	52 ± 5
2.0	40 ± 10
3.0	28 ± 6
4.0	21 ± 6
6.0	12 ± 6
Intraperitoneal administration	
0.5	9.5 ± 5.1
1.0	9.8 ± 4.8
2.0	7.7 ± 3.1
3.0	5.5 ± 2.1
4.0	4.5 ± 1.7
6.0	2.9 ± 1.1
Oral administration	
0.5	2.1 ± 0.6
1.0	2.1 ± 0.9
2.0	1.6 ± 0.8
3.0	1.2 ± 0.8
4.0	0.9 ± 0.9
6.0	0.4 ± 0.6

*Mean of three animals

previously [8, 14]. The mean area under the serum concentration vs. time curve for the oral treatment in the present study was 3.8% of the i.v. value. This is somewhat lower than reported values of 7 and 18% [8].

The difference in the results from p.o. and i.p. administration may reflect less than complete absorption and/or gut-wall metabolism, a process known to be important for some compounds [17].

Human serum I

Serum I levels were very low in humans after oral administration. A mean peak value of < 2 ng/ml was obtained after a 20-mg oral dose of I · HBr (Table II). This contrasts with peak drug levels of about 20 ng/ml after a 10-mg oral dose of hydrocodone bitartrate [15] and about 30 ng/ml after a 15-mg oral dose of codeine [18]. Intersubject variability in the present study was high,

TABLE II

SERUM CONCENTRATIONS OF I IN HUMANS AFTER A SINGLE 20-mg ORAL DOSE OF I · HBr

Hours	Serum I (ng/ml as free base)*
0.5	0.1 ± 0.1
1.0	0.7 ± 0.8
1.5	1.1 ± 1.3
2.5	1.8 ± 2.2
5.0	1.4 ± 1.6

*Mean of twelve subjects ± S.D.

indicating possible differences in absorption or metabolism of the drug. Four of twelve subjects had peak serum drug levels of > 2 ng/ml with one value of 7.9 ng/ml and seven had values < 1 ng/ml. This may account for the fact that Dixon et al. [8] were unable to detect I in the plasma of one subject using a radioimmunoassay with a sensitivity of about 1 ng/ml.

Preliminary work indicates that the GC method described above can also be used to analyze urine samples. Although it appears that serum drug levels may be too low in most cases to permit detailed kinetic analyses, this problem can perhaps be circumvented by using excretion data.

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Note

Analysis of the basic 5-nitroimidazole nimorazole in blood by reversed-phase high-performance liquid chromatography, and its application to pharmacokinetic studies in individual mice

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Nitrimidazole derivatives are widely used clinically as antimicrobial agents [1] and to sensitise hypoxic tumour cells to radiation [2]. In the latter case, monitoring of plasma or saliva drug concentrations has been used to estimate the potential toxic and therapeutic effects [3, 4].

The in vivo concentrations of two of the more widely used drugs, misonidazole (Roche, Welwyn Garden City, Great Britain) and metronidazole (May & Baker, Dagenham, Great Britain), can be determined by reversed-phase high-performance liquid chromatography (HPLC) [5, 6]. As part of a series of studies in this laboratory on the pharmacokinetics of hypoxic cell radiosensitisers, it has been found that whereas the above HPLC methods are readily adapted for the assay of other essentially unionised nitroimidazoles, the method is often highly unsatisfactory for more strongly ionised derivatives. These latter include a class of compounds of particular interest, the basic morpholine derivatives, e.g. nimorazole, used clinically mainly as an antitrichomonal agent [7] (Fig. 1).

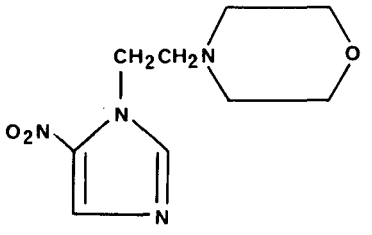


Fig. 1. Structural formula of nimorazole.

Reversed-phase HPLC, with aqueous methanol as the mobile phase and octadecylsilane as the stationary phase, gave poor chromatography with irreproducibility, broad asymmetrical peaks and poor resolution as the major problems. In the present paper a method is described for the estimation of nimorazole in whole blood by paired-ion reversed-phase HPLC [8].

In addition, the procedures were adapted to micro-scale to allow rapid analysis of 5-μl blood samples collected sequentially from individual mice, thus allowing detailed pharmacokinetic studies to be carried out with small quantities of drug. An application of the method is described, which demonstrates the dose-dependent pharmacokinetics of nimorazole in mice.

METHODS

Reagents

Nimorazole (4-[2-(5-nitroimidazol-1-yl)ethyl]-morpholine; nitrimidazine; Nagoxin) was supplied by Montedison Pharmaceuticals (Barnet, Great Britain), and 1-(2-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0269) by Roche.

Water was twice-distilled in glass. HPLC-grade methanol was obtained from Rathburn Chemicals (Walkerburn, Great Britain). Heptanesulfonic acid was obtained as a commercial preparation in aqueous solution containing acetic acid (PIC Reagent B-7) from Waters Assoc. (Milford, Mass., U.S.A.). The mobile phase was prepared by dissolving 15 ml of this reagent in 1 l of 30% methanol in water. This gave a heptanesulphonic acid concentration of 5 m*M* and a pH of 3.2. The mobile phase was passed through a 0.45-μm Millipore filter (for aqueous solvents) and de-aerated under vacuum before use.

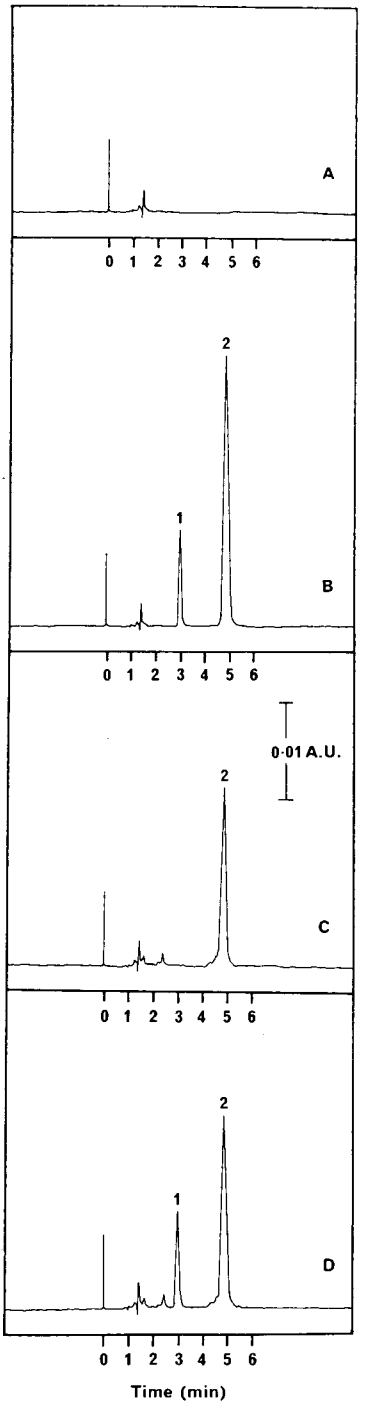
Sample preparation and chromatography

Blood samples (whole or diluted) were mixed thoroughly with 9 vol. methanol containing the standard Ro 07-0269 (11 μg/ml). After centrifugation (3000 *g*, 10 min), 10-μl samples of supernatant were chromatographed at ambient temperature using a Waters Model ALC/GPC-244 liquid chromatograph equipped with a U6K sample loop injector and a μBondapak C_{18} (octadecylsilane) column (30 cm × 3.9 mm I.D., particle size 10 μm) (Waters Assoc.). The mobile phase consisting of methanol—water (30:70) containing PIC Reagent B-7 was delivered at a constant flow-rate of 2 ml/min (pressure 20 MPa or 3000 p.s.i.). The absorbance of the column effluent was monitored at 313 nm using a Waters Model 440 absorbance detector coupled to a Servoscribe chart recorder (chart speed 10 mm/min).

Pharmacokinetics

Adult male BALB/c mice, weighing approx. 25—30 g were obtained from the breeding colony at N.I.M.R. (Mill Hill, London, Great Britain). They were allowed PRD nuts (Labsure Animal Diets, Poole, Great Britain) and tap water, both ad libitum.

For pharmacokinetic studies nimorazole was dissolved at appropriate concentrations in Hank's balanced salt solution and injected intraperitoneally in a volume of 40 ml/kg body weight. In the initial studies mice were bled at appropriate times by cardiac puncture under diethyl ether anaesthesia, and


A
0 1 2 3 4 5 6
2
1
B
0 1 2 3 4 5 6
0·01 A.U.
2
C
0 1 2 3 4 5 6
2
1
D
0 1 2 3 4 5 6
Time (min)

duplicate samples of heparinised whole blood (usually 0.1 ml) were then analysed. Subsequently, however, the method was scaled down to allow the analysis of sequential blood samples taken from the tail of individual mice. Duplicate 5-μl blood samples were collected in Microcap pipettes (Drummond, Broomall, Pa., U.S.A.) and mixed with 45 μl water. They were stored at $-20°$ and analysed as described above, with the Ro 07-0269 standard added in the methanol.

Ten duplicate 5-μl samples add up to a total volume of 0.1 ml. This represents about 5% of the blood volume in the mouse.

RESULTS

Fig. 2A shows the chromatogram of a methanol extract of pooled, control undiluted whole blood from a group of BALB/c male mice; the standard Ro 07-0269 was omitted. Similar results were obtained for extracts of whole blood collected at various times after mice received Hank's balanced salt solution, the vehicle used for nimorazole injection.

The chromatogram in Fig. 2B is that of a methanol extract of control whole blood spiked with nimorazole (peak 2) and containing the Ro 07-0269 standard (peak 1). These had retention times of 4.9 min and 3.0 min respectively, and the corresponding capacity factors were 3.27 and 2.00 respectively. Coefficients of variation for the retention times, calculated from 64 samples over 2 days, were <1% for both nimorazole and the Ro 07-0269 standard.

Fig. 2C and D show chromatograms of methanol extracts of whole blood from a mouse which received nimorazole (1 g/kg), by intraperitoneal (i.p.) injection in Hank's balanced salt solution, 4.3 h previously; the standard was omitted from Fig. 2C but included in Fig. 2D. Both chromatograms contain a peak corresponding to nimorazole, and also contain several smaller peaks which may be nimorazole metabolites. Comparison of Fig. 2A—D shows that the control blood contains no interfering peaks, and that the internal standard is resolved from the parent drug and its putative metabolites.

To determine the efficiency of extraction of nimorazole from whole blood, samples of blood and of water, containing various known concentrations of nimorazole, were analysed as described for blood in the Methods section. Comparison of the data for blood and water samples showed that over the concentration range studied (5—1000 μg/ml) the extraction efficiency was 100%. The extraction efficiency of the Ro 07-0269 was also 100%. A calibration plot of normalised peak height ratio (peak height nimorazole:peak height standard) against blood nimorazole concentration was linear over the range 5—1000

Fig. 2. HPLC chromatograms of methanol extracts of undiluted whole blood from BALB/c male mice. Chromatographic conditions: column, μBondapak C_{18} (30 cm × 3.9 mm I.D.; particle size 10 μm); mobile phase, methanol-water (30:70) containing PIC Reagent B-7 (Waters Assoc.); flow-rate, 2 ml/min; column pressure, 20 MPa or 3000 p.s.i.; temperature, ambient; detection, absorbance at 313 nm; sample volume, 10 μl; chart-speed, 10 mm/min. (A): Control whole blood. Ro 07-0269 standard omitted. (B): Whole blood spiked with nimorazole (500 μg/ml; peak 2). Peak 1 corresponds to the Ro 07-0269 standard (11 μg/ml methanol). (C): Whole blood from a mouse injected with 1 g/kg nimorazole i.p., 4.3 h previously. Ro 07-0269 standard omitted. (D): As (C) but contains Ro 07-0269 standard.

μg/ml (r = 0.999, p <0.001) and the intercept on the ordinate was not significantly different from zero (p >0.1).

The lower limit of sensitivity for nimorazole, as defined by a signal-to-noise ratio >3, was approx. 5 ng. Thus for a 10-μl injection the lower limit of sensitivity for nimorazole in undiluted whole blood was about 5 μg/ml. For the microprocedure the lower limit was approx. 50 μg/ml. The sensitivity could be improved by concentrating the methanol extract, but this proved unnecessary for the present studies.

Coefficients of variation were calculated for the ten independent replicate assays of the same blood sample. In a test to compare the coefficients of variation for the whole blood assay at different nimorazole concentrations, values obtained were 3.4% at 500 μg/ml and 3.9% at 33 μg/ml. In a separate test to compare the coefficients of variation for the two procedures, values

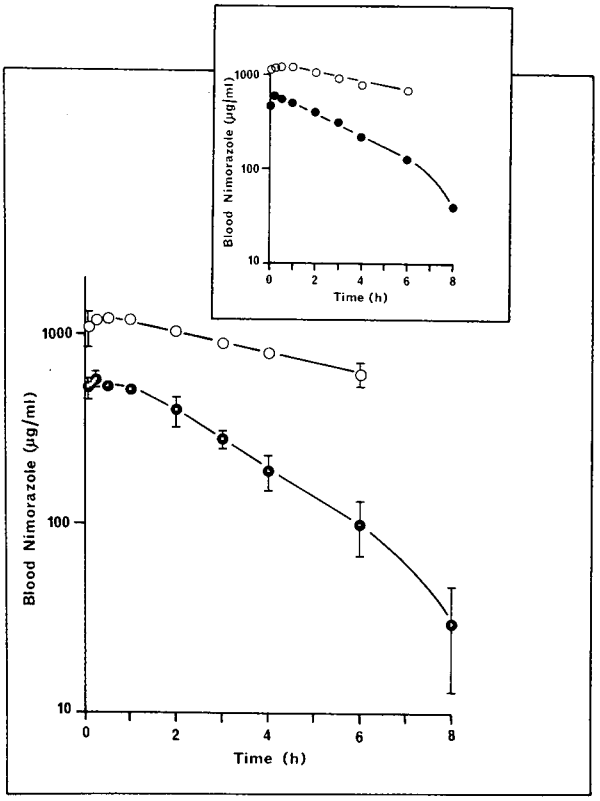


Fig. 3. Blood concentrations of nimorazole in BALB/c mice after i.p. injection of 1.31 g/kg (5 mmoles/kg, ○) or 0.655 g/kg (2.5 mmoles/kg, •). Three mice were studied at each dose; error bars show 2 S.E. Inset: data for a single mouse at each dose.

obtained were 5.1% for the whole blood assay and 3.4% for the microprocedure (500 μg/ml nimorazole in whole blood).

Typical values for height equivalents to a theoretical plate were 0.11 mm for both nimorazole and Ro 07-0269.

The HPLC method described above was used to investigate the pharmacokinetics of nimorazole in BALB/c male mice. Fig. 3 shows the mean concentrations of nimorazole in whole blood at various times after i.p. injection of 1.31 g/kg (5 mmoles/kg) and 0.655 g/kg (2.5 mmoles/kg) (three mice per group). Absorption from the peritoneum into the circulation is rapid and the elimination comparatively slow. Moreover, the elimination half-life is dose-dependent, and the low-dose data seem to reveal a deviation from first-order elimination kinetics.

The scaling down of the method allowed the pharmacokinetics to be determined in individual mice (e.g. see inset, Fig. 3). Apparent half-lives were calculated for data from 1—6 h inclusive using the method of least squares linear regression analysis. The values obtained were 2.47 h, 1.76 h and 2.01 h for the lower dose, and 6.89 h, 5.18 h and 4.77 h for the higher dose.

DISCUSSION

The advantages of HPLC for the assay of nitroimidazoles have been described previously [5]. Conventional reversed-phase HPLC was found to be unsuitable for the analysis of ionised nitroimidazoles, and the present paper describes a paired-ion method for the rapid analysis of the basic 5-nitroimidazole nimorazole present in whole blood at pharmacological or toxicological concentrations. The particular advantages of this method over conventional reversed-phase HPLC with methanol—water mixtures as the mobile phase are (1) improved reproducibility, (2) improved performance and peak symmetry and (3) increased resolution. Chromatograms from mice receiving nimorazole contained several small peaks not present in control samples. These were not seen using conventional reversed-phase HPLC. Although the peaks have not been identified it is likely that at least some of these are nimorazole metabolites. Two urinary metabolites have been found in man [7].

As would be expected, the method is not suitable for strongly acidic nitroimidazoles ($pK_a < 3$), e.g., some carboxylic acid derivatives and glucuronide metabolites. However, good results have been obtained using paired-ion chromatography at neutral pH with tetrabutylammonium phosphate as the counter-ion [9].

There is at the moment considerable interest in the design, synthesis and testing of new nitroimidazole compounds for use as radiosensitisers in the treatment of cancer [2, 10]. Most in vivo studies are carried out on mice, and comparative pharmacokinetic investigations in this species should represent an important aspect of the drug development programme. The sensitivity of the present HPLC method has allowed the sample preparation to be adapted to the

analysis of 5-μl blood samples and even less. This has two important advantages. Firstly, it allows sequential blood samples to be taken from the same mouse, and negates the need for anaesthetics and for the animal to be sacrificed at sampling. This allows pharmacokinetic investigations in individual mice, if necessary on several different occasions. Some differences in nimorazole half-life between individual mice are shown in the present paper.

The second advantage of this technique is that considerably less drug is required to obtain the necessary pharmacokinetic data. This is particularly important in the screening of nitroimidazole radiosensitisers, which have to be given in comparatively large doses, often greater than 5 mmoles/kg. Drug development costs could therefore be reduced with this technique.

The present HPLC method has been used to show that the elimination of nimorazole from mouse blood does not obey first-order kinetics, and that longer half-lives are seen at higher doses. Similar results have been obtained for these 2-nitroimidazole misonidazole in mice [11]. This demonstration of dose-dependent elimination kinetics has important implications for the selection and subsequent use of nitroimidazole misonidazole radiosensitisers.

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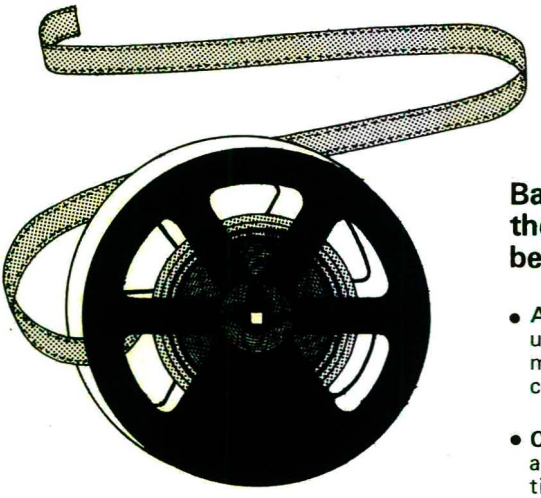

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