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CHROMBIO. 1461

AMINE METABOLITE PROFILE OF NORMAL AND UREMIC URINE USING GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method for the simultaneous analysis of phenolic amines and aliphatic amines in human urine is described. The amine metabolites in urine were extracted using Dowex 50W-X8 cationic resin, derivatized and analyzed by a gas chromatographic—mass spectrometric—computer system. The amine metabolites profile of 5 ml of urine was obtained with good gas chromatographic separation. The gas chromatographic method described here separates urinary phenolic amines, di- and polyamines and methylguanidine in a single chromatographic separation. The urinary levels of methylguanidine, putrescine, cadaverine, spermidine, *p*-tyramine, dopamine, and 3-methoxytyramine were quantitated by using a mass spectrometric technique. In uremic patients, only the urinary excretion of methylguanidine was increased in comparison with normal subjects, although the urinary excretion of other amines was decreased in uremic patients.

INTRODUCTION

Recently, gas chromatography—mass spectrometry (GC—MS) has been used to screen a number of metabolites in blood or urine. In particular, the analysis of organic acid in urine has been pursued by many investigators, and information

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has been obtained as to the diagnosis of patients with metabolic disorders [1—3]. However, very few studies have been devoted to the application of the profiling amine analysis to clinical diagnosis, since the amine levels were very low. It is important to analyze the urinary amines which are the intermediate metabolites of amino acids and possess biogenic activity.

LeGatt et al. [4] reported a new method for the simultaneous extraction and GC separation of trace amines: 2-phenylethylamine, *m*-tyramine, *p*-tyramine, *p*-octopamine, normetanephrine, and 3-methoxytyramine. Nelson et al. [5] analyzed 3-O-methylated catecholamine in human urine using ion-pair extraction and GC with electron-capture detection; they quantitated the amount of normetanephrine, metanephrine, and 3-methoxytyramine. Kawai and Tamura [6] quantitated urinary norepinephrine, epinephrine, and dopamine as the trifluoroacetyl derivatives using GC. The polyamines also have been analyzed using GC [7—9], ever since Russel et al. [10] reported an elevated polyamine concentration in the urine of cancer patients. However, a profiling analysis of all the amines, including phenolic and aliphatic amines, has not yet been attempted, except in our previous study [11].

The present study was undertaken to screen simultaneously phenolic amines and aliphatic amines in uremic urine using GC—MS.

MATERIALS AND METHODS

Urine samples

Twenty-four-hour urine samples were collected in containers containing 50 ml of 3 *N* hydrochloric acid and stored at −20°C until analyzed.

Sample preparation

As an internal standard, 40 nmol of N-3-aminopropyl-1,3-diaminopropane were added to 5 ml of urine. To hydrolyze the conjugated amines, 5 ml of concentrated hydrochloric acid were added to the samples and heated at 100°C for 16 h. The sample was evaporated to dryness and dissolved in phosphate buffer (pH 8) including 0.7 mol sodium chloride, and applied to Dowex 50W-X8 (H^+, 10 × 0.8 cm). After washing with 5 ml of water, 50 ml of phosphate buffer, and 30 ml of 1 *N* hydrochloric acid, the absorbed amines were eluted with 100 ml of 6 *N* hydrochloric acid. The eluate was evaporated to dryness with a rotary evaporator. For acylation, 200 μl of anhydrous ethyl acetate and 200 μl of pentafluoropropionic anhydride were added to the dry residue, and the amines were acylated at 70°C for 15 min. After cooling the sample was dried with a stream of nitrogen at room temperature and redissolved in 100 μl of anhydrous ethyl acetate. A 2-μl aliquot of this solution was subjected to GC—MS.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a JGC-20K gas chromatograph, a JMS D-300 double focusing mass spectrometer, and a JMA 2000 data processing system (JEOL, Tokyo, Japan). The gas chromatograph was equipped with a 3% OV-1 glass column (2 m × 2 mm I.D.). The column

temperature was programmed from 75 to 260°C at 6°C/min. The carrier gas was helium with a flow-rate of 30 ml/min. Electron-impact ionization mass spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 300 μA, ion source temperature 200°C, and accelerating voltage 3 kV.

Quantitative determination of amines

The calibration curves for the amines identified in the urine were obtained by adding a known amount of standard to 5 ml of deproteinized plasma. After addition of 40 nmol of N-3-aminopropyl-1,3-diaminopropane, the solution was hydrolyzed at 100°C for 16 h and applied to Dowex 50W-X8 resin column using the same procedure as with the urine sample. The calibration curves relating the concentration of methylguanidine, putrescine, cadaverine, *p*-tyramine, dopamine, 3-methoxytyramine, and spermidine to the ratio of the peak height of N-3-aminopropyl-1,3-diaminopropane, were obtained from the mass chromatogram. Ion *m/e* 246 was used for the quantitation of methylguanidine and ion *m/e* 176 for the quantitation of putrescine and cadaverine. Ion *m/e* 266 was used for the quantitation of *p*-tyramine, and ion *m/e* 428 for that of dopamine, and ion *m/e* 296 for quantitation of 3-methoxytyramine. Ion *m/e* 204 was used for the quantitation of spermidine, and for the monitoring of an internal standard, N-3-aminopropyl-1,3-diaminopropane.

RESULTS

The elution profile of putrescine, *p*-tyramine, 3-methoxytyramine, and dopamine on the Dowex 50W-X8 column was estimated. The washing buffer and elution buffer are the same as in the extraction procedure of Inoue and Mizutani [12], except for the elution volume of 6 *N* hydrochloric acid which was used for extraction of polyamine in tissue. The elution volume of putrescine was 13 ml of 6 *N* hydrochloric acid, but it was found that phenolic amines

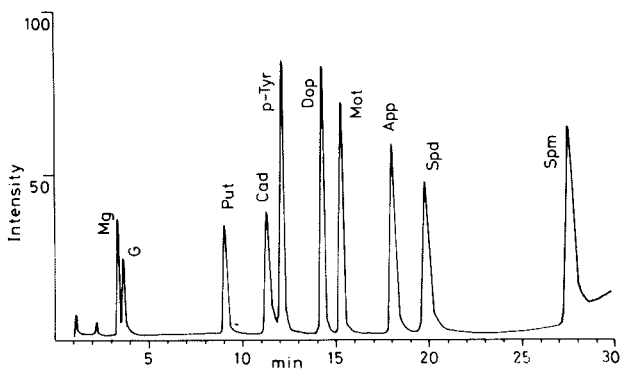

Fig. 1. Gas chromatogram of standard prepared with 50 nmol of each of the amines. Peaks: pentafluoropropyl derivatives of: Mg, methylguanidine; G, guanidine; Put, putrescine; Cad, cadaverine; p-Tyr, *p*-tyramine; Dop, dopamine; Mot, 3-methoxytyramine; App, N-3-aminopropyl-1,3-diaminopropane; Spd, spermidine; Spm, spermine. GC conditions were as follows: column, 3% OV-1 on Gas-Chrom Q (80—100 mesh), 2 m × 2 mm I.D.; column temperature, 75°C to 260°C at 6°C/min.

such as *p*-tyramine, 3-methoxytyramine, and dopamine were eluted with 100 ml of 6 *N* hydrochloric acid. The results suggest that it is possible to extract polyamines and phenolic amines simultaneously. Furthermore, sodium ion which was retained on the resin during the first washing was removed by washing with 1 *N* hydrochloric acid. The eluate without sodium ion was suitable for derivatization of the amines and GC analysis.

Fig. 1 shows a chromatogram of standard samples with 50 nmol of each of the amines. A good GC separation was obtained. The conditions were described under Gas chromatography—mass spectrometry.

Fig. 2 shows chromatograms of amines in the urine of a normal subject (Fig. 2A) and a patient with renal failure (Fig. 2B). Methylguanidine, guanidine, putrescine, cadaverine, *p*-tyramine, dopamine, 3-methoxytyramine, and spermidine were detected in normal and uremic urine. These amines were identified by comparison with mass spectra and retention times of pentafluoropropyl derivatives of authentic compounds in our laboratory.

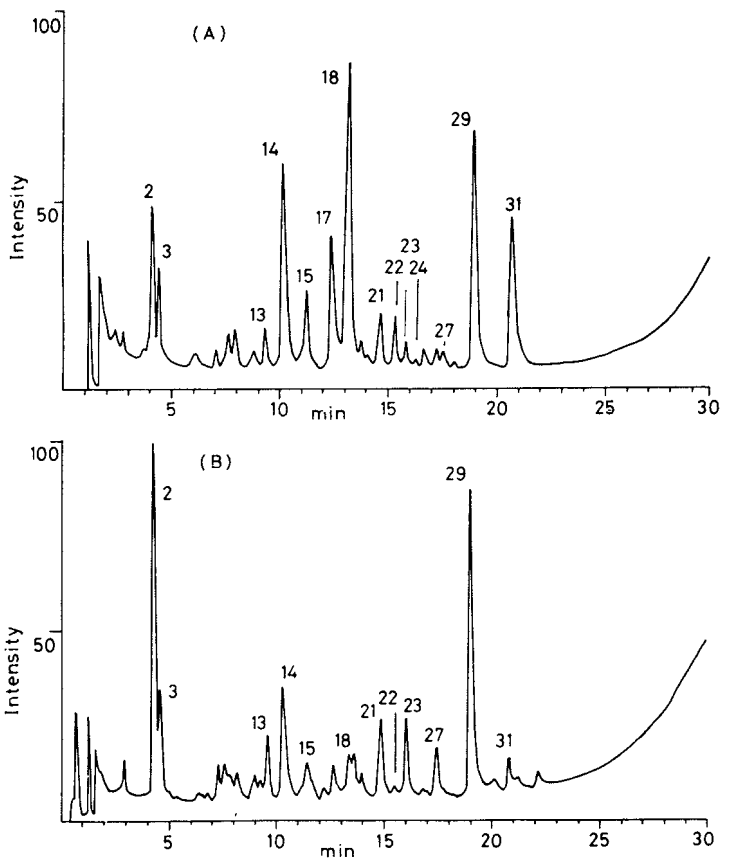

Fig. 2. Gas chromatograms of the urinary amines of (A) a normal subject and (B) a patient with renal failure. The extract was subjected to pertafluoropropyl derivatization and separated on a 3% OV-1 glass column.Peaks: 2, methylguanidine; 3, guanidine; 14, putrescine; 17, cadaverine; 18, *p*-tyramine; 22, dopamine; 24, 3-methoxytyramine; 29, N-3-aminopropyl-1,3-diaminopropane (internal standard); 31, spermidine.

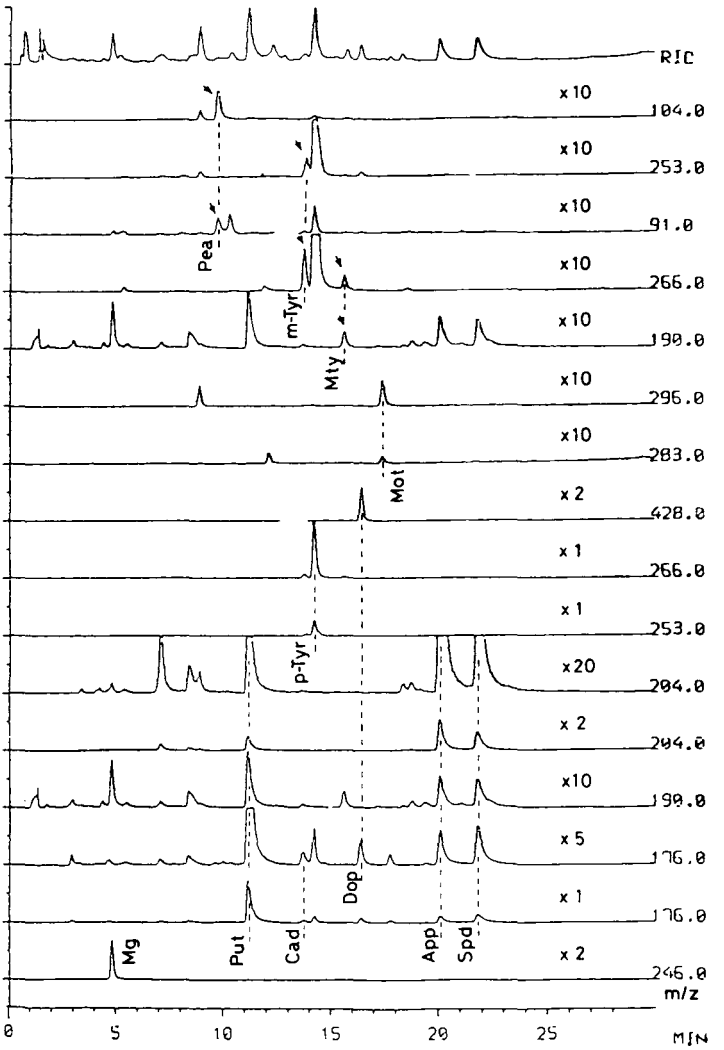

Fig. 3. Mass chromatogram of the urinary amines of a normal subject. Ions: *m/e* 246 for the detection of methylguanidine, *m/e* 176, 190 and 204 for putrescine and cadaverine, *m/e* 266 and 253 for *p*-tyramine, *m/e* 428 and 176 for dopamine, *m/e* 296 and 283 for 3-methoxytyramine, *m/e* 204, 190 and 176 for spermidine and N-3-aminopropyl-1,3-diaminopropane (internal standard), *m/e* 104 and 91 for 2-phenylethylamine (Pea), *m/e* 266 and 253 for *m*-tyramine (m-Tyr), *m/e* 190 and 266 for N-methyltyramine (Mty). For abbreviations, see Fig. 1.

The quantitation of urinary amines was performed using a mass chromatogram (Fig. 3, see Quantitative determination of amines). Table I shows the urinary excretion of seven amines in normal subjects and uremic patients. The levels of methylguanidine in urine of uremic patients were increased in comparison with that of normal urine, whereas those of other amines were decreased one-fourth to one-fifth.

TABLE I

URINARY EXCRETION OF SEVEN AMINES IN NORMAL SUBJECTS AND UREMIC PATIENTS, μmol/24 h (μmol/g CREATININE)

For abbreviations see Fig. 1.

	Put	Cad	Spd	p-Tyr	Dop	Mot	Mg	Creatinine (mg/dl)	Urine volume (l)
Control (n=9)	17.52±8.65	2.74±3.07	7.51±1.98	12.05±9.23	3.75±1.67	1.35±0.89	26.4±10.2		
$\bar{X}$±S.D.	(13.60±3.60)	(2.14±2.16)	(6.33±2.02)	(9.40±5.48)	(3.00±0.86)	(1.12±0.66)	(22.6±10.8)		
Uremia (n=7)									
I.S.	2.60(7.19)	1.36(3.76)	1.47(4.06)	2.39(6.60)	2.46(6.81)	0.48(1.32)	51.6(142.8)	47.6	0.76
M.S.	3.41(6.96)	0.18(0.37)	1.54(3.14)	3.42(6.97)	0.75(1.54)	0.34(0.70)	36.9(75.4)	92.5	0.53
T.I.	1.94(11.20)	0.53(3.44)	0.30(1.78)	0.74(4.28)	0.22(1.28)	0.09(0.55)	36.2(208.5)	36.2	0.48
S.Y.	1.49(6.88)	1.19(5.54)	0.45(2.12)	7.46(34.47)	0.24(1.14)	0.10(0.49)	40.2(186.0)	40.0	0.54
R.S.	3.70(16.27)	0.25(1.14)	0.59(2.64)	0.38(1.71)	0.12(0.57)	0.06(0.27)	31.3(139.3)	44.3	0.51
S.M.	4.68(8.49)	1.31(2.37)	1.13(2.06)	4.38(7.97)	0.81(1.47)	0.37(0.67)	39.6(72.1)	83.5	0.66
T.N.	1.82(3.31)	N.D.*	1.03(1.87)	1.50(2.73)	0.37(0.67)	0.58(1.05)	109.1(196.7)	38.5	1.44
$\bar{X}$ ±S.D.	2.80±1.08**	0.68±0.53***	0.93±0.45**	2.89±2.28§	0.71±0.75**	0.28±0.19§	49.2±25.0§		
	(8.61±3.80)	(2.37±1.86)	(2.52±0.76)	(9.24±10.5)	(1.92±2.02)	(0.72±0.32)	(145.8±51.5)		

* N.D. = not detected.

** $P<0.001$.

*** Not significant.

§ $P<0.05$.

DISCUSSION

Phenolic amines and aliphatic amines were profiled in urine and good recovery and reproducibility were obtained using GC—MS. MS which detects specific ions, in fact detected very small amounts of amines, although with less sensitivity than with selected ion monitoring. Fig. 3 shows a mass chromatogram of normal urine. The peaks at *m/e* 91 and *m/e* 104 appearing at a retention time of 9.8 min were considered to be the fragment ions of 2-phenylethylamine by comparing with the mass spectra and retention times of authentic compound. The peaks at *m/e* 190 and *m/e* 266 appearing at the retention time of 15.6 min were considered to be the fragment ions of N-methyltyramine by comparing with the mass spectra cited in the literature [13]. *m*-Tyramine was also detected at the retention time of 13.7 min in front of *p*-tyramine [13]. Many amine metabolites were extracted and separated using the present procedure.

In patients with renal failure, many metabolites which are normally excreted in urine are retained in blood, causing various uremic symptoms. Methylguanidine is noted to be one of the uremic toxins. It is also known that the levels of polyamines and tyramine are elevated in blood of uremic patients [14, 15]. The present finding that only the level of methylguanidine was increased in uremic urine is very striking. Stein et al. [16] also reported that the urinary excretion of methylguanidine was increased in uremia. It is considered that the increase of polyamine and tyramine in uremic blood results from decreased excretion, whereas increase of methylguanidine results form increased production.

The present finding as to the normal urinary *p*-tyramine level, 12.05 ± 9.23 μmol/24 h (9.40 ± 5.48 μmol/g creatinine), was about two times higher than in previous reports [13, 17—19] in which the unconjugated *p*-tyramine in the urine was quantitated. Our results that normal urinary putrescine and spermidine levels were 17.52 ± 8.65 μmol/24 h (13.60 ± 3.60 μmol/g creatinine) and 7.51 ± 1.98 μmol/24 h (6.33 ± 2.02 μmol/g creatinine) were in agreement with those of Marton et al. [20] and Denton et al. [7]. The fact that the normal urinary dopamine level was 3.75 ± 1.67 μmol/24 h (3.00 ± 0.86 μmol/g creatinine) was also in agreement with Kawai and Tamura's result [6]. However, our normal level of 3-methoxytyramine, 1.35 ± 0.89 μmol/24 h (1.12 ± 0.66 μmol/g creatinine), was 2 to 3 times higher than that of Nelson et al. [5].

The detection limit of these amines was about 0.2 nmol/ml in urine. Epinephrine, norepinephrine, metanephrine, normetanephrine, and synephrine could not be detected.

Scaro et al. [19] quantitated urinary free tyramine by using high-performance liquid chromatography with a fluorescence detector. They reported that patients with pheochromocytoma, neuroblastoma, and Parkinson's disease have elevated levels of urinary tyramine. In patients with pheochromocytoma or neuroblastoma, metabolic disorders of catecholamine have been reported by many investigators [21—24]. The present procedure for simultaneous quantitation of polyamines and phenolic amines such as 3-methoxytyramine and dopamine may be useful to study these diseases.

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CHROMBIO. 1453

ELECTRON-CAPTURE, CAPILLARY COLUMN GAS CHROMATOGRAPHIC DETERMINATION OF LOW-MOLECULAR-WEIGHT DIOLS IN SERUM

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SUMMARY

Research on alcoholism has revealed that concentrations of 1,2-propanediol, *d,l*-2,3-butanediol and *meso*-2,3-butanediol may be greater in the serum of chronic alcoholics than in the serum of social drinkers and nondrinkers. In connection with one of these studies, we developed methodology to determine these diols at the micromolar levels in 500 serum samples. The procedure consisted primarily of extraction of the serum with acetonitrile containing internal standard. The extract was then concentrated to dryness and reacted with *p*-bromophenylboric acid. The reaction mixture was injected into a gas chromatograph fitted with a capillary column and an electron-capture detector. The total coefficients of variation were best for 1,2-propanediol, 6.82 and 10.00%, and worst for *d,l*-2,3-butanediol, 13.64 and 19.22%. The observed means for the analytes were all within 10% of the spiked level.

INTRODUCTION

In recent years research on alcoholism has revealed that butanediol has been associated with alcoholism [1—3]. Preliminary work at the National Institute on Alcohol Abuse and Alcoholism (NIAAA) indicated that three diols, 1,2-propanediol, *d,l*-2,3-butanediol and *meso*-2,3-butanediol, may be associated with alcoholism. A joint study among the Centers for Disease Control (CDC), Harvard University's School of Medicine and NIAAA was designed to answer several questions regarding the concentrations of these low-molecular-weight diols in the blood serum of different populations. We developed a method to determine 1,2-propanediol, *d,l*-2,3-butanediol, and *meso*-2,3-butanediol at the micromolar levels in 500 serum samples and appropriate quality control samples. In trace analysis, procedures can be divided into several steps, which often include extraction, chromatography and detection. Previous gas chromatographic (GC) procedures that were developed at NIAAA for determining these diols included either direct injection of deproteinizated serum or injection of a methyl ethyl ketone extract. According to the authors, these procedures

suffered from inadequate sensitivity and extraction reproducibility. Although most reported procedures for ethylene glycol have similar deficiencies [4—6], a recent article for determining ethylene glycol [7] in blood served as a basis for our method. To determine the diols in which we are interested, we made many changes in the basic method. For example, we used a different extraction solvent and, as the derivatizing agent, a different boronic acid. We also added an internal standard and used capillary column chromatography with electron-capture detection. These changes made the analysis faster and more precise, sensitive and specific.

When our study was nearly completed, we learned that a procedure [8] similar to ours for determining ethylene glycol in serum had been submitted for publication. Our method, however, differed in several aspects: it determined different analytes; a different internal standard and a different derivatizing agent were used; and capillary column chromatography, with electron-capture detection, was employed.

EXPERIMENTAL*

Chemicals and reagents

The following were used: *p*-bromophenylboric acid and 1,2-butanediol (Aldrich, Milwaukee, WI, U.S.A.); propylene glycol (USP), ethyl acetate (spectranalyzed) and acetonitrile (Fisher Scientific, Fair Lawn, NJ, U.S.A.); *d,l*-2,3-butanediol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and 1,3-propanediol (Chem Service, West Chester, PA, U.S.A.). *meso*-2,3-Butanediol was fractionated from a 1:1 mixture of *meso* and racemic 2,3-butanediols (K & K Labs., Plainview, NY, U.S.A.) by aqueous liquid chromatography [9]. This mixture of 2,3-butanediols was used to fortify our quality control pools. The propylene glycol was further purified by distillation, boiling point 189°C.

Apparatus

A Hewlett-Packard Model 5713A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.), equipped with a constant-current ^{63}Ni electron-capture detector, a Varian Model 8000 autosampler (Varian, Walnut Creek, CA, U.S.A.) and a Hewlett-Packard 3390A integrator recorder were used. Instrument operating temperatures were as follows: detector, 350°C; injector, 200°C; column oven, 120°C. The carrier gas was helium (99.999 UHP), at a flow-rate of 1 ml/min; the detector make-up gas was argon—methane (95:5), at a flow-rate of 47 ml/min. The system was operated in a fully automated mode. Split mode of injection was incorporated with a split ratio of 1:150; the inlet splitter was packed with silanized glass wool. A 12-m fused-silica capillary column coated with methyl silicone fluid (Hewlett-Packard), was used. The injection volume was 1 μl.

Procedure

One milliliter of serum and 6 ml of acetonitrile, which contained the internal

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

standard (1,2-butanediol) at a concentration of 83 μM, were volumetrically pipetted into a 15-ml round-bottom tube. The tube was fitted with a PTFE-lined screw cap, vortexed for 3 min, and then centrifuged at 2400 g for 30 min. The extract was decanted to another 15-ml round-bottom tube. This tube was placed in a 70°C water bath, and the extract was concentrated to a reduced volume (ca. 0.2 ml) by using a stream of nitrogen. The nitrogen passed through a multiport manifold fitted with 18 needles, each of which was directed into a tube. Each extract was then reduced to dryness by passing a gentle stream of nitrogen through a 9-in. Pasteur pipet into the tube, which was hand-held at room temperature and continually rotated to disperse the residue.

One milliliter of a 1% p-bromophenylboric acid in ethyl acetate solution was added to each tube. The mixture was vortexed and allowed to stand at room temperature for a minimum of 30 min. The reaction mixture was again vortexed and then pipetted into automatic sampler vials. Samples of 1 μl were injected by the autosampler. Vials containing acetone were placed after each sample, and the automatic sampler was washed with acetone after each sample injection. Including the wash cycle and data reporting time, the analysis time was approx. 20 min.

Quantitation

Bovine sera spiked at six different concentrations, ranging from 20 to 1000 μM with each of the three analytes, were analyzed with each run. These standards were injected at both the beginning and the end of the gas chromatographic sequences. The areas for all three analytes and the internal standard were entered into a computer. Peak area ratios (analyte/internal standard) for each of the analytes in the standards were calculated and plotted on a log/log scale versus the concentration of the analyte in the corresponding standard. The respective calibration curves were constructed, and the best fitting line was found by using a least-squares regression method. The concentration of each of the analytes in the unknowns was computed.

Quality assurance

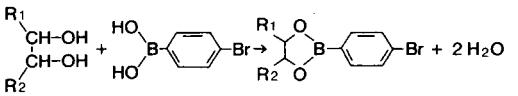
To estimate the precision and accuracy of our analyses, we prepared and analyzed quality control samples. The more concentrated quality control samples (pool 3) were prepared by spiking human serum with the analytes of interest in water; this more concentrated pool was then diluted with more serum to prepare pool 2. Pool 1 was the serum with no analytes added. The pools were each stirred overnight at 4°C and then filtered under sterile conditions. The filtrate was dispersed in 1-ml aliquots into vials and frozen. With each analytical run, which consisted of 20 unknown samples and 6 standards, 2 quality control samples from pools 2 and 3 were also analyzed.

RESULTS AND DISCUSSION

Procedure

The interaction of diols with traditional GC packing materials leads to poor separation and peak symmetry, especially in the less concentrated samples, and the end result is nonlinearity of response. Therefore, the analytes must be derivatized.

Several criteria for selecting the appropriate diol must be met. The derivative should not interact with the contents of the column, and it should be of sufficient molecular weight to be retained on the column. On the other hand, it must be capable of resolving the very similar butanediols and propanediol. Another factor in selecting the derivatizing agent is that it should not be monofunctional, which might lead to mixed mono- and di-derivatives. *p*-Bromophenylboric acid seemed to be an ideal candidate for meeting these criteria. The reaction with the diols of interest is shown in Fig. 1. In addition, the *p*-



for 1, 2 - propanediol $R_1 = CH_3$; $R_2 = H$
2, 3 - butanediols $R_1, R_2 = CH_3$
1, 2 - butanediol $R_1 = CH_3CH_2$; $R_2 = H$

Fig. 1. Reaction of the diols with *p*-bromophenylboric acid to form the cyclic *p*-bromophenylboronates.

bromophenylboronates exhibit increased stability and electron-capture detector sensitivity relative to the other boronates [10]. The use of electron-capture detection, as compared with flame ionization detection, permits greater sensitivity and selectivity. We used a detector temperature of 350°C because higher detector temperatures result in less detector contamination and because the dissociative capture mechanism which the boronates undergo [11] is more efficient at higher temperatures. The only disadvantage in using the boronates is that the derivatization of *meso*-2,3-butanediol might have slower kinetics than the *d,l*-form. The reason is that in the preferred conformation of the butanediols (that is, with the methyl groups *anti* to each other), the *meso* form also has its hydroxyl groups *anti* to each other, whereas the *d,l*-form has its hydroxyl groups *gauche* to each other (Fig. 2); this *gauche* conformation is

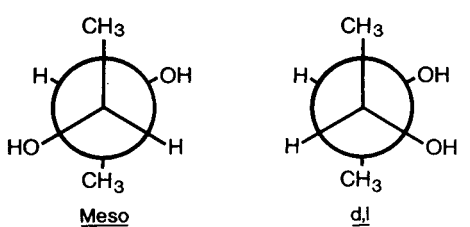


Fig. 2. The *anti* conformation of *meso*- and *d,l*-2,3-butanediol.

ideal for the formation of the bridged boronates. Although we did not study the kinetics, we saw no significant difference in the recoveries of the *meso*- compared with the *d,l*-2,3-butanediol derivative. Determining the percent recovery of each separate step was difficult because the diols apparently react with derivatizing agent that remains from previous injections on the head of the column or in the injector. This was shown by injecting the diols themselves into the

system after derivatized samples had been analyzed. The corresponding derivatized diols were then detected by electron capture and mass spectrometry. This on-column method and other methods for forming the boronates from diols were recently reported [12].

The electron-impact fragmentation patterns of these bromophenylboronate derivatives of the analytes of interest are very similar to the mass spectra of the corresponding phenylboronate derivative [13, 14]. As shown in Fig. 3, the molecular ion at *m/z* 254 for the *d,l*-2,3-butanediol derivative is pronounced. Furthermore, corresponding peaks are present at *m/z* 256 (primarily because of ^{81}Br) and at 253 and 255 (primarily because of ^{10}B). The base peak at *m/z* 239 is due to the loss of a methyl group. Other ions of prominent size are at *m/z* 182 (C_6H_4BBrO), 183 (C_6H_5BBrO), 103 (C_6H_4BOO), and 77 (C_6H_5).

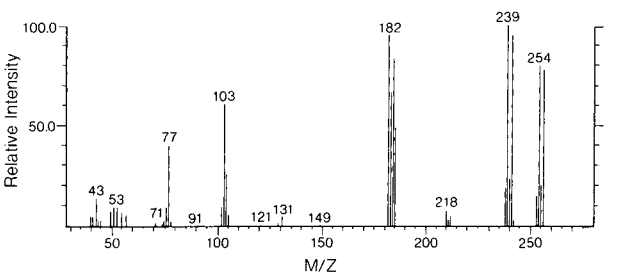


Fig. 3. The 70-eV electron-impact mass spectrum of the *p*-bromophenylboronate of *d,l*-2,3-butanediol.

Once we decided to use the boronate derivatives, the next problem was to free these water-soluble diols from serum. Extraction with semi-polar, water-immiscible solvents, such as methyl ethyl ketone and ethyl acetate, resulted in low and sporadic recoveries. We then used acetonitrile as both a deproteinizing and extracting solvent; the water was removed by using heat and a gentle stream of nitrogen. The volume of 6 ml of acetonitrile to 1 ml of water was used because these solvents form a 5.1:1 azeotrope which boils at 76.5°C [12]. This allows water to be rapidly removed at a lower temperature than would otherwise be possible. Nonetheless, this evaporation step could be one of the most error-prone steps in the analyses. For example, when the diols were spiked into water and concentrated, recovery was low. However, when the analytes were spiked into serum and concentrated to a low volume and then reduced to dryness by the hand-held procedure, the recoveries were good. Apparently, the serum lipids entrap the diols and prevent their loss. We were concerned about possible losses in this concentration step, and a primary reason for selecting 1,2-butanediol as the internal standard was that its reported boiling point (192—194°C) is close to those of the analytes of interest [14]. Another reason for selecting 1,2-butanediol is that it forms a five-membered cyclic boronate, as do the analytes of interest (Fig. 1); other diols, such as 1,3-propanediol, form a six-membered boronate and they reportedly have different stability properties from the five- and seven-membered rings [11]. 1,3-Propanediol was also not selected as the internal standard because it was found in the undistilled alcoholic beverages that we analyzed.

Precision and accuracy

We estimated precision and accuracy in our 26 runs of unknown samples by analyzing pools 2 and 3 in duplicate in each run. A gas chromatogram of pool 2 is shown in Fig. 4. The same derivatives were also separated at 120°C on a 1.83 m × 4 mm I.D. glass column packed with 3% SE-30 on 80—100 mesh Supelcoport; however, the packed-column separation resulted in less resolution and increased analysis time.

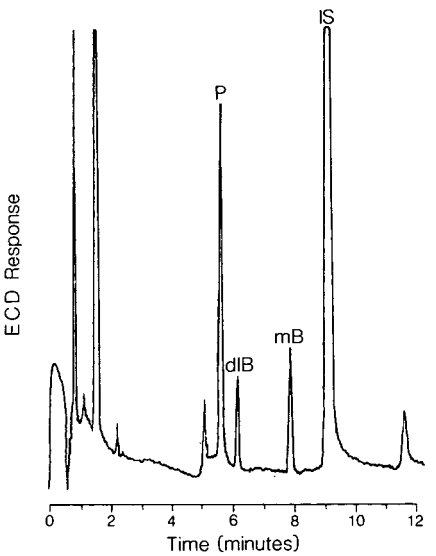


Fig. 4. Gas chromatogram of the *p*-bromophenylboronates of 1,2-propanediol (P); *d,l*-2,3-butanediol (dlB); *meso*-2,3-butanediol (mB); and 1,2-butanediol (IS) in Pool 2. Conditions as described in text.

As shown in Table I, the observed means for the analytes in the quality control pools in all instances were within 10% of the spiked levels as depicted by the percent bias. These results are not corrected for the very small amount of 1,2-propanediol found in the base pool. As expected, the coefficients of variation of the analysis for each analyte were lower for the more concentrated pool. Quality control charts for each analyte in each pool were generated by plotting the values for each run. The chart for *d,l*-2,3-butanediol in pool 2 is

TABLE I

ESTIMATE OF PRECISION AND ACCURACY OF THE METHOD

Analyte	Pool	Spiking level (μM)	Mean determined (μM)	Bias (μM)	Bias (%)	Standard deviation (μM)	C.V. (%)	95% Control limits (μM)	N
1,2-Propanediol	2	50	55	+ 5	+10.0	5.5	10	65.8/44.2	51
1,2-Propanediol	3	700	693	− 7	− 1.0	47.2	6.8	786/600	52
d,l-2,3-Butanediol	2	25	27	+ 2	+ 8.0	5.1	18.9	37.0/17.0	52
d,l-2,3-Butanediol	3	350	385	+35	+10.0	52.6	13.7	488/282	52
meso-2,3-Butanediol	2	25	24	− 1	− 4.0	3.4	14.2	30.7/17.3	52
meso-2,3-Butanediol	3	350	339	−11	− 3.1	32.6	9.6	403/275	52

shown in Fig. 5. The 95% and 99% control limits, which were used to determine if the method was in control, are calculated by subtracting from and adding to the mean the standard deviation times 1.96 and 2.58, respectively. In all of the analyses, we experienced the greatest deviation from the mean in the earlier runs. This deviation was decreased by lowering the column temperature.

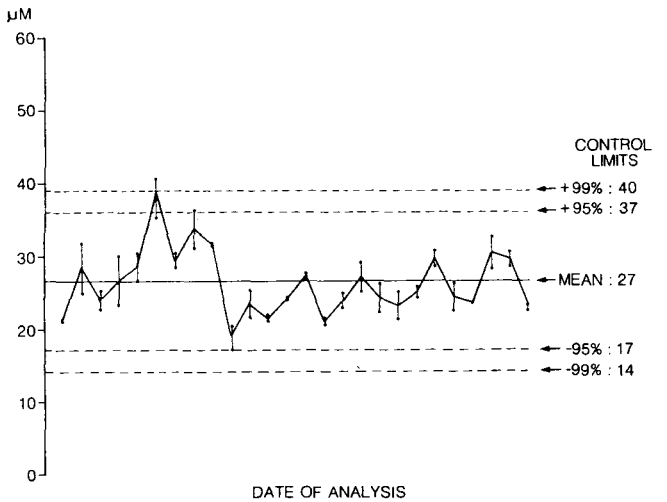


Fig. 5. Quality control chart for the observed values of *d,l*-2,3-butanediol in pool 2.

Recovery

Because our supply of the derivatizing agent was limited, we prepared and purified only the *p*-phenylboronate of *d,l*-2,3-butanediol. When this diol was added to serum at a concentration of 700 μM, the recovery was approximately 87%.

Linearity and reproducibility of standard curves

Log/log plots were used because of the large concentration range (20.0—1000 μM). The linearity of the standard curves for the 26 runs was very reproducible as evidenced by the mean correlation coefficients (± standard deviation) of 0.9968 (±0.0030), 0.9950 (±0.0044) and 0.9960 (±0.0044) for 1,2-propanediol, *d,l*-2,3-butanediol and *meso*-2,3-butanediol, respectively. The reproducibility of the standard curves was quite precise as indicated in Table II for 1,2-propanediol and *d,l*-2,3-butanediol. We calculated these values from the linear regression line using the area ratios for each point on the curve. These points represent 26 standard curves taken from each run over a one-month period.

Quantitation limit

Various definitions [15, 16] for the term "limit of quantiation" or "limit of determination" have been discussed. In this study, we spiked two serum pools with each analyte at 5.0 μM and at 10.0 μM. We took 19 aliquots from each

TABLE II

MEAN CONCENTRATIONS CALCULATED FOR STANDARDS FROM LINEAR REGRESSION LINES

Number of standard curves is 26 runs over a one-month period.

Target standard value	1,2-Propanediol* values found	*d,l*-2,3-Butanediol* values found
20	20 ± 2	21 ± 2
50	50 ± 3	50 ± 4
100	100 ± 10	99 ± 12
500	480 ± 20	490 ± 30
1000	1000 ± 110	1020 ± 70

*Mean ± standard deviation in μM.

pool and, in a separate analytical run, one analyst analyzed them under the same conditions that had been used for the unknowns. As shown in Table III, the accuracy, as reflected by percent bias, and the standard deviation were similar for either concentration. In this determination, the percent differences for the lower concentrations are not as clinically significant as for the higher concentrations. Nonetheless, because of the increasing relative standard deviation with lower concentrations, our lower limit of quantitation was set at 5.0 μM.

TABLE III

ESTIMATE OF PRECISION AND ACCURACY FOR THE METHOD AT LOW CONCENTRATIONS

Analyte	Spiked level (μM)	Observed mean	Bias (%)	Within-run standard deviation	C.V. (%)
1,2-Propanediol	10	12	20.00	1.0	8.33
1,2-Propanediol	5	5.5	10.00	1.5	27.27
d,l-2,3-Butanediol	10	9.5	− 5.00	1.6	16.84
d,l-2,3-Butanediol	5	4.6	− 8.00	2.0	43.48
meso-2,3-Butanediol	10	9.1	− 9.00	2.4	26.37
meso-2,3-Butanediol	5	4.4	−12.00	1.2	27.27

Application

The method described herein has been used for determining serum levels of the three analytes in the 500 samples prevously mentioned. Results of this joint study by the Harvard School of Medicine, the National Institute for Alcohol Abuse and Alcoholism, and the Centers for Disease Control will be reported soon.

This method could also be used for determining other diols and other difunctional compounds of clinical and toxicological interest.

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CAPILLARY GAS CHROMATOGRAPHIC ANALYSIS OF HUMAN SKIN SURFACE LIPIDS AFTER MICROSAMPLING ON GROUND-GLASS PLATELETS

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SUMMARY

Small amounts of human skin surface lipids, in the 1—20 μg range, sampled on ground-glass platelets are investigated using capillary gas chromatography.

A first system allows the separation of the neutral lipids, up to the triglyceride fraction. A second system reveals the distribution of the free fatty acids or of the free + glyceride fatty acids, after a methylation or transesterification step.

Examination of samples from nine subjects shows that the unsaturation of the free fatty acids increases during a four-day period of accumulation. Comparison of the free fatty acid fraction and the free + glyceride fatty acid fraction shows that the free fraction is more saturated than the latter. It is concluded that the bacterial lipases which cleave the fatty acids from the ester bond favor the liberation of straight-chain saturated fatty acids from sebum triglycerides.

This result is confirmed by comparison of the free fatty acid fraction with the glyceride fatty acid fraction separated from bulk samples of skin surface lipds from hair and scalp.

INTRODUCTION

Human skin surface lipids (SSL) are a very complex and rather unique mixture [1] which has been investigated using various sampling procedures and analytical techniques. The average class composition of the mixture is [2]: squalene 11—13%; wax esters 20—22%; sterol esters 2—3%; free fatty acids 30—33%; sterols 1—2%; glycerides 29—32%.

In the early seventies, Schaefer and Kuhn-Bussius [3, 4] described an original method for the quantitative determination of human SSL: during the application of a small ground-glass platelet upon the skin, lipids were deposited

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on the rough surface. The transparency of the glass, as measured using a photometric device, could be correlated with the amount of lipidic material deposited on it. Later other authors used this procedure to collect quantitative data, such as the casual level of SSL [5, 6] or the sebum excretion rate [7].

If considered as a sampling method for SSL, the ground-glass method has several positive features: the procedure is quick and easy to perform; the sampling site is not attacked by any solvent or surfactant; the sampled lipids are not diluted in a solution or an emulsion.

Therefore, it seemed very attractive to develop adequate methods for closer qualitative and quantitative examination of SSL deposited on ground-glass platelets. A thin-layer chromatographic (TLC) method, which separates the mixture into five basic classes (cholesterol, free fatty acids, triglycerides, wax esters and squalene) has been reported recently [8]. However, this analytical approach does not give any information about the distribution of the individual components within the basic classes, and does not consider the sterol ester fraction. Here we describe chemical derivatization procedures of these microsamples, followed by capillary gas chromatography.

EXPERIMENTAL

Sampling procedures

The ground-glass microsampling procedure was performed according to the description of Saint-Léger and Bague [8]. Bulk sampling of SSL was performed from individual subjects by washing the scalp and hair with a surfactant solution. The lipids were extracted from the emulsion using diethyl ether [9].

Fractionation of the bulk samples

The bulk samples from individual subjects were fractionated into the free fatty acid fraction and the glyceride esterified fatty acid fraction according to the method of Boré et al. [10]. A pool of SSL (from several subjects) was fractionated using preparative TLC and the following basic classes of components were recovered: wax esters; sterol esters; triglycerides.

The separation was performed on silica gel G precoated plates with a thickness of 250 μm (Uniplate® from Anachem, Luton, Great Britain). The plates were cleaned by elution with diethyl ether. Then 150 μl of chloroform containing 23 mg of human SSL were deposited on a 16-cm length with a Linomat III (from Camag, Muttenz, Switzerland). A first elution was performed using hexane, followed, after drying of the plate, by elution with benzene. The eluted strips were revealed at their extremities by carbonisation, scraped out and the lipidic fractions extracted with diethyl ether. Each fraction was then rechromatographed using the same conditons, in order to ensure minimal contamination from its neighbours.

Prechromatographic treatment of microsamples

The loaded ground-glass platelet is placed into a 1-ml Reacti-Vial® (from Pierce Eurochem B.V., Rotterdam, The Netherlands) and rinsed by addition of 400 μl of diethyl ether or dichloromethane. For absolute quantitation of

the chromatogram, a standard solution of an adequate internal standard is used instead of pure solvent.

After homogenisation, the platelet is withdrawn from the vial; half of the solution is transferred into a second vial and both solutions are evaporated to dryness under a nitrogen stream at room temperature.

Direct chromatographic analysis without any derivatization can be performed after redissolution of the residue in 50 μl of diethyl ether or dichoromethane.

For derivatization with diazomethane, 200 μl of the reagent (3 g of diazomethane in 250 ml of diethyl ether prepared according to ref. 11 are added to the evaporation vial. The mixture is allowed to stand at room temperature for a few minutes and then evaporated to dryness at room temperature under a stream of nitrogen. The residue is dissolved in 50 μl of diethyl ether or dichloromethane and injected.

For transesterification of the glycerides, 50 μl of reagent [Meth-Prep II from Applied Science Labs., State College, PA, U.S.A.: 0.2 *N* methanolic solution of (*m*-trifluoromethylphenyl)trimethylammonium hydroxide] are added to the evaporation vial. The mixture is allowed to react at room temperature during 15 min and then injected.

Gas chromatography of the fatty acids

A borosilicate glass capillary (90 m × 0.3 mm I.D.) was preconditioned using the $BaCO_3$ procedure of Grob et al. [12—14]. The tube was then dynamically wall-coated with free fatty acid phase (FFAP) stationary phase.

The operating conditions were:
injector and flame ionization detector temperature, 230°C; column temperature, programmed from 150°C to 210°C at 3°C min^{-1}; carrier gas, helium; inlet pressure, 2 bars; injected volume, 3 μl; split ratio, 1:10.

The internal standard for absolute quantitation was *n*-heptadecanoic acid. Five micrograms of internal standard are a convenient amount for platelets loaded with 1—30 μg of SSL. Possible substitutes to *n*-heptadecanoic acid as an internal standard are *cis*-9-hexadecenoic acid (palmitoleic acid) or 2-hydroxypalmitic acid.

Gas chromatography of the neutral lipids

A borosilicate glass capillary (9 m × 0.3 mm I.D.) was preconditioned using the $BaCO_3$ procedure of Grob et al. [12—14]. The tube was then dynamically coated with OV-1 stationary phase.

The operating conditions were: injector and temperature, 350°C; column temperature, programmed from 200°C to 330°C at 4°C min^{-1}; carrier gas, helium; inlet pressure, exponentially programmed from 0.2 to 2 bars in 50 min using an FP 222 flow programmer from Dani, Monza, Italy. The injected volume was 5 μl in the splitless mode according to the procedure of Grob et al. [15, 16].

Evaluation of the analytical procedure

The free fatty acid and glyceride fatty acid percentages of a bulk sample of SSL were determined by gravimetry after fractionation. The sample was found

to contain 25 ± 2% of free fatty acids and 35 ± 2% of glyceride fatty acids.

Standard solutions of this sample in diethyl ether or dichloromethane (from 50 to 1000 μg/ml) were used to test the reproducibility and linearity of the analytical procedure. Therefore, aliquots (10–20 μl) of the standard solutions were deposited on ground-glass platelets with a microsyringe and, after evaporation of the solvent, the platelets were analyzed using the procedure described above.

RESULTS AND DISCUSSION

Chromatogram of the neutral lipids

The chromatogram of the neutral lipids was obtained after injection of an underivatized sample or after derivatization with diazomethane. The esterified fractions (waxes, sterol esters and triglycrides) which have been isolated by TLC were treated with the diazomethane reagent in the same manner. This showed that no side-reaction such as transmethylation was occurring to a detectable extent during this treatment.

A typical chromatogram of human SSL microsampled from the scalp is shown in Fig. 1. The following compounds or classes of compounds are successively eluted: free fatty acis — these compounds are not properly resolved using these chromatographic conditions; they will be separated on another column; squalene; cholesterol; wax esters, cholesteryl esters and triglycerides — the elution range of these classes of compounds was determined using the fractions isolated from TLC.

No attempt was made to identify single compounds in the wax ester fraction since these species will be further studied by gas chromatography—mass

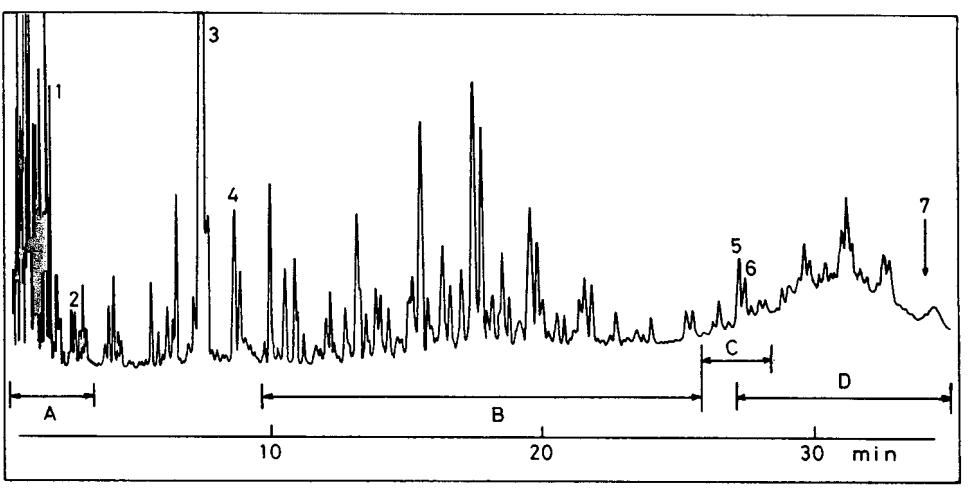

Fig. 1. Chromatogram of the neutral lipids of SSL on an OV-1 capillary column. (A) Elution range of the free fatty acid fraction. (B) Elution range of the wax esters. (C) Elution range of the sterol esters. (D) Elution range of the triglycerides. 1 = Stearic acid, 2 = arachidic acid, 3 = squalene, 4 = cholesterol, 5 = cholesteryl palmitoleate, 6 = cholesteryl palmitate, 7 = elution position of tristearin.

spectrometry. Cholesteryl palmitoleate and palmitate proved to be the main components in the sterol ester fraction.

The elution ranges of the sterol esters and the triglycerides overlap partially. The latter fraction extends up to compounds which are somewhat heavier than tristearin. Using flow programming in conjunction with temperature programming, complete elution of that fraction could be achieved, as shown by the return to baseline at the end of the chromatogram.

Chromatogram of the free fatty acid fraction

The free fatty acids are converted into the corresponding methyl esters with diazomethane. A typical chromatogram is shown in Fig. 2. The chain lengths are within the C_{12} to C_{20} range, with the straight-chain saturated and monounsaturated species being prominent in the mixture. The monounsaturated chains from C_{14} to C_{17} bear their double bond at the 6-position [17] whereas the peak corresponding to the C_{18} species is of composite structure, indicating a mixture of positional *cis*-isomers [17]. The elution level of *cis*-9-octadeconoic acid (oleic acid) has been determined by addition of the reference compound.

Heptadecanoic acid is a minor component in the mixture (1–2%). It is possible to use it as an internal standard provided it is added to the sample in a sufficient amount, the endogenous contribution then being negligible (5 μg of internal standard for SSL samples in the 1—30 μg range).

cis-9-Hexadecenoic acid (palmitoleic acid) is another possible choice as an

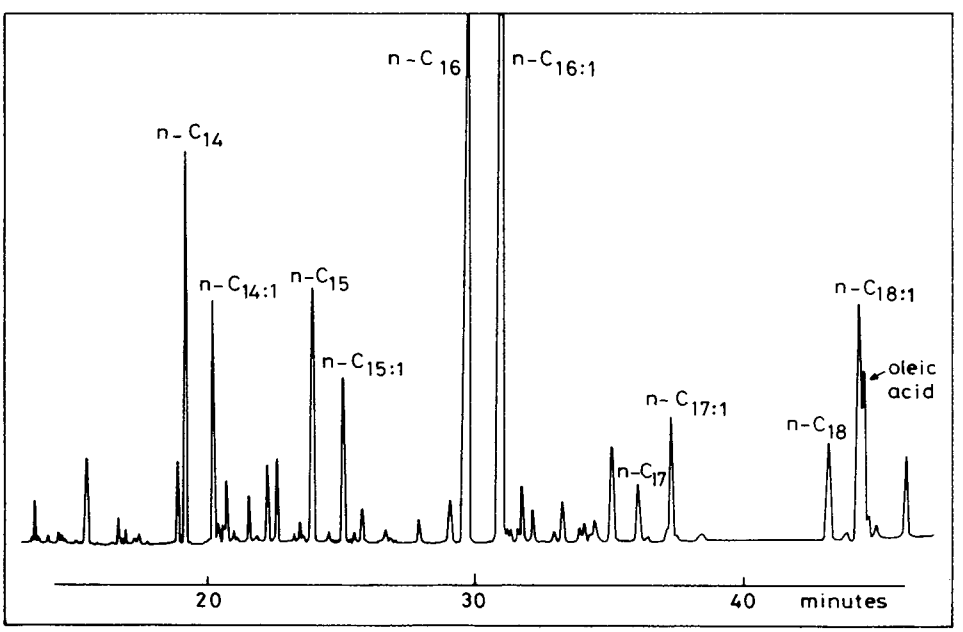

Fig. 2. Chromatogram of the fatty acids of SSL on an FFAP capillary column. The *n*-monoenic acids with chain length from 14 to 17 carbon atoms are 6-unsaturated. A number of positional isomers are found at the C_{18} level, the 8- and 9-unsaturated species being predominant. The other peaks of the chromatogram, between *n*-C_{14} and *n*-C_{18} arise from branched-chain, mainly saturated, species [17].

internal standard, it being eluted just after its positional isomer. The separation of the isomers, however, is very critical, requiring a column of good efficiency.

α-Hydroxy-palmitic acid is also possible internal standard; on our column we found it to be eluted just before heptadecanoic acid, at a level where no compound of endogenous origin interferes. However, since this hydroxy compound is not homologous to the fatty acids, its retention relative to the acids may be not very reproducible from column to column, or may change as a column ages. As a consequence, interferences with endogenous compounds then become possible.

Derivatization with Meth-Prep II®

The effect of this reagent upon the different species found in human SSL was investigated using isolated fractions. The triglycerides proved to be quantitatively transersterified by the reagent, within 15 min at room temperature. No significant transformation of the waxes could be observed after contact with the reagent during 15 min at room temperature. However, after 12 h at room temperature, the waxes were quantitatively cleaved into the corresponding fatty alcohols and methyl esters. The cholesteryl esters proved to behave in a manner comparable to the waxes. The free fatty acids were quantitatively derivatized by the reagent to methyl esters, using the same conditions as for triglyceride transesterification.

As a consequence, using both derivatizing reagents and different conditions, separate classes of fatty acids can be determined in human SSL. Derivatization with diazomethane yields the methyl esters of the free fatty acids from SSL. Derivatization with Meth-Prep II® during 10 min yields the free + glyceride fatty acids methyl esters. Prolonged derivatization with Meth Prep II® results in complete recovery of all fatty acid moieties which are found in SSL as methyl esters.

Evaluation of the analytical procedure

Fifteen ground-glass platelets were loaded with 15 μg of SSL, and the amounts of free and free + glyceride fatty acids were determined. The experimental values were free fatty acids 3.70 μg (standard deviation 0.16), and free + glyceride fatty acids 8.89 μg (standard deviation 0.55). These are in good agreement with the composition determined by gravimetry.

The linearity of the analytical procedure was tested by successive analyses of platelets loaded with 1, 2, 3, 5, 10, 15, 20 and 30 μg of SSL. A linear relationship between amounts of free fatty acids and SSL, and amounts of free + glyceride fatty acids and SSL was found, the correlation coefficient of the regression curve being 0.99.

From each chromatogram we also determined the $C_{16}/C_{16:1}$ ratio. The relative standard deviation was 4% for both the free and the free + glyceride fatty acid fractions. Previous studies [9, 17] showed that this ratio is a good reflection of the balance between saturated and unsaturated species in the considered fraction.

In situ evolution of free fatty acids

The free fatty acids of human SSL are believed to originate through enzymatic hydrolysis of the triglycerides by bacterial lipases [2, 18]. There-

fore, an increase in the amount of free fatty acids is expected during accumulation of SSL, resulting from the cumulative effect of continuous excretion and increasing degree of hydrolysis.

An experimental population of nine subjects was investigated to check if the expected evolution can be observed using the ground-glass platelet sampling method. After a cleaning shampoo, SSL aged one, two, three and four days were recovered from neighbouring sites on the scalp and subsequently analyzed for amount and composition of the free fatty acid fraction.

The results are given in Table I. It can be seen that, in some cases, no determination at all could be performed, the amount of free fatty acids being less than 0.1 μg per platelet. This apparent failure of the sampling procedure is probably a consequence of non-uniform distribution of SSL on the scalp.

For most subjects, the amount of free fatty acids (FFA) seems to increase as SSL accumulate on the scalp; however, this evolution as observed through the microsampling methodology is far from being even over the four-day period.

In Table II, we report the values of $\Delta(\mu g\ FFA) = (\mu g\ FFA)_{4\ days} - (\mu g\ FFA)_{1\ day}$ relative to the eight cases for which these data could be obtained. The mean value is 4.16 μg, with a very wide standard deviation (4.25); so it can hardly be stated from these data that the amount of free fatty acids is actually increasing with time of accumulation.

This observation also suggests that, due to non-uniform distribution of SSL, the amount of material which is collected on a single platelet with a diameter of 6 mm does not reflect the average status on the complete site.

The distribution pattern of the free fatty acids seems to undergo an evolution during accumulation of SSL on the scalp, with the relative proportion of unsaturated compounds being increased. This situation can be characterized by the determination of the ratio $C_{16}/C_{16:1}$ as reported in Table I.

In Table II we also report the values of $\Delta(C_{16}/C_{16:1}) = (C_{16}/C_{16:1})_{4\ days} - (C_{16}/C_{16:1})_{1\ day}$ relative to the eight cases for which these data could be obtained. The mean value is -0.50 with a standard deviation of 0.32, which shows that the relative proportion of unsaturated species in the free fatty acid fraction increases during accumulation of SSL on the scalp.

TABLE I

IN SITU EVOLUTION OF THE FREE FATTY ACIDS (FFA) FRACTION DURING ACCUMULATION OF SSL ON THE SCALP

Subject	1 day		2 days		3 days		4 days	
	μg FFA	$C_{16}/C_{16:1}$	μg FFA	$C_{16}/C_{16:1}$	μg FFA	$C_{16}/C_{16:1}$	μg FFA	$C_{16}/C_{16:1}$
1	5.5	1.66	*	*	13	1.11	*	*
2	3.5	2.66	*	*	3.5	1.50	3.5	1.69
3	1	1.15	2.5	0.99	5	0.87	4.2	0.65
4	4.5	1.43	3	1.53	6	1.32	3	0.92
5	3	1.57	4	1.34	12	0.88	15	1.0
6	8	2.00	3.5	1.50	17	1.81	11.6	1.28
7	1	1.48	0.2	1.37	*	*	3.8	0.96
8	1	1.06	2.5	1.08	10.8	0.95	6.5	1.22
9	1.8	1.36	2.6	1.37	5.7	1.20	9.5	0.97

*No determination could be performed, the amount of FFA being less than 0.1 μg per platelet.

TABLE II

VARIATIONS OF AMOUNT AND COMPOSITION OF THE FREE FATTY ACID (FFA) FRACTION WITHIN THE 1—4-DAY PERIOD

$\Delta(\mu g\ FFA) = (\mu g\ FFA)_{4\ days} - (\mu g\ FFA)_{1\ day}$
$\Delta(C_{16}/C_{16:1}) = (C_{16}/C_{16:1})_{4\ days} - (C_{16}/C_{16:1})_{1\ day}$

Subject	$\Delta(\mu g\ FFA)$	$\Delta(C_{16}/C_{16:1})$
2	0	—0.97
3	3.2	—0.50
4	—1.5	—0.51
5	12	—0.57
6	3.6	—0.72
7	2.8	—0.52
8	5.5	0.16
9	7.7	—0.39
Mean	4.16	—0.50
S.D.	4.28	0.32

This result suggests that the enzymatic hydrolysis which cleaves the fatty acid moieties from the triglycerides is not a random process but is actually affecting first the saturated chains.

Another explanation could, however, be proposed, which relates the increasing unsaturation of the free fatty acids to a 6-dehydrogenase from a microbial source. Biotransformations by the enzymes of the skin surface microflora have already been observed, such as 9-hydroxylation of fatty acids [19]. However, a 6-dehydrogenation was never reported; on the contrary the rather uncommon Δ^6 unsaturation in the acidic chains of the SSL is generally considered to be highly characteristic of the sebaceous production.

Comparison of free fatty acids with free + glycerides fatty acids from a single microsample

If the bacterial lipases which hydrolyse the triglycerides proceed in a structure-dependent manner, then there must be a difference between the composition of the free fatty acid fraction and the triglyceride fatty acid fraction. A difference is also to be expected between the composition of the free fatty acid fraction and the free + glyceride fatty acid fraction, and these two fractions can be investigated starting from a single platelet, using the two derivatization procedures described in this paper.

These determinations were performed over a population of nine subjects, and the results are given in Table III. The degree of hydrolysis of the samples can be calculated considering the respective amounts of the free fatty acids and the free + glyceride fatty acids within the investigated population of Table III, this degree of hydrolysis ranges from 0.32 to 0.65. In all cases, the free fatty acid fraction proved to be more saturated than the corresponding free + glyceride fatty acid fraction; from the first fraction to the other, the $C_{16}/C_{16:1}$ ratio decreases by a mean value of 0.38, with a standard deviation of 0.18.

TABLE III

COMPARISON BETWEEN THE AMOUNT AND THE COMPOSITION OF THE FREE FATTY ACID (FFA) FRACTION AND THE FREE + GLYCERIDE FATTY ACID FRACTION DETERMINED FROM THE SAME MICROSAMPLE

Subject	Fatty acids (μg)		$C_{16}/C_{16:1}$ ratio	
	FFA	Free + glyceride FA	FFA	Free + glyceride FA
1	5.5	9.4	1.82	1.51
2	3.8	6.6	1.94	1.76
3	3.2	10	2.05	1.56
4	6.8	13.7	1.50	0.99
5	6.5	11.8	1.56	0.97
6	7.3	18	1.62	1.02
7	10.3	20.2	1.32	1.10
8	6.2	9.5	1.73	1.39
9	5	9.5	1	0.85

TABLE IV

COMPARISON BETWEEN THE FREE FATTY ACID (FFA) FRACTION AND THE GLYCERIDE FATTY ACID (FA) FRACTION OF BULK SAMPLES OF SSL COLLECTED FROM SCALP AND HAIR

	Subject	$C_{16}/C_{16:1}$	
		FFA	Glyceride FA
1-day-old samples	1	0.81	0.56
	2	1.21	0.60
	3	0.82	0.53
	4	1.29	0.73
	5	1.21	0.68
4-day-old samples	6	0.80	0.49
	7	0.63	0.48
	8	0.63	0.47
	9	1.04	0.68
	10	0.88	0.58

These results show that the enzymatic hydrolysis is structure-discriminative, and cleaves first the saturated moieties. As this point seems of importance, the validity of the conclusion was checked using another sampling procedure and a different prechromatographic approach.

Investigation on SSL from bulk samples

SSL from scalp and hair were sampled by washing with a surfactant solution according to ref. 9. The free fatty acid fraction and the glyceride fatty acid fraction were recovered according to the procedure of Boré et al. [10].

The chromatographic profiles of the two fractions were compared over an experimental population of ten subjects. Both 1-day-old and 4-day-old SSL

were investigated; the results are given in Table IV. Here again it can be seen that the bacterial lipases perform a structure discriminatory hydrolysis which affects more readily the saturated species. This, however, does not necessarily mean that the lipases actually recognize the saturated structure of the acyl moiety to be cleaved. Another explanation could be that the lipases are selective toward the position (α and β) of the acyl group within the triglyceride. In this case, the favored release of saturated acids would be the consequence of a non-random distribution of the saturated and unsaturated chains between the α and β sites. A definitive conclusion on this point needs further experimentation using in vitro methodology.

CONCLUSIONS

The mcirosamples of human SSL, as obtained using the ground-glass platelet technique, can be very closely examinated by gas chromatographic methods.

The amount and distribution of the fatty acids in the free fatty acid fraction and in the free + glyceride) fatty acid fraction can be determined from a single sample containing 1—20 μg of fatty acid using two derivatizing reagents.

These examinations show that lipolytic activity linked to the presence of bacteria in the sebaceous gland favors the liberation of straight-chain saturated fatty acids from sebum triglycerides.

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GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF URINARY ACETYLPOLYAMINES

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SUMMARY

A gas chromatographic method was developed for the determination of monoacetylputrescine, monoacetylcadaverine, N^1-acetylspermidine and N^8-acetylspermidine in human urine. The amines were isolated from urine by silica gel column chromatography. 1,10-Diaminodecane was used as internal standard. The amines were reacted with ethyl chloroformate in aqueous medium to four ethyloxycarbonyl derivatives prior to application to gas chromatography using a flame ionization detector. Separation and determination of the derivatives were carried out on a Uniport HP column (1.0 m) impregnated with 0.5% SP-1000 under temperature-programmed conditions. The monoacetylpolyamines could be measured accurately at the nanomole level. The method was used for the determination of the monoacetylpolyamines in urine of healthy volunteers. The values obtained were in the range of the published data.

INTRODUCTION

The importance and clinical significance of polyamine determinations in human patients for diagnosis and biochemical monitoring of the progression of neoplastic diseases for the evaluation of the efficacy of chemotherapy have been reviewed [1—3]. In most studies evaluating the relationship between cancer and urinary polyamine excretion, the urine was hydrolyzed with 6 *N* hydrochloric acid prior to analysis [1]. However, the polyamines are excreted in the urine both of normals and cancer patients predominantly as their monoacetylated derivatives [4—9] such as monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine. The increase of monoacetylpolyamine levels in the urine of cancer patients [7—10] is not in contradiction with the earlier observations obtained from the analysis of hydrolyzed urines of cancer patients. Abdel-Monem and co-workers [11, 12] have reported an attractive finding that the ratios of N^1-acetylspermidine to N^8-acetylspermidine in 24-h urine of

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cancer patients are higher than those of healthy volunteers, and have suggested that the ratio of the two isomeric monoacetylspermidines may provide a more reliable marker for cancer than the urinary concentrations of total polyamines obtained after hydrolysis. This proposal has recently been emphasized by the results obtained from analyses of urines of additional cancer patients [13], although an inconsistent result has been reported [14].

A number of methods are available for the determination of total polyamines in urine hydrolysates [15—22]. For the determination of monoacetylpolyamines in urine pre-separation by ion-exchange [22] or silica gel [13] column chromatography has been applied, followed by dansylation and subsequent thin-layer chromatography [23] or high-performance liquid chromatography (HPLC) [13]. Recently, an HPLC method using a reversed-phase column and post-column derivatization with *o*-phthalaldehyde has been developed [24]. This method excludes the determination of monoacetylputrescine in urine. More recently, two methods based on ion-exchange column chromatographic separations followed by post-column derivatization with *o*-phthalaldehyde have been developed [25, 26]. By the method of Mach et al. [25] it is not possible to separate the two isomeric monoacetylspermidines. Prussak and Russell [26] completely separated all monoacetylpolyamines including monoacetylcadaverine in urine.

To our knowledge, no method using gas chromatography (GC) or gas chromatography—mass spectrometry (GC—MS) exists which permits the separation of the urinary monoacetylpolyamines, especially of the two isomeric monoacetylspermidines. On the other hand, many GC and GC—MS methods suitable for the determination of urinary total polyamines have been reported [7,21,22,27—31].

In the present paper, a GC method for the determination of urinary monoacetylpolyamines and of monoacetylcadaverine is reported which has been developed on the basis of our previous investigations [21,32—34]. The method requires a simple procedure for isolation of monoacetylpolyamines from urine. Silica gel column chromatography was used prior to derivatization with ethyl chloroformate, which was followed by GC separation of the ethyloxycarbonyl (EOC) derivatives.

MATERIALS AND METHODS

Chemicals

The hydrochlorides of 1,3-diaminopropane, putrescine, cadaverine and spermidine were purchased from Nakarai Chemicals (Kyoto, Japan). 1,10-Diaminodecane was from Sigma (St. Louis, MO, U.S.A.). The monoacetylpolyamine hydrochlorides were prepared in our laboratory according to the method described by Tabor et al. [35].

Ethyl, *n*-propyl, *n*-butyl and isobutyl chloroformates were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Silica gel (Wakogel C-200) was obtained from Wako Junyaku Co. (Osaka, Japan). Before use, it was treated according to the procedure of Grettie et al. [36]. Sodium chloride and anhydrous sodium sulphate were purified as follows to remove contaminants which showed peaks on the gas chromatogram: the salts were dissolved in water and

the solutions extracted three times with an equal volume of chloroform. The resulting solutions were evaporated to dryness in vacuo in an all-glass apparatus. Sodium chloride was dried in an oven at 100°C and sodium sulphate on a clean hot-plate at ca. 200°C. All organic solvents and inorganic chemicals were of the highest purity commercially available. Deionized water was used after distillation in an all-glass apparatus.

Sample preparation

Twenty-four-hour urine samples from healthy volunteers ranging in age from 21 to 52 years were collected in glass bottles under toluene. No restrictions of food and fluid intake were imposed on the volunteers. After the 24-h collection was complete, the volume of urine was measured and an aliquot was transferred to a polyethylene bottle and stored frozen until analyzed.

A 5-ml aliquot of the urine sample was taken and mixed with 2.5 ml of the internal standard solution (25 nmol/ml), and then was adjusted to pH 9.0 with 1.0 *N* sodium hydroxide solution. The precipitate was removed by centrifugation. The clear supernatant was transferred to a 10-ml volumetric flask and was brought to volume with water (pH 9.0).

Isolation of monoacetylpolyamines from urine

The monoacetylpolyamines were isolated and concentrated from urine by using a silica gel column. The procedure was performed according to the procedure of Grettie et al. [36] with some modifications. The pre-treated silica gel (2.0 g) was suspended in 10 ml of 0.1 *N* hydrochloric acid solution and transferred to a glass column (9 mm I.D.) with a stop-cock, which had a glass-wool plug in the constriction. The column was washed with 20 ml of water. A 4-ml aliquot (corresponding to 2 ml of a 24-h urine collection) was applied to the column, which was then washed with 30 ml of water. The fraction eluted with 35 ml of 0.1 *N* hydrochloric acid solution was collected and was evaporated to dryness at 40°C under vacuum.

Preparation of derivatives

The residues of the acetylpolyamine fraction were dissolved in 2 ml of water. These solutions were transferred to reaction vials (50 mm × 21 mm O.D.; Mighty Vial, No. 3, Maruemu Co., Osaka, Japan) with PTFE-lined caps. After addition of 0.5 ml of 10% sodium hydroxide solution and 0.2 ml of ethyl chloroformate, the mixtures were shaken at a frequency of 300 min^{-1} with a shaker for 10 min at room temperature. The resulting EOC derivatives were extracted three times with 2 ml of chloroform after saturation of the reaction mixtures with sodium chloride (1 g). The chloroform layers were collected, taking care to avoid aqueous droplets. The combined extracts were dried over anhydrous sodium sulphate (0.2 g) and evaporated to dryness at 65°C in a gentle stream of nitrogen. At this stage, the excess of reagent was also removed. The residues were dissolved in 50 μl of ethyl acetate; 4-μl aliquots of these solutions were analyzed by GC. The alkyloxycarbonyl derivatives other than EOC derivatives were prepared in the same way except that 0.1 ml of each reagent was used instead of ethyl chloroformate.

Gas chromatography

GC analyses were carried out on a Shimadzu 4CM gas chromatograph equipped with a flame ionization detector and a temperature programmer. Before packing, the glass column (1.0 m × 3 mm I.D.), the quartz wool which was placed in each end of the column, and the support (100—120 mesh Uniport HP; Gasukuro Kogyo Co., Tokyo, Japan) were silanized using 5% dimethyldichlorosilane in toluene [37]. The column packing, 0.5% SP-1000 on silanized Uniport HP, was prepared in our laboratory by using 1-butanol—chloroform (1:1, v/v) as a coating solvent according to the filtration method [37]. The packed column was conditioned at 280°C for at least 20 h with a nitrogen flow-rate of 30 ml/min. The operating conditions were as follows: nitrogen flow-rate, 40 ml/min; hydrogen flow-rate, 50 ml/min; air flow-rate, 0.8 l/min; injection and detector temperatures, 285°C; oven temperature, programmed to rise linearly at 6°C/min from 150°C to 280°C and held at 280°C for 5 min; chart speed, 0.5 cm/min; sensitivity, 10^2 (× 10^6 Ω); range, 4—8 (× 0.01 V).

Gas chromatography—mass spectrometry

The mass spectrometer coupled to a gas chromatograph was a Shimadzu LKB 9000, operated under the following conditions: trap current, 60 μA, ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion source temperature, 290°C; separator temperature, 285°C. GC analyses were performed under the following conditions: column (1.0 m × 3 mm I.D.), packed with 0.5% SP-1000 on silanized Uniport HP; helium flow-rate, 25 ml/min; oven temperature, programmed from 150°C to 280°C at 6°C/min.

Preparation of calibration curves and calculation

Calibration curves for the polyamines and their monoacetyl derivatives in the range 5—25 nmol were constructed using 12.5 nmol of the internal standard. In a series of reaction vials, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the standard solution (25 nmol/ml) were placed, and to each solution 0.5 ml of the internal standard solution (25 nmol/ml) was added. The total volume was made up to 2 ml with water. These mixtures were treated in the same manner as the urine samples. The peak height ratios of the samples to the internal standard were calculated, and plotted against the known quantities of the amines. For the determination of the monoacetylpolyamines in urine, the peak height ratios obtained from urine samples were compared to those of one standard, usually containing 10 nmol of monoacetylcadaverine, N^1-acetylspermidine and N^8-acetylspermidine and 20 nmol of monoacetylputrescine. This comparison was done for correcting daily variations; the linearity of each calibration curve was found to be reproducible.

Identification of chromatographic peaks from urine

Chromatographic peaks from urine samples were identified by co-injecting authentic EOC derivatives and, in some representative samples, by GC—MS of the EOC derivatives obtained from 10 ml of urine. Moreover, the EOC derivatives obtained from some samples were hydrolyzed with 6 *N* hydrochloric acid at 110°C for 4 h and analyzed by GC after re-derivatization in order to evaluate whether overlapping peaks were present at the positions of the EOC derivatives of monoacetylpolyamines.

Recovery experiment

To determine recovery of the overall procedure, four 24-h urine specimens were fortified with the standard solutions at the levels of 5, 7.5 and 10 nmol/ml urine. The overall recovery was calculated from triplicate analyses of these samples and of the corresponding urine specimens.

RESULTS AND DISCUSSION

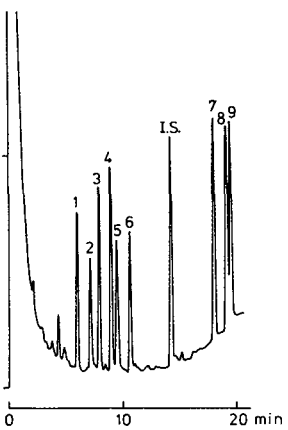
The reaction conditions for the preparation of the alkyloxycarbonyl derivatives of nine amines including monoacetyl-1,3-diaminopropane, monoacetylputrescine, monoacetylcadaverine, N^1-acetylspermidine and N^8-acetylspermidine as well as their non-conjugated forms were established on the basis of previous investigations [21,31—33]. At the initial stages of this work, we encountered the problem that the extraction of the alkyloxycarbonyl derivatives of monoacetyl-1,3-diaminopropane, monoacetylputrescine and monoacetylcadaverine from the aqueous reaction mixture was less efficient than that of the other derivatives when diethyl ether was used as solvent. However, this problem was solved by salting out the compounds with sodium chloride and by using chloroform instead of diethyl ether. Preparation of the derivatives could be performed within 20 min without special equipment, and several samples could be treated simultaneously. This derivatization method has, therefore, no obvious disadvantage over the dansylation procedure [13, 22].

The initial objective was to obtain a complete GC separation for each amine and our efforts were, therefore, directed towards testing the applicability of four alkyl chloroformates (ethyl, *n*-propyl, *n*-butyl and isobutyl), which are obtained from commercial sources. Each derivative was prepared from a standard solution containing all amines and was separated by GC using various columns in order to select the most suitable derivative and separation conditions. As a result, it was found that a 0.5% SP-1000 column (1.0 m) provided the desired separations with reasonable retention times. However, spermine could not be eluted from this column. Quantitatively measurable peaks were obtained of the EOC derivatives of all amines at the nanomole level.

A chromatogram showing separation of nine amines is reproduced in Fig. 1. A chromatographic run was completed within 20 min. In the case of urine analyses an additional 5 min were required for elution of other peaks. Formation of isobutyloxycarbonyl derivatives, which was developed by us [21] for the determination of free polyamines in urine hydrolysates and utilized by Bakowski et al. [38] for the analysis of plasma polyamines in the prognosis of response to chemotherapy of tumours, was not suitable for separation of spermidine, N^1-acetylspermidine and N^8-acetylspermidine.

The structures of all EOC derivatives were verified by GC—MS. Molecular ions with the expected *m/e* were observed for each derivative, suggesting that all primary and secondary amino groups were substituted by ethyloxycarbonyl groups, but the amide NH groups were not.

In order to examine the stability of the EOC derivatives, the amines were derivatized in the nanomole range, dissolved in ethyl acetate and stored at room temperature over a period of two weeks. No decomposition could be observed with any of the compounds, the peak heights remaining identical.



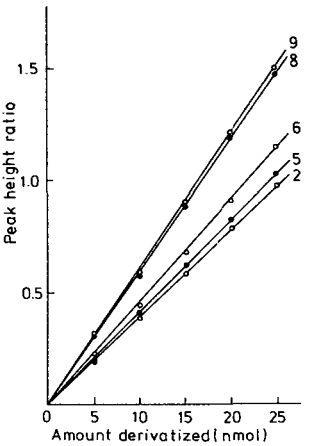


Fig. 1. GC separation of the EOC derivatives of a mixture of standard compounds. Each peak represents approximately 1.2 nmol. Peaks: 1=1,3-diaminopropane; 2=monoacetyl-1,3-diaminopropane; 3=putrescine; 4=cadaverine; 5=monoacetylputrescine; 6=monoacetylcadaverine; 7=spermidine; 8=N^1-acetylspermidine; 9=N^8-acetylspermidine; I.S.=internal standard (1,10-diaminodecane; this peak represents approximately 1.0 nmol). For details of the GC conditions see the Methods section.

Fig. 2. Calibration graphs for the monoacetylpolyamines. Internal standard: 1,10-diaminodecane, 12.5 nmol. Each line number corresponds to each peak number in Fig. 1.

A variety of compounds were evaluated for use as internal standard. 1,10-Diaminodecane was selected since it showed the same chromatographic behavior as the other amines on the silica gel columns and it did not co-chromatograph with other peaks derived from urine under the GC conditions used.

The calibration curves of the amines were constructed by ethyloxycarbonylating increasing amounts of the amines (5—25 nmol) and a fixed amount (12.5 nmol) of internal standard. Linear relationships were obtained for all amines. In Fig. 2, however, only the calibration curves of monoacetylpolyamines are shown. Each point in this figure represents the mean value obtained from six repetitive analyses of the same amounts of the various amines, and the relative standard deviation of the peak height ratios was 3.2% or less. The same is true for the free polyamines. This suggests that the reproducibility of both derivatization and GC analysis is adequate for quantitative determinations.

On the basis of these results, the applicability of the method in the determination of monoacetylpolyamines in urine was investigated. A clean-up procedure was needed to concentrate the compounds of interest to eliminate interfering urinary compounds. Several column chromatographic methods employing cation-exchange resin [23], silica gel [13, 36] and CM-cellulose [39] were evaluated. The silica gel column chromatography, which was originated by Grettie et al. [36], was found to be the most promising with respect to time requirement, simplicity and selectivity. Monoacetylpolyamines including monoacetylcadaverine were clearly separated from interfering urinary compounds, as is shown in Fig. 3A. Unfortunately, it turned out that a constituent which overlaps with putrescine could not be eliminated under our column chromato-

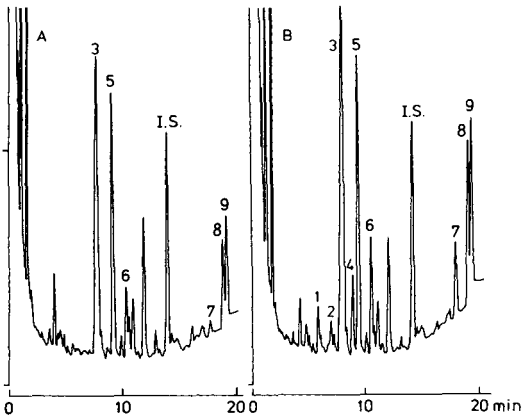


Fig. 3. Representative gas chromatograms obtained from a urine sample (A) and the same urine sample fortified with 5 nmol of each amine (B). Peaks: the same as in Fig. 1. I.S. = internal standard, 12.5 nmol of 1,10-diaminodecane. For details of the GC conditions see the Methods section.

graphic conditions. The peaks corresponding to monoacetylpolyamines were identified by analyzing urine samples fortified with standard solution (Fig. 3B) or by co-injecting authentic EOC derivatives. Further confirmation was carried out by GC—MS of some representative samples. The results showed that all peaks of interest were almost uniform.

Each quintuple of four different urine samples was treated as described in the Methods section in order to evaluate the overall reproducibility of peak height ratios. The results are summarized in Table I. They indicate that the reproducibility of the method is satisfactory. The overall recoveries of monoacetylpolyamines are summarized in Table II. It could be concluded that recovery was almost satisfactory. The overall recovery for cadaverine and spermidine was good. However, the concentrations of these amines were not determined in urine since their amounts were found to be small.

The 24-h excretion of monoacetylpolyamines in urine was estimated with the present method in fifteen normal individuals. The results are summarized in Table III. The concentrations of monoacetylputrescine, N^1-acetylspermidine

TABLE I

REPRODUCIBILITY OF PEAK HEIGHT RATIOS

Four different urine samples were treated as described in the text (n=5).

Subject	Ac-Put*		Ac-Cad		N^1-Ac-Spd		N^8-Ac-Spd	
	Mean**	S.D.(%)	Mean	S.D.(%)	Mean	S.D.(%)	Mean	S.D.(%)
A	1.232	4.4	0.211	3.8	0.427	4.2	0.468	3.2
B	1.236	2.8	0.276	1.0	0.324	5.6	0.422	2.4
C	2.064	2.5	0.655	2.4	0.649	5.7	0.750	4.5
D	1.399	1.6	1.474	2.4	0.476	2.7	0.419	3.1

*Abbreviations: Ac-Put, monoacetylputrescine; Ac-Cad, monoacetylcadaverine; N^1-Ac-Spd, N^1-acetylspermidine; N^8-Ac-Spd, N^8-acetylspermidine.

**Peak height ratios relative to the internal standard.

TABLE II

OVERALL RECOVERY OF MONOACETYLPOLYAMINES

Polyamines and their monoacetyl derivatives were added to 24-h urine specimens and subsequently these samples were treated as described in the text. Three independent determinations were carried out for each urine specimen. Data are shown for those monoacetylpolyamines which are usually found in urine.

Compound*	Recovery (%)**					
	Amount added (nmol/ml urine)					
	5		7.5		10	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
Ac-Put	84.0 ± 6.2	73.6—93.4	82.2 ± 8.0	75.9—93.4	86.6 ± 5.8	76.7—95.5
Ac-Cad	97.8 ± 6.0	91.6—104.7	92.5 ± 4.2	85.2—99.6	101.0 ± 5.9	92.5—108.6
N^1-Ac-Spd	102.0 ± 4.6	95.3—108.5	100.5 ± 5.4	86.9—106.1	101.6 ± 5.0	90.6—107.5
N^8-Ac-Spd	101.0 ± 3.9	93.4—105.1	100.5 ± 5.1	95.2—112.3	100.9 ± 5.5	93.2—108.4

*For abbreviations, see footnote to Table I.
**Calculated from the results of four different urine specimens.

TABLE III

24-h URINARY EXCRETION OF MONOACETYLPOLYAMINES IN NORMAL SUBJECTS

Subject No.	Concentration (μmol per 24 h)*					
	Ac-Put**	Ac-Cad	N^1-Ac-Spd	N^8-Ac-Spd	N^1-Ac-Spd + N^8-Ac-Spd	Ratio***
Male						
1	14.68	1.52	3.61	3.45	7.06	1.05
2	9.48	1.58	2.68	2.85	5.53	0.94
3	18.11	5.61	4.76	5.34	10.10	0.89
4	24.58	2.87	6.34	3.41	9.75	1.86
5	11.11	0.99	2.03	1.84	3.87	1.10
6	12.19	7.32	2.80	3.04	5.84	0.92
7	13.33	0.60	3.26	2.82	6.08	1.16
Mean ± S.D.	14.8 ± 5.1	2.9 ± 2.6	3.6 ± 1.5	3.3 ± 1.1	6.9 ± 2.3	1.1 ± 0.3
Female						
1	11.82	2.69	2.59	3.48	6.07	0.74
2	10.29	8.91	2.68	1.96	4.64	1.37
3	15.55	10.41	2.79	3.49	6.28	0.80
4	7.83	0.55	3.04	2.57	5.61	1.18
5	8.12	0.89	2.18	1.82	4.00	1.20
6	14.67	1.93	3.36	3.09	6.45	1.09
7	7.98	13.94	4.76	3.00	7.76	1.59
8	11.58	9.44	3.66	2.37	6.03	1.54
Mean ± S.D.	11.0 ± 3.0	6.1 ± 5.2	3.1 ± 0.8	2.7 ± 0.6	5.9 ± 1.1	1.2 ± 0.3

*Mean values of duplicate determinations.
**For abbreviations, see footnote to Table I.
***Ratios of N^1-Ac-Spd to N^8-Ac-Spd.

and N^8-acetylspermidine obtained in this study are in the same range as those published previously [11,14,23]. The ratios of N^1-acetylspermidine to N^8-acetylspermidine are also in agreement with the reported values, the mean value being close to one. The concentrations of monoacetylcadaverine in some samples were considerably elevated compared with the results of Abdel-Monem et al. [11]. These samples were hydrolyzed with 6 *N* hydrochloric acid at 110°C for 4 h, and the hydrolysates were re-derivatized and analyzed. The results suggest that the peak corresponding to monoacetylcadaverine was uniform in our GC. This was further confirmed by GC—MS; the spectra obtained from the urine samples were identical with those obtained from the authentic EOC derivative of monoacetylcadaverine. It has been pointed out that cadaverine is presumably produced by bacteria in the lumen of the large intestine from dietary protein or lysine [5]. It is likely that the observed differences are due to differences in the intestinal bacterial flora and/or the diet taken by volunteers. However, it is not clear which organ is responsible for the formation of monoacetylcadaverine.

CONCLUSSION

In the present work we have described a GC method for the determination of urinary monoacetylpolyamines. This is the first GC method which permits the separation of N^1-acetylspermidine and N^8-acetylspermidine. The method includes three steps: isolation of the monoacetylpolyamine fraction from urine, derivatization with ethyl chloroformate and determination by GC. The clean-up step using a silica gel column [36] is obligatory for the determination of urinary monoacetylpolyamines since urine contains many interfering substances. The preparation of the EOC derivatives can be carried out rapidly and simply, and no precaution is necessary in the handling and storage of these derivatives. A GC run takes 25 min.

The sensitivity of the method is modest compared with the method using fluorescence detection. However, the method is sufficiently applicable to the analysis of urinary monoacetylpolyamines. The reproducibility of the method for the measurement of monoacetylpolyamines in urine samples was reasonable, the relative standard deviations ranging from 1.0 to 5.7% (Table I). It was observed that the recoveries of monoacetylputrescine varied somewhat depending on the urine specimen, although individual specimens gave constant recoveries. This reflects the relatively large standard deviations of monoacetylputrescine (Table II). There are two serious limitations to the method: putrescine determination in urine is hampered by interfering peaks, and spermine can not be eluted from the GC column that is suitable for the analysis of other amines.

We believe that the method described here can be considered as an alternative routine method in biochemical and clinical research requiring the analysis of urinary monoacetylpolyamines.

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PRACTICAL GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF AMINO ACIDS IN HUMAN SERUM

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SUMMARY

Application of the gas—liquid chromatographic method previously reported by us was made to the analysis of the 22 amino acids including asparagine and glutamine in serum. The method permitted that aqueous serum samples obtained after deproteinization with perchloric acid were directly subjected to derivatization without any further clean-up procedure such as ion-exchange chromatography. The N-ethyloxycarbonyl methyl esters, which were prepared in the same manner as the N-isobutyloxycarbonyl methyl esters, were introduced for the determination of leucine, isoleucine, arginine and tyrosine. Both derivatives were prepared by two-step procedures involving alkyloxycarbonylation in aqueous media and esterification with diazomethane, and simultaneously analyzed by using the dual set of columns with the same thermal conditions. The advantages of this method are that the sample pretreatment and derivatization are very simple and rapid, and that both asparagine and glutamine along with other amino acids in serum can be determined.

INTRODUCTION

Extensive research investigations conducted for the development of amino acid analysis by gas—liquid chromatography (GLC) have resulted in the appearence of certain promising derivatives and derivatization methods. An enormous number of approaches for the preparation of volatile derivatives suitable for the quantitative analysis of amino acids has been reviewed [1—3]. The N-perfluoroacyl alkyl esters including the N-trifluoroacetyl (TFA) methyl esters [4—8], the N-TFA *n*-butyl esters [9—18], the N-heptafluorobutyryl (HFB) *n*-propyl esters [19—23], the N-HFB isoamyl esters [24,25], the N-HFB isobutyl esters [26—37] and the N-pentafluoropropionyl isopropyl esters [38,39] have been studied the most extensively, some of which have been practically employed for the successful quantitation of amino acids in biological samples.

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However, it has been recently pointed out that these derivatives possess certain negative aspects when applied to routine laboratory usage [40]. Notably, it would be supposed to be substantially disadvantageous that derivative preparations require conditions free from moisture and high reaction temperature, and that the amides, asparagine and glutamine, can not be differentiated from their corresponding acids due to hydrolysis in the drastic esterification phase. On the other hand, the N-acetyl *n*-propyl esters which are considered to be more stable to moisture than the N-perfluoroacyl alkyl esters have been successfully applied to biological samples [41—44]. Also in this case it was not possible to distinguish the amides from their acids.

The amides, especially glutamine, play important roles in a wide variety of physiological processes [45—49], so that a suitable method which allows simultaneous estimation of the amides and the free acids is desired. Therefore, the GLC method is inferior to the classical ion-exchange chromatographic method [50,51] because the latter allows determination of asparagine and glutamine in the presence of the free acids together with other amino acids. However, the ion-exchange chromatographic method is characterized by more lengthy analysis times if these amides should be analyzed, and some problems have been mentioned for analysis of glutamine [52]. The difficulty in the GLC methods described above still remains to be solved, although a few attempts using the esterification under restricted conditions have been made [45,48,53].

Recently, another approach has been proposed on the basis of the formation of 1,3-oxazolidinone derivatives of amino acids [40]. This method maybe has potential since it permits analyzing both asparagine and glutamine in the presence of all other serum amino acids.

Although the N-dinitrophenyl methyl esters possess more desirable features with regard to ease of derivative preparation, they have only been applied to analysis of limited amino acids in serum owing to their low volatility [54].

Some years ago, we proposed a new GLC approach [55], in which the volatile derivatives, N-isobutyloxycarbonyl (isoBOC) methyl esters were prepared under mild conditions by reaction with isobutyl chloroformate in aqueous alkaline media, followed by esterification with diazomethane. Recently, we have reported [56] that improvements of the previous method have made it possible to perform the determination of all the protein amino acids including asparagine and glutamine in sub-microgram amounts. In this paper, we describe the application of the method to the determination of amino acids in human sera.

EXPERIMENTAL

Reagents and materials

Standard amino acids and kainic acid as an internal standard were obtained from Nakarai Chemicals (Kyoto, Japan). Two standard solutions, one containing the 21 amino acids except ornithine listed in Table I and the other containing the 21 amino acids plus ornithine, were prepared in 0.1 *M* hydrochloric acid at individual concentrations of 50 μg/ml. An internal standard solution (50 μg/ml) was prepared in water. Ethyl and isobutyl chloroformates were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The reagents (anhydrous sodium sul-

phate, sodium chloride, reagents for generation of diazomethane), arginase solution and water were the same as in the previous paper [56]. It is recommended that freshly distilled, peroxide-free diethyl ether is used to protect the amino acids easily oxidized, especially methionine. All other chemicals and solvents were the purest grades available from standard commercial sources.

Sample preparation

Human blood was drawn from the cubital vein, left to stand for 10 min in an ice-bath and then centrifuged to remove blood particles.

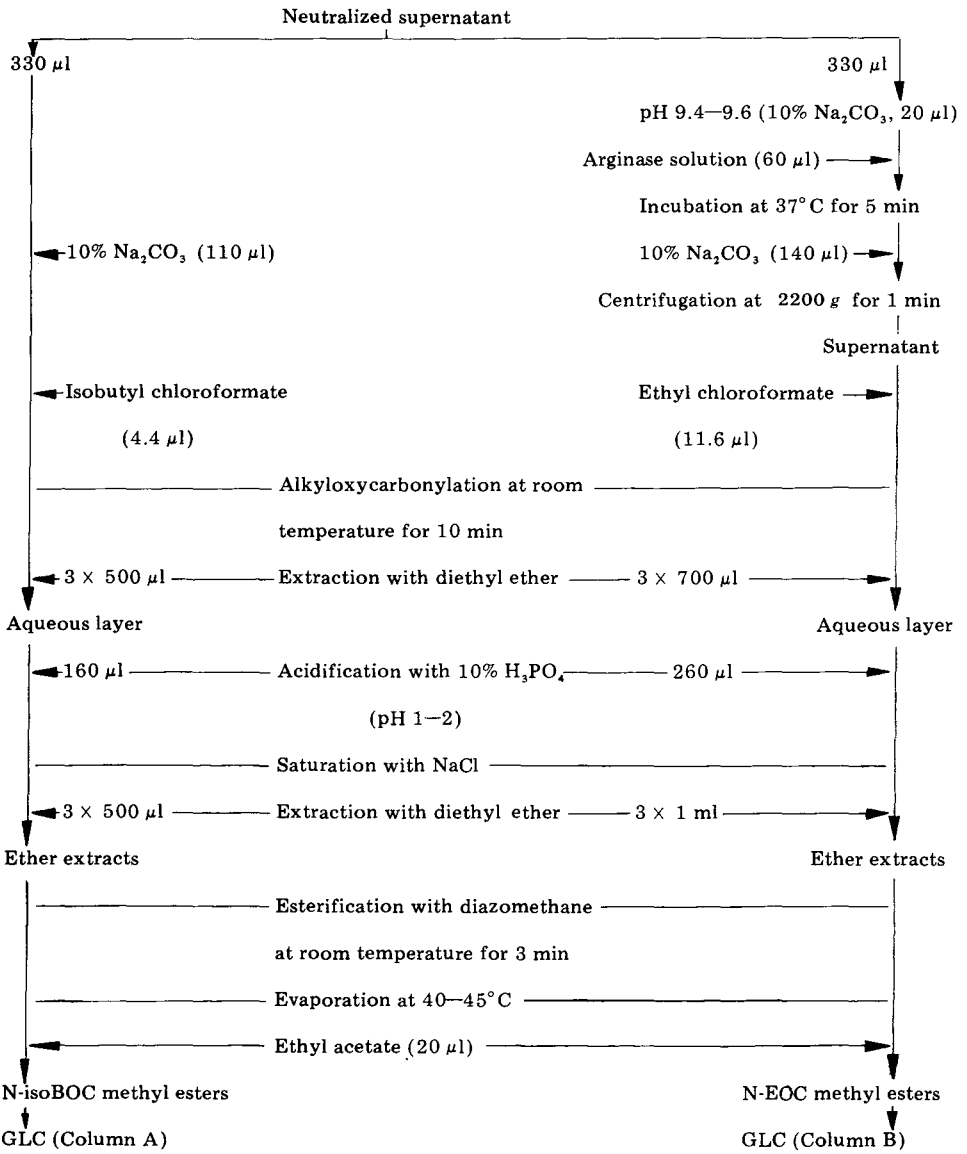

Fig. 1. Schematic flow diagram of the procedure.

To 200 μl of serum in a 2-ml glass tube (6.5 cm × 9 mm I.D.) were successively added 100 μl of the internal standard solution, 100 μl of water and 80 μl of 10% orthophosphoric acid. The solution was gently mixed for 10 sec with an equal volume of chloroform with a Vortex-type mixer and centrifuged at 2200 *g* for 5 min. The upper layer was transferred to another tube, taking care not to collect any chloroform droplets, and 80 μl of 12% perchloric acid were added with gentle swirling to precipitate proteins. Vigorous mixing should be avoided at this stage. After centrifugation for 1 min at 2500 *g*, the supernatant was carefully transferred to a small glass tube and neutralized with 10% sodium carbonate (ca. 120 μl).

Each 330-μl portion of the neutralized supernatant was taken in two 2-ml vials fitted with PTFE-lined screw caps (Wheaton No. 224950, Wheaton Scientific, Millvilles, NJ, U.S.A.) and they were treated according to the flow diagram shown in Fig. 1. Leucine, isoleucine, arginine and tyrosine were determined as their N-ethyloxycarbonyl (EOC) methyl esters, the reason for which is described in the Results and Discussion section. Since the colloidal precipitates were produced when 10% sodium carbonate was added to the incubation mixture obtained after arginase treatment, they were removed by centrifugation at 2200 *g* for 1 min and the supernatant was transferred to another vial with a PTFE-lined screw cap. This procedure contributed to obtaining reproducible results in the determination of tyrosine. Ethyl and isobutyl chloroformates (11.6 μl and 4.4 μl, respectively), were taken up by means of 10-μl syringes (Hamilton, Reno, NV, U.S.A.). Both isobutyloxycarbonylation and ethyloxycarbonylation were performed with a shaker (Iwaki KM Shaker VS Type, KK Iwaki, Tokyo, Japan), which was operated at 300 rpm (up and down). Extraction with diethyl ether was achieved with a Vortex-type mixer for 30 sec. After the first ether extracts were discarded, the reaction mixture was brought to pH 1—2 with 10% orthophosphoric acid and then saturated with sodium chloride. The combined ether extract was esterified with diazomethane [57]. The residue obtained after evaporation of the solvent was dissolved in 20 μl of ethyl acetate and thereafter a few grains of anhydrous sodium sulphate were added. From the resulting solution two 4—5 μl aliquots were taken and each was injected simultaneously into the gas chromatograph with two analytical columns, Column A and B.

Gas—liquid chromatography

The Shimadzu 4CM gas chromatograph used in this study was equipped with dual hydrogen flame ionization detectors, a double-column oven with on-column injection ports and a temperature programmer, and each electrometer was individually connected to a one-pen recorder. Two columns (each 1 m × 3 mm I.D.) were employed consisting of silanized glass tubes packed with 100—120 mesh re-silanized Uniport HP coated with 1.605% Poly-I-110/Poly-A-101A/FFAP (2000:1300:105, w/w/w) mixed phase (Column A) and 1.0% Poly-A-101A/FFAP (1:1, w/w) mixed phase (Column B), and were simultaneously operated. Materials for GLC were obtained as follows: the polyamide stationary phases Poly-I-110 and Poly-A-101A and the polyester stationary phase FFAP from Applied Science Labs. (State College, PA, U.S.A.), and the support Uniport HP from Gasukuro Kogyo (Tokyo, Japan). The column packings were

prepared and conditioned exactly as described in the previous paper [56]. The GLC conditions for analyses were identical for both columns with the exception of the nitrogen flow-rates. The columns were programmed from 80°C to 280°C at 10°C/min and maintained at 280°C for 5 min. The nitrogen flow-rates for Columns A and B were 35 and 25 ml/min, respectively. Quantitation of leucine, isoleucine, arginine and tyrosine was performed on Column B, and that of the other amino acids on Column A.

Calibration graphs, recovery rates and calculations

For the construction of calibration graphs, the proportional amounts of the standard corresponding to 0.5—10 μg (10—200 μl of the standard solution) and 100 μl of the internal standard (10 μg) were put into 2-ml vials, and the total volume of each vial was made up to 330 μl with water. The solutions were subjected to the procedure shown in Fig. 1. The standard solution not containing ornithine was employed only in the procedure involving the arginase treatment. The peak height ratios of amino acids with respect to the internal standard were calculated and were plotted against the quantities of amino acids.

To evaluate the recovery rates, five 200-μl portions of the serum sample, supplemented with 5—15 μg of each amino acid, were analyzed by the present procedure. The parent serum sample was processed at the same time. The recovery rates were calculated by subtracting the amounts obtained from the parent serum from those obtained from the same serum fortified with amino acids.

The calibration graph for ornithine obtained on Column B was used for the determination of total ornithine obtained after arginase treatment because the conversion yield of arginine to ornithine was above 95% in the range 0.5—10 μg of arginine under the conditions used. The concentration of arginine was determined by the following equation:

$$\text{Arginine (mg/dl)} = (\text{Orn}_{\text{total}} - \text{Orn}) \times 1.318 \times 500$$

where $\text{Orn}_{\text{total}}$ = amount of total ornithine obtained using Column B after arginase treatment, and Orn = amount of ornithine obtained using Column A without arginase treatment.

In the analysis of serum amino acids, one standard point, usually at the 5-μg level of amino acids, was incorporated in each series of determinations and the peak height ratios obtained from the standard were employed for the calculation of amino acid concentrations in sera.

RESULTS AND DISCUSSION

Generally, in most of the GLC methods proposed for the determination of amino acids in serum, the denaturants such as picric acid [12,17,39,58], sulfosalicylic acid [33] and trichloroacetic acid [15] were employed to remove proteins by precipitation. An alternative technique, based on dilution of the sample with acetic acid and without involving protein precipitation, has also been used [40,42,44,58,59]. However, in all cases a clean-up procedure using the ion-exchange resin column prior to derivatization was necessary, not only to exclude the excess of denaturants which usually interfere with the following GLC analysis, but also to isolate the amino acids.

On the other hand, our derivatization method has the advantage that amino acids in aqueous solution can be easily converted to their N-isoBOC amino acids, which are selectively extracted from the acidified reaction mixture into diethyl ether, without any step to exclude water (moisture) being necessary prior to derivatization. Taking this into account, attempts were carried out to develop a less time-consuming and less laborious sample pretreatment. Inorganic denaturants would be expedient for this purpose since it is not necessary in our method to remove them. The use of perchloric acid finally proved to be convenient for the effective and reliable removal of serum proteins in combination with prior extraction of lipids with chloroform from the acidified serum. Not only did this improve the clean-up of amino acids from serum but it was also satisfactory with respect to time requirements.

In the preliminary experiments, we tried to analyze all of the serum amino acids as their N-isoBOC methyl esters. However, it was found that the recovery of tyrosine from serum was not satisfactory, being below 70%. A similar phenomenon was also observed even when serum was subjected to the picrate precipitation followed by cation-exchange column chromatography according to the method of Zumwalt et al. [12] and the amino acids thus obtained were analyzed as their N-isoBOC methyl esters. Therefore, it may be tentatively concluded that the incomplete derivatization of tyrosine mainly resulted from its adsorption to some specific components which were not removed by these clean-up processes. This problem was solved by introducing the N-EOC methyl ester derivatives, which could be prepared by the reaction with ethyl chloroformate in the same fashion as in the N-isoBOC methyl esters. The recovery rates of tyrosine were sufficiently improved (see Table II), suggesting that ethyl chloroformate is more reactive than isobutyl chloroformate. As a consequence

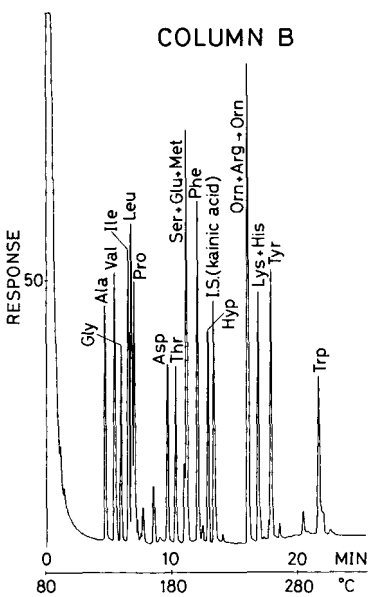

Fig. 2. Gas chromatographic separation of the N-EOC methyl esters of amino acids on Column B. Sample preparation and GLC conditions as described in Experimental.

of these observations, we decided to adopt the N-EOC methyl esters for the determination of leucine, isoleucine and arginine along with tyrosine in serum on Column B since in our method the former three amino acids essentially required a separate analysis [56]. Fortunately, it was found that the N-EOC methyl esters of these four amino acids provided good separations on Column B, as shown in Fig. 2. Alternatively, an attempt to use the N-EOC methyl esters for the simultaneous analysis of all serum amino acids failed because the N-EOC amino acids (not being esterified) of threonine, serine and hydroxyproline could not be extracted quantitatively from the acidified aqueous reaction mixture, particularly when working with the low levels. In addition asparagine and glutamine only gave negligible peaks presumably due to higher solubility of their N-EOC amino acids in aqueous solution.

After trying a number of compounds, kainic acid was chosen as the internal standard. Both derivatives of kainic acid were well-separated from those of the amino acids both on Column A and B, as shown in Figs. 3 and 4, and no peak was observed in the serum samples with the same retention time as that of the internal standard. Addition of the internal standard to serum at the first step in the overall procedure made the method reliable.

The total reaction volume in the alkyloxycarbonylation was reduced to below 1 ml while the concentration of sodium carbonate was maintained at

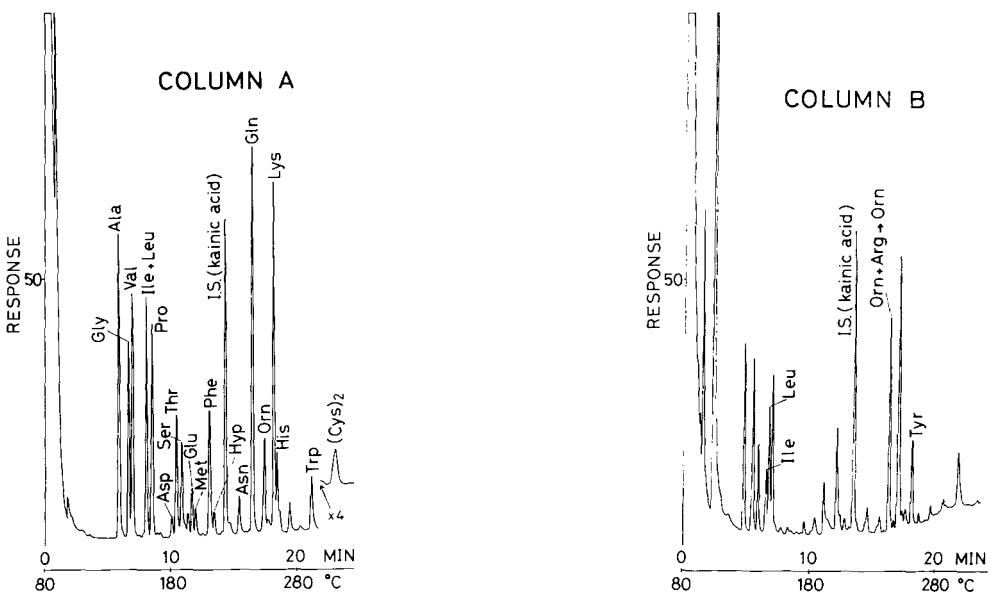

Fig. 3. Representative gas chromatogram of the N-isoBOC methyl esters of amino acids in a serum sample analyzed on Column A. Sample preparation and GLC conditions as described in Experimental.

Fig. 4. Representative gas chromatogram of the N-EOC methyl esters of amino acids in a serum sample analyzed on Column B. Prior to derivatization, a serum sample was treated with arginase, and only the peaks labelled by their names were determined. Sample preparation and GLC conditions as described in Experimental.

2.5% (w/v). From the experiments with the standard mixture containing 30 μg of each amino acid, it was found that the amounts of reagents equivalent to ca. 1% (v/v) for isobutyl chloroformate and ca. 2% (v/v) for ethyl chloroformate against the total reaction volume were enough to accomplish the reaction quan-

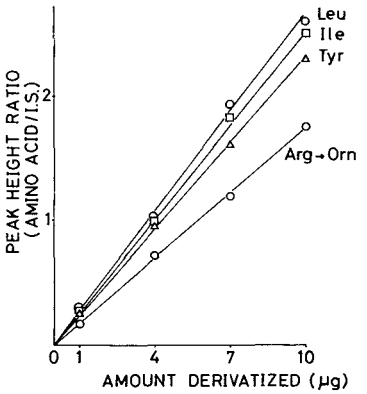

Fig. 5. Calibration graphs for the amino acids analyzed as their N-EOC methyl esters on Column B after arginase treatment followed by derivatization. Internal standard: 5 μg of kainic acid.

TABLE I

OVERALL REPRODUCIBILITY OF ANALYSES OF AMINO ACIDS IN HUMAN SERUM

Five 200-μl aliquots of serum were individually analyzed throughout the entire procedure.

Amino acid*	Abbreviation	Mean (mg/dl)	C.V. (%)
Alanine	Ala	2.81	1.67
Glycine	Gly	1.84	2.83
Valine	Val	2.57	2.98
Proline	Pro	2.41	3.69
Aspartic acid	Asp	0.23	6.15
Threonine	Thr	1.28	4.80
Serine	Ser	1.35	4.84
Glutamic acid	Glu	0.76	3.88
Methionine	Met	0.23	5.68
Phenylalanine	Phe	1.20	1.51
Hydroxyproline	Hyp	0.29	3.93
Asparagine	Asn	0.89	6.74
Glutamine	Gln	11.0	4.40
Ornithine	Orn	0.50	4.60
Lysine	Lys	2.02	5.37
Histidine	His	1.31	4.23
Tryptophan	Trp	0.35	5.50
Cystine	$(Cys)_2$	1.03	7.27
Isoleucine	Ile	0.65	4.74
Leucine	Leu	1.22	4.02
Arginine	Arg	2.47	1.65
Tyrosine	Tyr	1.45	1.43

*Prior to derivatization, arginine was converted to ornithine by treatment with arginase, and the total ornithine (ornithine from arginine plus ornithine originally present) together with isoleucine, leucine and tyrosine were analyzed as their N-EOC methyl esters on Column B. The other amino acids were analyzed as their N-isoBOC methyl esters on Column A.

titatively and reproducibly. It is, therefore, apparent from the above experiments that quantitative analysis was not affected even by more elevated amino acid levels in sera under the experimental conditions described.

The use of large excess of arginase (ca. 24 units/vial) in this study shortened the incubation time to 5 min, while maintaining the quantitative conversion of arginine to ornithine. However, in this case the precipitates were produced by subsequent addition of sodium carbonate solution, and this should be centrifuged off in order to ensure the quantification of tyrosine.

Calibration linearity for leucine, isoleucine, arginine and tyrosine obtained in the presence of all other amino acids was satisfactory as depicted in Fig. 5 and its between-run and within-run reproducibility throughout the overall procedure consisting of arginase treatment, derivatization and GLC analysis on Column B was sufficient enough to perform quantitative analyses. Similarly good results were obtained for other amino acids which were analyzed on Column A as their N-isoBOC methyl esters.

To check the reproducibility of this method, five 200-μl portions of the same serum sample were taken through the complete procedure. Table I lists the results with the mean concentration and the relative standard deviation for

TABLE II

RECOVERY RATES OF AMINO ACIDS ADDED TO HUMAN SERUM

Serum samples fortified with the known amounts (μg/200 μl of serum) were analyzed throughout the entire procedure.

Amino acid*	Recovery (%)						Mean	C.V. (%)
	5 μg		10 μg		15 μg			
	A**	B	A	B	A	B		
Alanine	101.9	94.2	98.4	93.1	98.4	101.7	98.0	3.75
Glycine	104.3	96.1	98.8	90.9	100.1	100.6	98.5	4.63
Valine	98.1	91.0	97.1	92.4	97.6	99.4	95.9	3.54
Proline	95.3	92.5	97.6	93.0	99.9	97.7	96.0	3.03
Aspartic acid	103.7	98.9	97.3	96.9	96.5	101.7	99.2	2.95
Threonine	103.9	99.2	99.3	100.7	106.6	102.5	102.0	2.84
Serine	105.2	102.9	98.1	106.1	95.1	106.1	102.3	4.52
Glutamic acid	94.2	98.9	101.2	93.8	101.5	99.6	98.2	3.46
Methionine	95.8	91.2	96.5	90.5	105.4	99.8	96.5	5.76
Phenylalanine	89.1	83.9	94.3	90.2	99.7	96.4	92.3	6.14
Hydroxyproline	100.3	103.7	103.7	100.8	98.1	105.5	102.0	2.69
Asparagine	103.4	110.8	95.9	108.9	94.8	108.6	103.7	6.70
Glutamine	101.7	100.0	92.3	92.2	100.2	106.5	98.8	5.67
Ornithine	99.0	93.7	100.8	97.3	92.1	102.5	97.6	4.14
Lysine	94.6	90.6	101.1	95.6	93.6	99.6	95.9	4.06
Histidine	106.9	108.4	102.5	109.2	106.3	101.4	105.8	3.00
Tryptophan	83.2	81.2	95.4	84.8	99.4	94.0	89.7	8.40
Cystine	101.4	110.5	110.4	106.7	107.5	104.2	106.8	3.33
Isoleucine	102.9	101.3	101.4	99.1	94.8	98.7	99.7	2.87
Leucine	93.0	98.6	102.2	101.1	96.0	92.5	97.2	4.20
Arginine	99.8	101.9	102.2	102.7	96.4	104.2	101.2	2.71
Tyrosine	102.7	100.5	95.6	99.0	96.3	94.2	98.1	3.30

*See footnote to Table I.

**A and B are different serum samples.

each amino acid. As can be seen, the method allows the determation of amino acids in serum with a relatively high degree of reproducibility.

When serum samples fortified with cysteine in addition to the 22 amino acids were analyzed by the present method, the cysteine peak mostly disappeared and the recovery of cystine was increased. It was proved by almost all investigators that cysteine could be easily oxidized to cystine, but not quantitatively, and this would explain the increase of recovery rate for cystine. Therefore, in this study cysteine was excluded. The recovery rates were determined by comparison with their respective standards and the results are shown in Table II. Good recoveries with reasonable relative standard deviations could be obtained for all amino acids investigated.

Ten human serum samples obtained from normal adult volunteers, who were not restricted in food intake, were analyzed according to the present method to discern whether the levels of amino acids determined are in agreement with those reported by others using an amino acid analyzer technique [42,60] and a GLC method [42]. The results are given in Table III, and typical chromatograms both on Column A and B obtained from a serum sample are illustrated in

TABLE III

AMINO ACID ANALYSIS OF HUMAN SERUM BY THE PRESENT METHOD AND COMPARISON WITH THE LITERATURE VALUES

Samples were taken from ten normal adult subjects and each was analyzed in duplicate. Values for the amino acid analyzer and GLC methods were from refs. 60 and 42, respectively.

Amino acid	Concentration (mg/dl)				
	Present method*		Amino acid analyzer		GLC method
	Range	Mean	Range	Mean	Range
Alanine	2.45—4.64	3.32	2.31—4.65	3.46	2.9—4.3
Glycine	1.75—2.70	2.15	1.37—2.30	1.84	0.9—2.8
Valine	2.20—3.14	2.50	2.34—3.63	2.80	2.1—4.1
Proline	1.31—2.77	1.86	–	2.36**	1.9—3.0
Aspartic acid	0.17—0.50	0.32	0.13—0.28	0.17	0.2—1.5***
Threonine	0.96—2.25	1.58	1.31—2.38	1.87	1.0—1.7
Serine	0.96—1.88	1.33	0.95—1.79	1.30	0.7—1.9
Glutamic acid	0.52—1.03	0.75	0.22—1.27	0.43	4.8—12.9***
Methionine	0.27—0.47	0.34	0.39—0.73	0.55	0.3—0.8
Phenylalanine	0.96—1.48	1.19	0.74—1.34	0.97	0.3—2.2
Hydroxyproline	0.17—0.38	0.26	–	–	0.1—0.8
Asparagine	0.67—1.09	0.81	0.63—1.19	0.82	–
Glutamine	9.60—11.4	10.6	7.31—12.1	9.21	–
Ornithine	0.61—1.09	0.85	0.46—1.15	0.74	0.1—1.2
Lysine	1.68—3.10	2.26	1.68—3.95	2.51	1.9—4.1
Histidine	1.11—1.59	1.29	0.90—1.72	1.33	0.6—1.8
Tryptophan	0.62—1.17	0.87	–	1.11**	0.5—2.4
Cystine	0.24—1.60	0.73	1.20—1.97	1.44	0.6—1.9
Isoleucine	0.66—1.07	0.88	0.70—1.34	0.96	0.3—1.7
Leucine	1.45—1.99	1.64	1.36—2.40	1.80	0.7—2.1
Arginine	0.95—2.87	2.05	1.24—2.26	1.65	0.9—2.7
Tyrosine	0.72—1.23	1.12	0.94—2.07	1.40	0.6—1.5

*See footnote to Table I.
**Values cited from ref. 61.
***Values include the corresponding amides.

Figs. 3 and 4. The ranges of serum amino acid values found by the present method correspond reasonably well with the results reported by others, although the population of individuals taking part in this study was rather small. Only a few and small extraneous peaks are detected on the chromatograms, thus indicating that the procedure provides adequate purification and separation of amino acids in sera. This method is by no means inferior to other GLC methods, as it required only small volumes of serum (200 μl).

In most GLC methods proposed to date, asparagine and glutamine are completely converted to their corresponding acids during acid-catalyzed esterification, so that no distinction between the amides and the acids is possible, whereas in our method both of the amides emerged as symmetrical and well-separated peaks, and could be determined separately from the respective acids and also from all other amino acids. Purification of the support is most important for a precise analysis of both amides since their derivatives still keep the intact amide groups in their molecules. The purification procedure for Uniport HP used has been reported in a previous paper [56].

CONCLUSION

We have developed a GLC method for the determination of the 22 amino acids including asparagine and glutamine in human serum, in which a serum treatment, involving neither ion-exchange column chromatography nor subsequent eluate evaporation, was introduced so that the first step of our derivattization method could be carried out in aqueous medium. Only 20 min was needed for the alkyloxycarbonylation and esterification, and the GLC analysis could be performed in 25 min. This method has the advantage of determining both asparagine and glutamine in serum. Recently, another method has been reported which also allows determination of asparagine and glutamine in serum [40].

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REVERSED-PHASE LIQUID CHROMATOGRAPHIC INVESTIGATION OF UV-ABSORBING LOW-MOLECULAR-WEIGHT COMPOUNDS IN SALIVA

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SUMMARY

The reversed-phase mode of high-performance liquid chromatography was used to determine the intra- and inter-individual levels of UV-absorbing low-molecular-weight compounds in saliva. Many of the compounds known to occur in serum were also found in saliva; however, concentrations in saliva are lower. Both the intra- and inter-individual levels of these compounds vary significantly; in most cases, the inter-individual variance is 2—3 times the intra-individual variance.

Caffeine and its metabolites in saliva are also reported. A greater number of metabolites were found in the saliva of habitual coffee drinkers. After caffeine was administered orally, paraxanthine, theobromine, theophylline, 1-methylxanthine, and 1-methyluric acid were found in the saliva of an individual who did not drink coffee regularly. In this subject, the serum half-life for caffeine was 3.49 h and the saliva half-life was 3.27 h. The half-life of caffeine in an habitual coffee drinker who had refrained from caffeine products for four days was 4.39 h.

INTRODUCTION

Since dosages required for optimal effects can vary widely among patients, drugs with a narrow therapeutic range, such as the methylated xanthines, must be monitored periodically to ensure that an effective and nontoxic dose has been prescribed. However, frequent collection of blood is inconvenient and can add discomfort, especially with pediatric and geriatric patients. Saliva, on the other hand, is convenient; sample collection is noninvasive and can

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be repeated often. Therefore, saliva has been investigated as a matrix to monitor caffeine [1] and theophylline [2, 3].

Numerous methods have been developed to measure the methylated xanthines in biological matrices [1—5]. In recent years, high-performance liquid chromatography (HPLC) has been shown to be ideally suited for the assay of these compounds [6—13]. Many of these methods have been applied directly to saliva analysis [1—3, 9, 12—14]. However, little information has been reported on the metabolites of the methylated xanthines in saliva. Moreover, the UV-absorbing low-molecular-weight endogenous constituents of saliva have not been fully studied; knowledge of these compounds could provide useful clinical data and may aid in the identification of possible interfering substances.

In this paper, the reversed-phase mode of HPLC was used to determine the intra- and inter-individual levels of the UV-absorbing low-molecular-weight endogenous compounds in the saliva of a group of subjects. In addition, caffeine and its metabolites in saliva are reported. The effects of diet and sample preparation on the chromatographic profiles of saliva constituents were also examined.

EXPERIMENTAL

Chromatographic equipment

A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with two M 6000A pumps, M 660 solvent programmer, U6K injector, and M 440 dual-wavelength UV detector was used. A Houston Instrument (Austin, TX, U.S.A.) two-pen recorder was used to trace the 280-nm and 254-nm signals and to calculate peak-height ratios (PH-280/PH-254). A scanning UV—VIS spectrophotometer (SF 770 Spectroflow Monitor) and fluorescence detector (FS 970 L.C. Fluorometer) manufactured by Schoeffel Instrument Division (Kratos, Westwood, NJ, U.S.A.) were used to aid in the identification of the saliva constituents. The output of the ancillary detectors was recorded on a Kratos dual-pen recorder. Peak areas and retention times were obtained with an HP 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Chromatographic conditions

The low-molecular-weight UV-absorbing compounds in saliva were separated on commercially available columns (300 × 3.9 mm I.D.; Waters) packed with 10-μm octadecyl-silica (Waters). A Waters precolumn (25 × 3.9 mm I.D.) packed with 25—37-μm octadecyl-silica (Whatman, Inc., Clifton, NJ, U.S.A.) was used to protect the analytical column.

A screening separation of the endogenous compounds was done with a 5-min isocratic elution with 0.02 *M* potassium dihydrogen phosphate, pH 5.7, followed by a 35-min linear gradient to 24% methanol. Paraxanthine, theophylline, theobromine, caffeine, and 8-chlorotheophylline (as an internal standard) were separated isocratically with 0.01 *M* dibasic ammonium phosphate (pH 4.5)—methanol—acetonitrile (91:6:3, v/v). In addition, the chromatographic conditions developed by Orcutt et al. [9] were used to aid in the identification of the caffeine metabolites. Flow-rates were 1.5 ml/min and column temperature was ambient. All eluents were degassed by sonication and helium purge.

Chemicals and chromatographic standards

The phosphate and acetate buffers were obtained from Fisher Scientific Company (Fair Lawn, NJ, U.S.A.). Glass-distilled methanol and acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were used throughout. Standard compounds and enzymes were from Sigma Chemical Company (St. Louis, MO, U.S.A.) and Vega Biochemicals (Tucson, AZ, U.S.A.). Caffeine tablets (100 mg, Bristol-Meyers Co., New York, NY, U.S.A.) were used in the caffeine metabolism studies. The chemical reagents for qualitative identifications were obtained from Fisher and other sources.

Sample collection

Saliva samples were obtained from donors with no known disease or abnormalities. The subjects were not maintained on strict diets; however, inventories of foods, beverages, and drugs were kept. Samples were taken in the morning after a 12-h fasting period unless otherwise stated. The saliva was collected without the aid of saliva-stimulating agents.

Serum was obtained by subcubital venipuncture drawing of blood into 5-ml tubes and clot formation was allowed for 15—20 min at room temperature, after which the tubes were centrifuged at 1145 relative centrifugal force (RCF) for 5—15 min. The serum was then removed from the packed materials.

Sample preparation

Serum samples were processed according to protocol developed elsewhere [15, 16]. Sera were filtered through membrane cones (CF-25, Amicon Corp., Lexington, MA, U.S.A.) at 640 RCF for 20 min to remove material with molecular weights greater than 25,000.

The effects of handling procedures on the chromatographic profiles of saliva were examined. The techniques investigated were filtration with membrane cones, centrifuging at 1145 RCF for 5—10 min and removal of the upper layer, and direct injection of whole saliva. To test each method, 20 ml of saliva from one subject were collected, vortexed, and divided into six lots; of the six lots, three were used for blank determinations. For the remaining three lots, 100 μl of saliva and 100 μl of 10^{-5} M standard solution were mixed and processed.

Peak identification

The low-molecular-weight UV-absorbing constituents of the saliva matrix were identified from the combined data of retention time, UV spectra, fluorescence, enzyme reactions, and chemical reactions [16—18].

RESULTS

Chromatography

Fig. 1 shows a chromatographic profile of saliva constituents. Based on the combined data from the identification techniques, the endogenous compounds present in the majority of saliva samples from fifteen subjects were identified as creatinine, uric acid, tyrosine, hypoxanthine, uridine, xanthine, kynurenine,

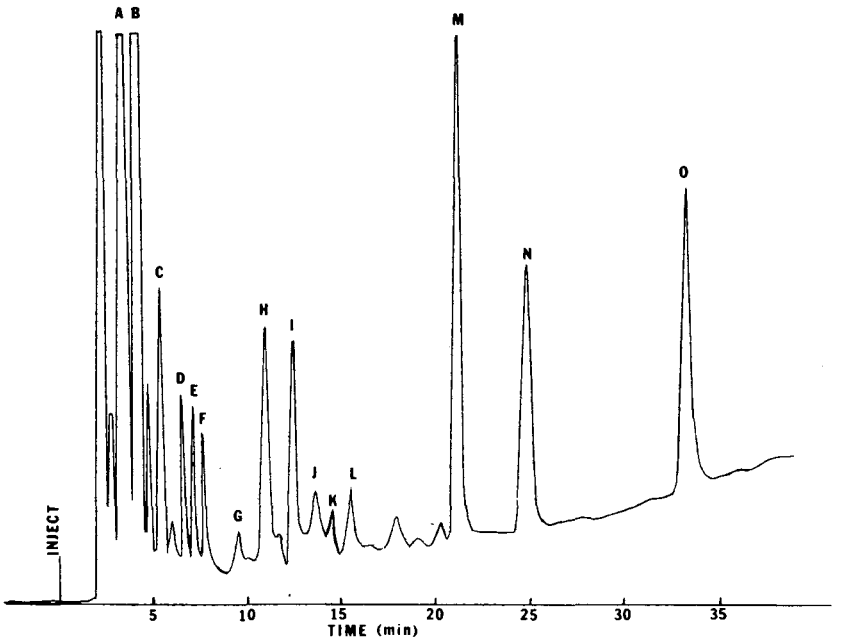

Fig. 1. Chromatogram of a saliva sample (100 μl). Conditions are listed in the text. Peaks: A = creatinine; B = uric acid; C = tyrosine; D = hypoxanthine; E = uridine; F = xanthine; G = kynurenine; H = 5-hydroxytryptophan; I = inosine; J = guanosine; K = hippuric acid; L = tryptophan; M = theobromine; N = paraxanthine/theophylline; O = caffeine.

5-hydroxytryptophan, inosine, guanosine, hippuric acid, and tryptophan. The exogenous compounds theobromine, paraxanthine/theophylline, and caffeine were also found to occur frequently in the saliva profiles.

Sample handling

A study of serum processing techniques prior to chromatography has been reported [15, 16]. Filtration through membrane cones was the preferred method to remove the high-molecular-weight materials in serum [16]. How-

TABLE I

PERCENTAGE RECOVERY OF STANDARD COMPOUNDS

Compound	Direct injection		Ultrafiltration	
	Whole saliva	Saliva	Saliva	Serum
Tyrosine	38.7	35.5	19.8	86.3
Xanthine	72.3	70.6	73.5	92.7
Inosine	52.8	51.6	50.6	96.0
Guanosine	76.5	75.8	79.4	61.5
Tryptophan	34.4	33.9	18.1	11.9
Theobromine	90.9	88.1	86.2	79.5
Paraxanthine	88.7	86.9	89.2	86.5
Caffeine	87.3	84.7	87.6	84.1

ever, filtering the saliva samples is not required since only a small percentage of whole saliva is high-molecular-weight material. In addition, differences in recoveries of the constituents of saliva and serum were noted (Table I). The recovery differences could be due to the pH of the two matrices.

Using the precolumn to trap high-molecular-weight material, we found the direct injection of whole saliva convenient for 40 serial assays; purging with 100% methanol is recommended after the last sample. However, when many samples (> 300) must be analyzed and time is an important factor, centrifuging the whole saliva and injection of the upper layer eliminates the need for frequent purging of the system. The differences between recoveries of these two methods are shown in Table I.

Constituents of saliva and serum

Many of the compounds found in serum [16, 19—21] were also noted in saliva. However, saliva contains lower concentrations of these compounds (Table II).

TABLE II

AVERAGE CONCENTRATIONS OF SALIVA COMPOUNDS

Compound	Saliva (μM; mean ± S.D.)	Serum range (μM)
Creatinine	3.71 ± 2.52	71.0—133
Uric acid	44.8 ± 21.5	155—429
Tyrosine	6.63 ± 2.92	44.0—71.0
Hypoxanthine	1.09 ± 1.00	1.56—12.8
Uridine	0.214 ± 0.231	<0.100—5.39
Xanthine	1.18 ± 0.814	0.542—4.70
Kynurenine	0.331 ± 0.289	55.0—151
5-Hydroxytryptophan	0.272 ± 0.174	—
Inosine	1.13 ± 0.610	<0.100—11.4
Guanosine	0.216 ± 0.168	<0.100—1.98
Hippuric acid	0.159 ± 0.125	<0.100—1.57
Tryptophan	1.55 ± 0.713	9.16—17.0

Reproducibility of saliva constituents

By monitoring five subjects for several months, the intra-individual levels of the saliva constituents were determined; these values are for samples taken after the donors had fasted for 12 h (Table III). Qualitatively, the saliva profiles are reproducible. Each individual appears to maintain a characteristic pattern of peaks in their profile. However, the concentrations of these compounds can vary greatly from day to day. These variations could be linked to foods consumed prior to sampling; however, no direct correlations were determined. Furthermore, the inter-individual levels of the endogenous saliva compounds are distributed over a larger range than the intra-individual concentrations (Table III). The inter-individual variance, in most cases, is 2—3 times that of the intra-individual variance.

TABLE III

VARIATIONS IN SALIVA CONCENTRATIONS OVER THREE MONTHS

Compound	Intra-individual		Inter-individual	
	Range (μM)	C.V. (%)	Range (μM)	C.V. (%)
Creatinine	3.01—4.42	40.3	2.45—5.01	69.4
Uric acid	34.8—56.3	38.1	27.8—61.8	51.7
Tyrosine	6.03—7.23	17.6	3.71—9.55	44.8
Hypoxanthine	0.970—1.21	23.1	0.589—1.59	92.6
Uridine	<0.100—0.325	100	<0.100—0.554	100
Xanthine	1.08—1.28	17.5	0.773—1.59	69.3
Inosine	0.949—1.32	33.0	0.825—1.44	54.2
Tryptophan	1.33—1.75	26.8	1.19—1.91	46.4

Methylated xanthines in saliva

The conditions of Orcutt et al. [9] were used to determine the methylated xanthines and methylated uric acids in saliva. A great number of these compounds were found in the saliva of habitual coffee drinkers. In one subject, 1,3-dimethyluric acid, 1-methyluric acid, 7-methylxanthine, 3-methylxanthine, and 1-methylxanthine were observed; however, the concentrations of these compounds were small compared to the levels of paraxanthine, theophylline, theobromine, and caffeine.

Metabolites of caffeine in saliva

Two habitual coffee drinkers were used to measure the rate of metabolism of caffeine from coffee. The subjects had refrained from consuming caffeine-containing products for 24 h. A cup of coffee (approx. 150 mg of caffeine) was given at the start of the experiment. Saliva samples were collected immediately before and after drinking the coffee, and thereafter at intervals spanning 20 h. The chromatographic conditions for the separation of paraxanthine, theophylline, theobromine, and caffeine were used.

The change in caffeine, paraxanthine, and theobromine concentrations in saliva are shown in Fig. 2. Within the first hour, caffeine concentrations in saliva decrease rapidly. A maximum concentration is reached at 4 h, followed by a steady decrease with a calculated half-life of 5.7 h. These changes in caffeine with time were similar in both subjects. The increase in caffeine in saliva after 4 h is explained by the redistribution of caffeine from blood back to saliva. The increase of paraxanthine and theobromine in saliva immediately after drinking coffee is due to their presence in the coffee. Relatively steady levels of paraxanthine and theobromine are observed from 2 to 9 h. After 9 h, paraxanthine and theobromine decrease at approximately the same rate. Theophylline levels followed those of paraxanthine.

To follow the metabolism of caffeine from an oral administration, a subject who was not an habitual coffee drinker was given 100 mg of caffeine to hold in the oral cavity. Saliva samples were collected at set intervals over 9 h. None of the metabolites of caffeine are observed in the saliva in the first 4 h. However, when the same subject, several days later, ingested a 100-mg tablet, paraxanthine is seen after 20 min and reaches a maximum concentration at 5 h (Fig. 3);

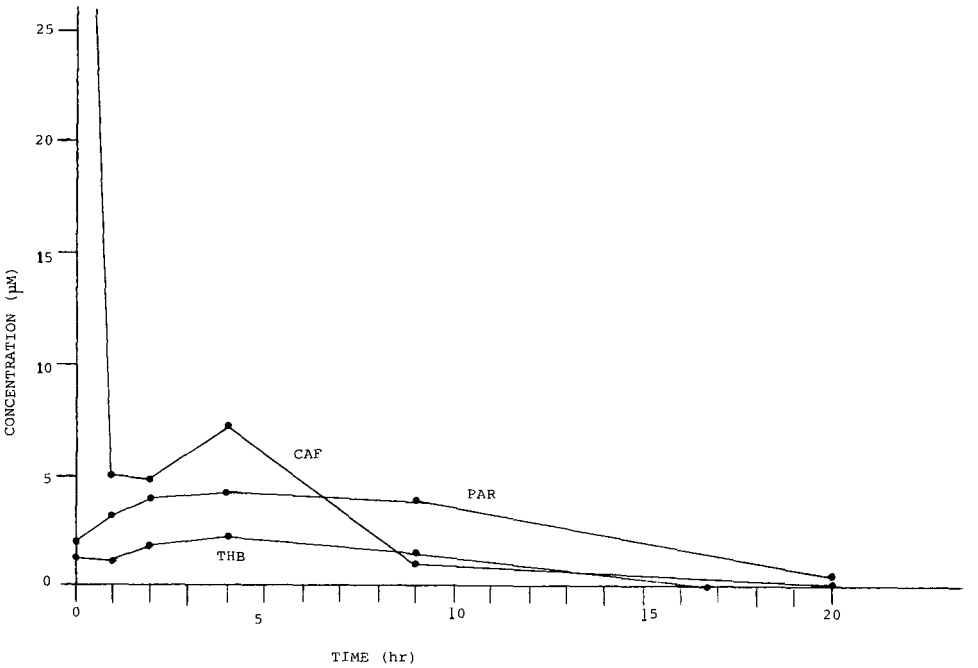

Fig. 2. Time curves for caffeine (CAF), paraxanthine (PAR) and theobromine (THB) in the saliva of an habitual coffee drinker.

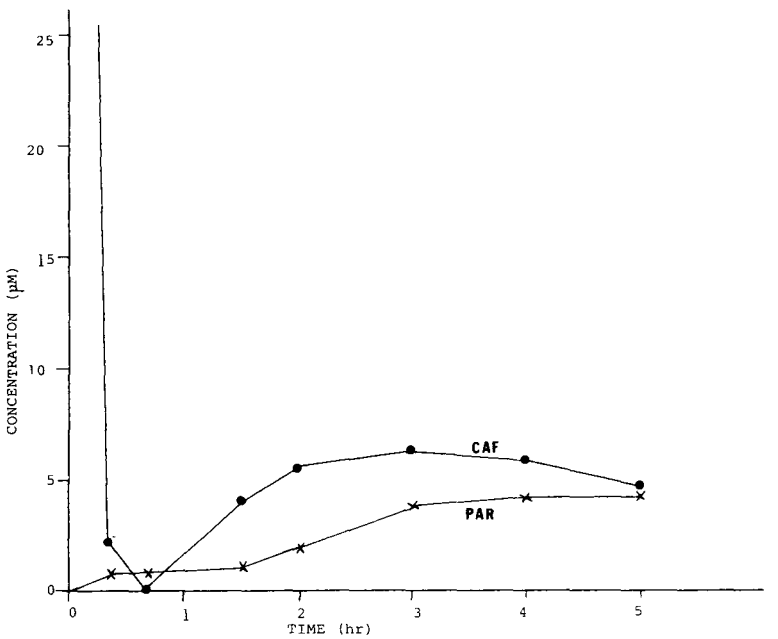

Fig. 3. Time curves for caffeine (CAF) and paraxanthine (PAR) in saliva after an oral administration of caffeine.

caffeine levels peak at 3 h and the drug has a half-life of 4.39 h. Theobromine concentrations reach maximum levels 5 h after caffeine administration. Maximum theophylline concentrations occur at approximately 3 h; the levels of theophylline are less than those of theobromine and paraxanthine. The caffeine metabolites 1-methyluric acid and 1-methylxanthine were also observed.

Caffeine in saliva and serum

Fig. 4 illustrates the changes with time in concentrations of caffeine and paraxanthine in saliva and serum of an habitual coffee drinker. The subject had avoided all products containing caffeine for four days prior to testing. Both the saliva and serum profiles obtained before the administration of 100 mg of caffeine showed no caffeine or metabolites.

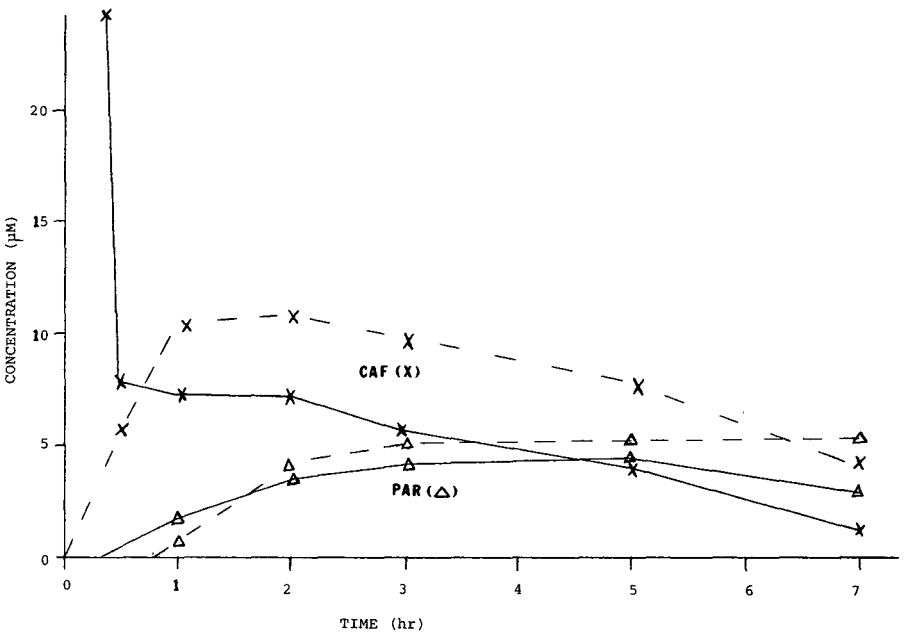

Fig. 4. Time curves for caffeine (CAF) and paraxanthine (PAR) in saliva (——) and serum (— — —) after an oral administration of caffeine to a decaffeinated habitual coffee drinker.

Between 1 and 2 h after administration of caffeine, a maximum in caffeine concentration is observed in saliva and serum. The rate of appearance of caffeine in serum is much slower than the initial disappearance of caffeine from saliva. However, the time curves for caffeine in saliva (half-life = 3.27 h) and serum (half-life = 3.49 h) are very similar after 2 h. For paraxanthine, the rate of appearance in serum follows the rate in saliva. After 50 min paraxanthine is observed in both saliva and serum; it is believed that caffeine is converted to paraxanthine in the liver [22, 23]. Comparing the data of Figs. 3 and 4, the kinetics of caffeine differ in individuals who drink coffee habitually and those who do not; Christensen and Whitsett [24] previously reported on these differences and also noted the influence of smoking on the rate of caffeine metabolism.

DISCUSSION

The saliva profiles of low-molecular-weight UV-absorbing compounds are similar to serum profiles. The major difference, however, is that saliva contains much lower concentrations of these compounds than serum. Furthermore, there appears to be a much greater variation in the intra-individual profiles of saliva than serum [13]. In addition, the tremendous variation in the inter-individual saliva profiles warrants great care in drug monitoring and metabolic studies.

The major metabolites of caffeine found in urine were shown to be 1-methyluric acid and 1-methylxanthine [22]. It was also suggested that paraxanthine, which was found in small amounts in urine, may be the intermediate in the formation of 1-methylxanthine [22]. After an oral dose of caffeine, the major metabolites of caffeine in saliva were found to be paraxanthine, theobromine, theophylline, 1-methylxanthine, and 1-methyluric acid. The other possible metabolites [22, 23] were not readily observed.

The decay of caffeine in saliva was found to consist of two different rates (Fig. 4). The first rate of disappearance of caffeine in saliva is very rapid, as seen within the first 30 min, and the second rate is much slower. The exponential rates of caffeine in saliva and serum can be expressed as

$$-dX(L)/dt = A(L1)X(L) \quad (1)$$

$$-dX(L)/dt = A(L2)X(L) \quad (2)$$

$$-dX(R)/dt = A(R)X(R) \quad (3)$$

where $X(L)$ and $X(R)$ represent the caffeine concentrations in saliva and serum, respectively. $A(L1)$ and $A(L2)$ are the first and second decay constants in saliva and $A(R)$ is the rate of decay in serum. When the redistribution rate constant is denoted by $A(D)$ the following expressions between the rates could be expected

$$\text{if } A(D) >> A(L1) \text{ then } A(L2) \approx A(R) \quad (4)$$

$$\text{if } A(D) << A(L1) \text{ then } A(L2) \approx A(L1) \quad (5)$$

From Fig. 4, the rate constant $A(L2)$ approximates $A(R)$ and not $A(L1)$. Applying the least-squares method to the observed kinetics of caffeine in Fig. 4, we find $A(L1) = 8.5$, $A(L2) = 0.24$, and $A(R) = 0.15$; thus, $A(L1) >> A(L2) > A(R)$. Therefore, caffeine disappearance in saliva proceeds according to the conditions expressed in relationship 4. However, the exact quantitative solution would require compartment analysis of the two matrices.

The rates of caffeine metabolism differ in individuals who are habitual coffee drinkers from those who are not. Caffeine in saliva peaks at 3 h and has a 4.4-h half-life in persons with no coffee habit. For individuals who drink coffee regularly, a 3.3-h half-life is found after a four-day fast from caffeine and a 5.7-h half-life when caffeine is removed from the diet for only 24 h. The 4.4-h half-life in saliva (and serum) is in good agreement with the 4-h half-life reported by Cook et al. [1] and the 4.7-h half-life reported by Christensen and Whitsett [24]; in addition, it compares well with the 4.3-h half-life Tse and Szeto [11] found for caffeine in the male beagle dog.

The rates and levels of caffeine are similar in saliva and serum; a simple relationship is evident between the two matrices from the log—linear decline of caffeine in saliva and serum. This would suggest, as reported elsewhere [25], that wherever such relationships exist, saliva can be a useful matrix to monitor. However, in monitoring drugs such as theophylline, the presence of possible interfering substances such as paraxanthine should be noted; especially when the interfering substance has a long half-life and its distribution correlates between the two matrices.

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DETERMINATION OF 17-OXOSTEROID GLUCURONIDES AND SULFATES IN URINE AND SERUM BY FLUORESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING DANSYL HYDRAZINE AS A PRE-LABELING REAGENT

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SUMMARY

A fluorescence high-performance liquid chromatographic method is described for the direct determination of conjugated 17-oxosteroids in biological fluids without hydrolysis. Conjugated 17-oxosteroids are extracted with Sep-Pak C_{18} cartridge, labeled with dansyl hydrazine in trichloroacetic acid—benzene solution and then separated by high-performance liquid chromatography on reversed-phase μBondapak C_{18} column using 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1, v/v) as the mobile phase. The eluate is monitored by a fluorophotometer at 365 nm (excitation) and 520 nm (emission). Linearities of fluorescence intensities (peak heights) with the amounts of various conjugated 17-oxosteroids were obtained between 10 pmol and 100 pmol. This method is sensitive, reliable and useful for the simultaneous determination of conjugated 17-oxosteroids in urine and serum.

INTRODUCTION

The measurement of steroids in serum and urine samples has been used in clinical laboratories as a clinical indicator of adrenal function [1]. Many methods have been reported for analysis of metabolic profiles of steroids in urine by gas chromatography [2—6] and gas chromatography—mass spectrometry [7, 8]. As the steroids excreted in urine are mainly in the conjugated form, the analytical methods generally involve hydrolysis of the conjugates prior to chromatographic analysis. Therefore, important information about metabolic pathways such as subtle changes in type or site of conjugation may be overlooked by the use of such procedures.

Recently, high-performance liquid chromatography (HPLC) has been studied

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for the separation of conjugated steroids [9—13], mainly estrogen glucuronide and estrogen sulfate. Conjugated 17-oxosteroids were separated by reversed-phase HPLC [13], which showed poor efficiency, however.

In the previous paper [14], we reported a highly sensitive HPLC method using dansyl hydrazine as a fluorescent labeling reagent for the determination of urinary 17-oxosteroids after enzymatic hydrolysis and extraction of the liberated steroids.

In this paper, we describe a highly sensitive fluorescence HPLC method for the direct determination of conjugated 17-oxosteroids in urine and serum samples without hydrolysis.

EXPERIMENTAL

Materials

Androsterone glucuronide (AN-G)* and androsterone sulfate (AN-S) were obtained from Sigma (St. Louis, MO, U.S.A.). Etiocholanolone glucuronide (ETIO-G) and etiocholanolone sulfate (ETIO-S) were purchased from Makor (Jerusalem, Israel). Dehydroepiandrosterone sulfate (DHEA-S), androsterone (AN), etiocholanolone (ETIO) and dehydroepiandrosterone (DHEA) were those used in previous studies [14]. β-Glucuronidase and arylsulphatase were from Boehringer Mannheim-Yamanouchi (Tokyo, Japan). Sep-Pak C_{18} cartridges from Waters Assoc. (Milford, MA, U.S.A.) and Amberlite XAD-2 from Rohm and Haas Co. (Philadelphia, PA, U.S.A.) were used. All other reagents and solvents were of analytical reagent grade from commercial sources.

Instruments and chromatographic conditions

We used an Hitachi Model 635 high-performance liquid chromatograph equipped with a Kyowa Seimitsu KHP-UI-130 injection valve, a stainless-steel column, a Jasco Model FP-110 fluorescence spectrophotometer equipped with a mercury lamp and a micro flow cell. A reversed-phase μBondapak C_{18} column (Waters, 300 × 3.9 mm I.D., particle size 10 μm) was used for the separation of all conjugated 17-oxosteroids. The solvent system used was 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1, v/v) at a flow-rate of 1 ml/min. A Zorbax SIL column (250 × 4.6 mm I.D., DuPont, Wilmington, DE, U.S.A.) was also used for free steroids liberated by enzymatic hydrolysis. The detector wavelength was set at 365 nm and 505 nm for excitation and emission, respectively.

Reagent solutions

Dansyl hydrazine solution. A 0.2% (w/v) solution was prepared by dissolving 20 mg of dansyl hydrazine in 10 ml of benzene, and stored in a refrigerator until use.

Trichloroacetic acid—benzene solution. A 0.5% (w/v) solution was prepared by dissolving 50 mg of purified trichloroacetic acid in 10 ml of benzene.

Steroid stock solutions. AN-G, ETIO-G, DHEA-S, AN-S and ETIO-S were

*The following trivial names are used: androsterone (AN), 3α-hydroxy-5α-androstan-17-one; dehydroepiandrosterone (DHEA), 3β-hydroxy-androst-5-ene-17-one; etiocholanolone (ETIO), 3α-hydroxy-5β-androstan-17-one.

dissolved separately in methanol to make each stock solution (0.5 μmol/ml), and stored at −20°C until use.

Extraction method

Urine sample. A 0.5-ml aliquot of urine was diluted to 1.0 ml with redistilled water, applied onto a Sep-Pak C_{18} cartridge and washed successively with 5 ml of water and 3 ml of 20% ethanol. Conjugated 17-oxosteroids were eluted with 2 ml of methanol and the eluent was then evaporated to dryness at 40°C under a stream of nitrogen gas. The resultant residue was assayed as described below.

Serum sample. A 0.2-ml volume of serum was diluted to 1.0 ml with 0.025 *M* phosphate buffer (pH 7.0), applied onto a Sep-Pak C_{18} cartridge and washed with 4 ml of water. Conjugated 17-oxosteroids were eluted with 2 ml of methanol and the eluent was then evaporated to dryness under a stream of nitrogen gas at 40°C. The resultant residue was assayed by the following procedure.

Labeling reaction

The residue in the test tube was dissolved by adding 0.2 ml of 0.5% trichloroacetic acid—benzene solution, admixed with 50 μl of 0.2% dansyl hydrazine solution, left to stand for 20 min at 60°C, and then evaporated to dryness under a stream of nitrogen gas. The labelled residue was dissolved in 200 μl of methanol and an aliquot of the solution was injected into the chromatograph described above.

RESULTS

Effect of eluent composition

For a reversed-phase μBondapak C_{18} column, many solvent systems were examined to obtain the complete separation of 17-oxosteroid glucuronides and sulfates. The effect of the methanol concentration on the capacity factors was investigated. The mixture of methanol and water containing 0.075 *M* sodium acetate and 2% acetic acid was used as mobile phase because glucuronides and sulfates were separated completely from the peak of excess dansyl hydrazine by addition of acetic acid and sodium acetate to the mobile phase. As shown in Fig. 1, the most suitable k' value was obtained by the use of 65% methanol but AN-S and ETIO-G did not separate. As shown in Fig. 2, sodium acetate concentration is important for the separation of glucuronides and sulfates. At low sodium acetate concentration, the sulfates are eluted first and then the glucuronides. The addition of acetic acid to the mobile phase gave a distinct effect on the separation of glucuronides and excess dansyl hydrazine but the capacity factors did not change to a large extent. Therefore, 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1) was used as eluent. The chromatogram presented in Fig. 3 shows good separation of standard conjugated 17-oxosteroid mixture including DHEA-S, ETIO-S, AN-S, ETIO-G and AN-G.

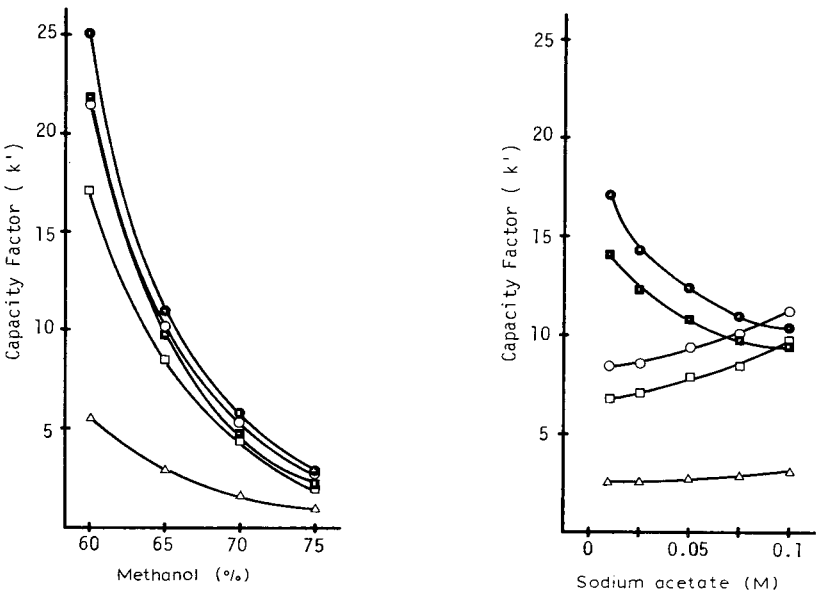

Fig. 1. Effect of methanol concentration on capacity factor. Eluent: methanol—water + 2% acetic acid + 0.075 *M* sodium acetate. (•) AN-G; (▪) ETIO-G; (○) AN-S; (□) ETIO-S; (△) DHEA-S.

Fig. 2. Effect of sodium acetate concentration on capacity factor. Eluent: methanol—water—acetic acid (65:35:2) + sodium acetate. (•) AN-G; (▪) ETIO-G; (○) AN-S; (□) ETIO-S; (△) DHEA-S.

Working curves and sensitivities

Typical working curves are shown in Fig. 4. Linearity of fluorescence intensity (peak height) with the injected amounts of conjugated 17-oxosteroids were obtained between 10 pmol and 100 pmol, and detection limits were about 1.0 pmol. When 0.2 ml of serum sample was used for the assay, the detection limit for DHEA-S was about 8 μg/dl from this working curve.

Recovery and reproducibility

Recovery tests were carried out by determining urine samples spiked with a mixture of known amounts of the five conjugated 17-oxosteroids and pooled serum sample with DHEA-S. As shown in Table I, conjugated 17-oxosteroids were recovered in the range 94.9—105.2% with C.V. (%) in the range 1.7—3.4%.

Typical chromatograms from urine and serum samples

Typical chromatograms obtained from normal human and patient urine samples and normal human serum are shown in Fig. 5; urinary conjugated 17-oxosteroids and serum DHEA-S were clearly separated and identified by comparison with authentic samples.

Comparison with results obtained by the deconjugation method

The reliability of the newly devised HPLC method for the direct determination of urinary and serum conjugated 17-oxosteroids was assessed by com-

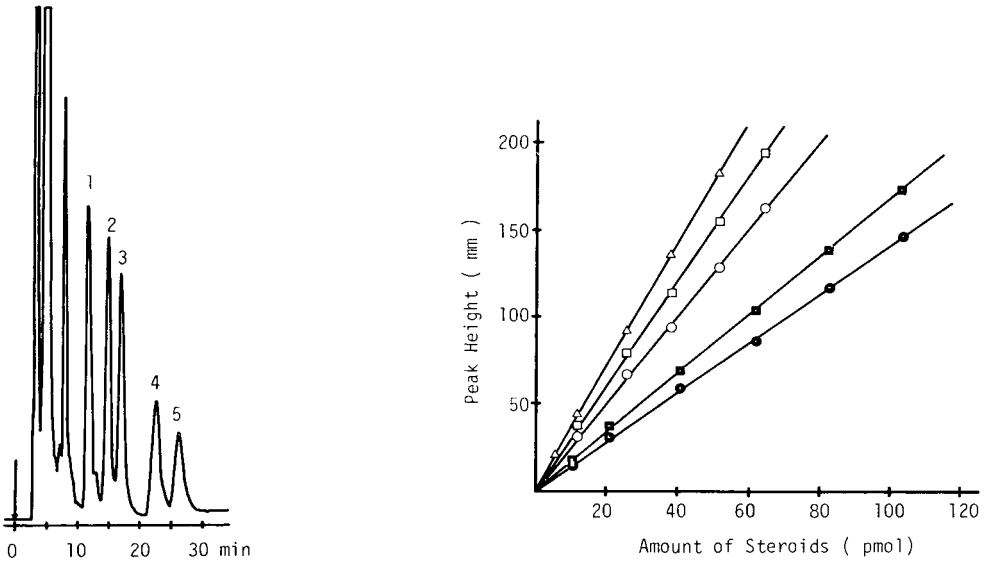

Fig. 3. Chromatogram of dansyl hydrazone derivatives of conjugated 17-oxosteroid standard mixture. Peaks: 1 = dehydroepiandrosterone sulfate, 2 = etiocholanolone sulfate, 3 = androsterone sulfate, 4 = etiocholanolone glucuronide, 5 = androsterone glucuronide. μBondapak C_{18} (300 × 3.9 mm I.D.) column; mobile phase, 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1), 1 ml/min; JASCO FP-110 fluorescence detector (excitation 365 nm, emission 520 nm).

Fig. 4. Standard curves for conjugated 17-oxosteroids. (•) AN-G; (▪) ETIO-G; (○) AN-S; (□) ETIO-S; (△) DHEA-S.

TABLE I

RECOVERIES OF CONJUGATED STEROIDS ADDED TO HUMAN URINE AND SERUM

Urine (0.5 ml) with 0.75 nmol of each of the five conjugated 17-oxosteroids added was used. Serum (0.2 ml) with 0.5 nmol of added DHEA-S was used.

	Steroid	*n*	Recovery (%)	C.V. (%)
Urine	Androsterone glucuronide	4	95.3	2.2
	Etiocholanolone glucuronide	4	101.2	3.4
	Androsterone sulfate	4	101.0	1.7
	Etiocholanolone sulfate	4	105.2	3.2
	Dehydroepiandrosterone sulfate	4	96.8	3.4
Serum	Dehydroepiandrosterone sulfate	4	94.9	2.0

paring the results with those obtained by the previous method, involving enzymatic hydrolysis of urine samples with β-glucuronidase or arylsulphatase. The results obtained by both methods are illustrated in Table II.

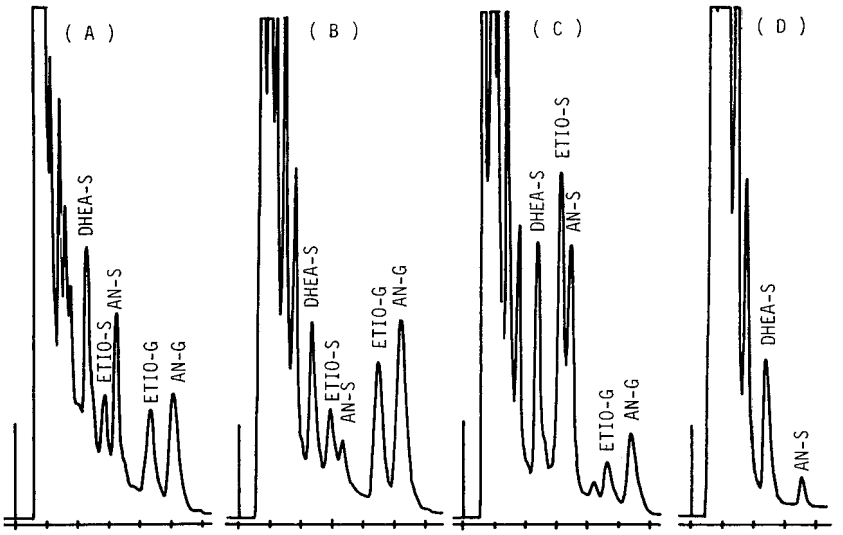

Fig. 5. Typical chromatograms of human urine and serum samples. (A) Normal human urine (male, aged 21). (B) Ovarian tumor patient's urine (female, aged 21). (C) Thyroiditis patient's urine (female, aged 50). (D) Normal human serum (male, aged 21).

TABLE II

COMPARISON BETWEEN THE DIRECT METHOD* AND THE METHOD WITH HYDROLYSIS** FOR CONJUGATED 17-OXOSTEROIDS IN URINE

Steroid	Regression line***	r	n
Androsterone glucuronide	$Y = 1.35\,X - 0.15$	0.894	20
Etiocholanolone glucuronide	$Y = 0.83\,X - 0.01$	0.948	20
Androsterone sulfate	$Y = 1.03\,X + 0.04$	0.972	16
Etiocholanolone sulfate	$Y = 1.01\,X - 0.06$	0.987	16
Dehydroepiandrosterone sulfate	$Y = 1.12\,X + 0.30$	0.914	18

*The method proposed in this paper.
**The previous method in the previous paper [14].
***X = the values obtained by the proposed method; Y = the values obtained by the previous method.

DISCUSSION

Urinary steroids, metabolized and excreted by conjugation with glucuronic acid or sulfuric acid, provide information on the physical condition of the organism because the state of conjugation depends on the structure and metabolic origin of the steroid metabolites. Several reports have been published for the determination of metabolic profiles of urinary steroids [2–8]. In most cases [2–6], hydrolysis of conjugated steroids is the first step in the assay method so that subtle changes in type or site of conjugation will be lost. Therefore, group separation of conjugates prior to hydrolysis based on ion-exchange triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) [8]

and DEAE-Sephadex [15] has been developed. However, this method is time-consuming and less suitable for routine assay. A method that permits the direct determination of conjugated steroids without hydrolysis would probably give additional information on the pathological state.

Recently, HPLC has been studied for the separation of conjugated steroids [9—13] because they are not sufficiently volatile to allow their direct gas chromatographic separation. Lafosse et al. [13] reported the separation of conjugated 17-oxosteroids; however, they could not separate different types of conjugates, glucuronide and sulfate, and their determination range was at the microgram level. In the previous paper [14], we reported a highly sensitive fluorescence HPLC method for the determination of 17-oxosteroids, the liberated free steroids by hydrolysis, in serum and urine.

In this paper, we have developed a fluorescence HPLC method for the direct determination of conjugated 17-oxosteroids in urine or serum without hydrolysis.

Amberlite XAD-2 has been commonly used to extract conjugated steroids from urine [8, 16], and recently the extraction method with Sep-Pak C_{18} cartridge was reported by Shackleton and Whitney [17] and Axelson and Sahlberg [18]. In this assay, when Amberlite XAD-2 was used, unknown peaks appeared occasionally and interfered with the separation of conjugated steroids. Urinary conjugated steroids could be extracted with no chromatographic interferences by using Sep-Pak C_{18} cartridge. This extraction method was superior with respect to speed, simplicity and recovery for conjugated 17-oxosteroids.

The derivatization conditions of conjugated 17-oxosteroids with dansyl hydrazine were examined and the optimal conditions were similar to those of free 17-oxosteroids as described in the previous paper [14]. We chose chromatographic conditions which, in the shortest possible assay time, gave acceptable resolution between the dansyl hydrazones of conjugated steroids and the fluorescent coexisting substances in urine or serum samples. As shown in Fig. 3, good separation could be achieved on μBondapak C_{18} column using 0.01 *M* sodium acetate in methanol—water—acetic acid (65:35:1) as the eluent. The sensitivity of this method was superior to other HPLC methods using a UV detector.

As shown in Table II, the correlation between the values of urinary conjugated 17-oxosteroids obtained by the present method and by the previous method [14] involving enzymatic hydrolysis prior to chromatographic separation is not good for three of them (AN-G, ETIO-G and DHEA-S) as indicated by the regression line and *r* value. Since there are many kinds of conjugated oxosteroids in urine, it is preferable that the peaks should be isolated and analyzed by an independent method. In this study, the peaks in the chromatogram of the sample were identified by their retention times as compared with the chromatogram of the standard solution measured at the same time. The differences between the present and previous methods may be due to interfering substances or efficiency of enzymatic hydrolysis.

In conclusion, the present method gives, with a very short assay time, direct information on the different concentrations of conjugated 17-oxosteroids in urine and serum and on the rate of conjugation with glucuronic acid or sulfuric

acid. The extraction with Sep-Pak C_{18} cartridge represents a simple procedure with almost complete recovery of conjugated 17-oxosteroids. The newly developed fluorescence HPLC method may be clinically useful in the routine assay of urine and serum conjugated 17-oxosteroids.

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CHROMBIO. 1425

CORRELATION BETWEEN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND AUTOMATED FLUORIMETRIC METHODS FOR THE DETERMINATION OF DOPAMINE, 3,4-DIHYDROXYPHENYLACETIC ACID, HOMOVANILLIC ACID AND 5-HYDROXYINDOLEACETIC ACID IN NERVOUS TISSUE AND CEREBROSPINAL FLUID

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SUMMARY

The correlation between automated fluorimetric methods and high-performance liquid chromatography is described for the determination of homovanillic acid and 5-hydroxyindoleacetic acid in cerebrospinal fluid, and for dopamine, 3,4-dihydroxyphenylacetic acid and homovanillic acid in striata of rat brain. The automated fluorimetric methods for 3,4-dihydroxyphenylacetic acid and homovanillic acid showed a good correlation with the high-performance liquid chromatographic methodology. The fluorimetric determination for dopamine was somewhat less reliable than the high-performance liquid chromatographic assay. The fluorimetric assay for 5-hydroxyindoleacetic acid correlated poorly with the chromatographic method.

INTRODUCTION

Several authors have expressed some doubt about the specificity of the fluorimetric methods used for the analysis of neurotransmitters and metabolites in biological samples [1—3]. Fluorimetric methods can suffer from aspecific fluorescence and quenching. The degree to which these phenomena interfere with the assays depends to a great extent on the quality of the purification procedures. The availability of an automated fluorimetric assay in our laboratory, as well as high-performance liquid chromatographic (HPLC) methodology offered the interesting possibility for a correlation study. Here, we describe the correlation between a fluorimetric method and an HPLC method for the determination of homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in cerebrospinal fluid (CSF) and for dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and HVA in striata of rat brain.

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EXPERIMENTAL

Materials

Materials and their sources were as follows. Dopamine·HCl, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid were from Fluka (Buchs, Switzerland). All other chemicals were of analytical reagent grade and were purchased from E. Merck (Darmstadt, G.F.R.). All aqueous solutions were prepared from deionized water distilled in glass. Stock solutions of DA, DOPAC, HVA and 5-HIAA consisted of 100 μg/ml of 0.01 *M* formic acid and were stored in portions in the freezer (—80°C). Solutions of reference compounds were freshly prepared every week from a portion of the stock solution after appropriate dilution with 0.01 *M* formic acid.

Animals and dissection

Female albino rats weighing 150—200 g (Wistar; C.D.L., Groningen, The Netherlands) were used. Haloperidol (Serenase; Janssen, Beerse, Belgium) was administered intraperitoneally (1 mg/kg). Rats were killed by decapitation and the brains rapidly removed. After dissection, which was completed within 3 min, the tissue samples were frozen on solid CO_2. Samples were kept at —80°C until assayed.

Isolation procedure on Sephadex G-10

CSF samples (0.5 ml), adjusted to pH 2—3 with 50 μl of formic acid (98%), were applied to Sephadex G-10 columns (5 × 70 mm) prepared in long-size Pasteur pipettes as described previously [4, 5]. Tissue samples were homogenised in 1 ml of 0.1 *M* perchloric acid (PCA). Following centrifugation (15 min, 4000 *g*, 4°C) the supernatants were put on the Sephadex columns. At least 80 columns can be handled in one run with the help of automated pipettes. Before use the columns were washed with 3.0 ml of 0.02 *M* ammonia and 3.0 ml of 0.01 *M* formic acid. After the samples had passed through the columns, 2.5 ml of 0.01 *M* formic acid were added. DA was then eluted with 1.0 ml of 0.01 *M* formic acid followed by 1.5 ml phosphate (0.005 *M* $Na_2HPO_4 \cdot 2H_2O$). DOPAC and HVA were subsequently eluted with 1.1 ml of the phosphate solution. The DOPAC- and HVA-containing fraction was acidified with 50 μl of 6 *M* formic acid. 5-HIAA was subsequently eluted with 2.5 ml of 0.02 *M* ammonia. The 5-HIAA-containing fraction was collected in a test tube to which 50 μl of 6 *M* formic acid were added. HVA and 5-HIAA could be collected in one fraction if, after the 1.5-ml of phosphate wash, the acids were eluted with 2.5 ml of 0.02 *M* ammonia. The columns were stored in 0.02 *M* ammonia. The isolation procedure is summarized in Fig. 1.

Chromatography

A Waters Model 6000 A liquid chromatograph was employed in conjunction with an electrochemical detector. The detector was based on the rotating disc electrode principle [6] and it was used in combination with a Bioanalytical Systems potentiostat Type LC-2A. The detector potential was set at 500 mV or 700 mV (for HVA assay) versus a Hg/Hg_2Cl_2 reference electrode. The

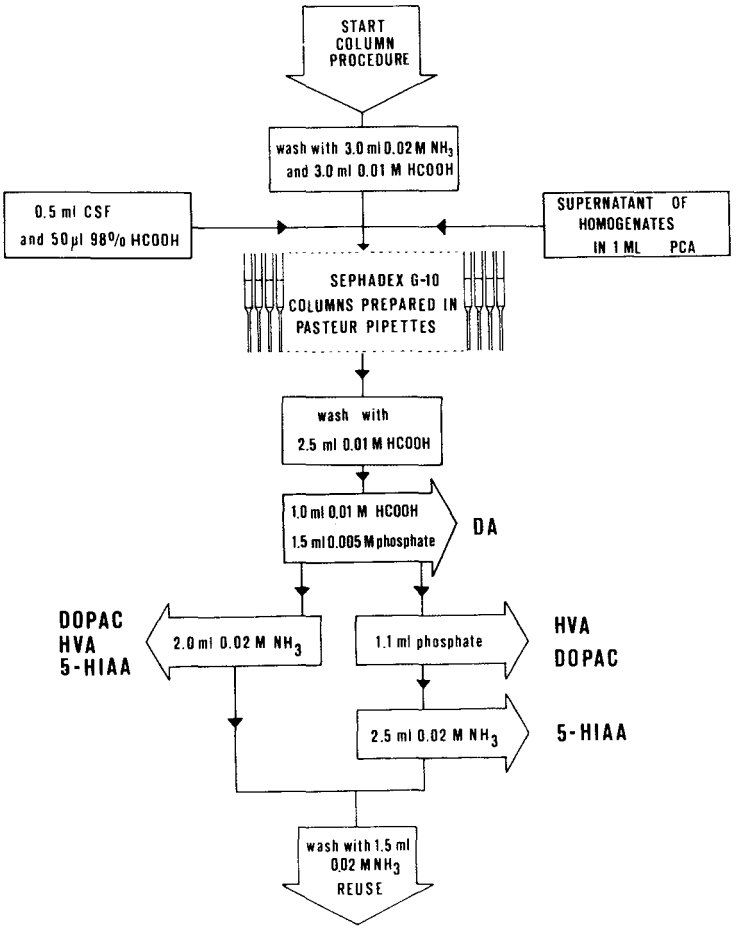

Fig. 1. Flow chart for the isolation procedure.

column (150 × 4.6 mm I.D.) was packed with a slurry of Nucleosil 5C-18 (particle size 5 µm) (Macherey-Nagel, Düren, G.F.R.) reversed-phase material in methanol—carbon tetrachloride (20:80, v/v). The slurry (10%, w/w), degassed in an ultrasonic generator, was pumped into the chromatographic column with a pressure of 40 MPa (the highest possible flow-rate). Columns were washed by passing 200 ml of methanol and further equilibrated with the mobile phase. Analyses were performed at a flow-rate of 60 ml/h at room temperature. The mobile phases consisted of a mixture of McIlvaine buffer (prepared from 0.1 *M* citric acid and 0.2 *M* $Na_2HPO_4 \cdot 2H_2O$), 0.1 m*M* EDTA and methanol. For the assay of DA a mobile phase of pH 5.5 with 3% methanol was used. The eluent for the DOPAC, HVA and 5-HIAA assay was adjusted to pH 3.5 and contained 20% methanol. Injections were made with a high-pressure injection valve (Rheodyne) fitted with a 50-µl or 100-µl sample loop. The concentrations in the biological samples were calculated with the aid of calibration curves obtained after the injection of pure standards. The analytical recoveries of HVA and 5-HIAA were determined by analysing spiked CSF

samples. If the Sephadex eluates had to be preserved until the next day, ascorbic acid (final concentration 10^{-4} *M*) was added to protect 5-HIAA and the samples were stored at 4°C.

Automated fluorimetry

DA, DOPAC and HVA were assayed according to our previously described method [4, 5]. 5-HIAA was determined as an *o*-phthaldialdehyde derivative [7].

RESULTS AND DISCUSSION

HVA and 5-HIAA in CSF

CSF samples were purified on Sephadex G-10 and subsequently analyzed using HPLC with amperometric detection. Fig. 1 shows that the CSF samples can be purified in two different ways. It is evident from Fig. 2 (B, C) that the collection of HVA and 5-HIAA fractions is not necessary, since the two compounds are well separated under the chromatographic conditions used.

Pooled CSF samples were spiked with 50 ng of HVA and 50 ng of 5-HIAA. The analytical recoveries (± S.D.) of the combined fractions were (data collected from three different experiments): HVA, 94.6 ± 7.2% (n = 12); and 5-HIAA, 95.0 ± 4.9% (n = 12). HVA was determined in a series of CSF samples with the HPLC method and automated fluorimetry. To exclude variation in the methods due to the purification step, eighteen CSF samples were purified on Sephadex G-10 and identical aliquots were used for both the automated fluorimetric method and the HPLC method for HVA. A good correlation was found (r = 0.990, Fig. 3).

The fluorimetric detection for HVA is based on an oxidation-induced dimerization of the HVA molecule, which results in the formation of a very specific fluorophore.

The blank procedure records the background fluorescence of each of the

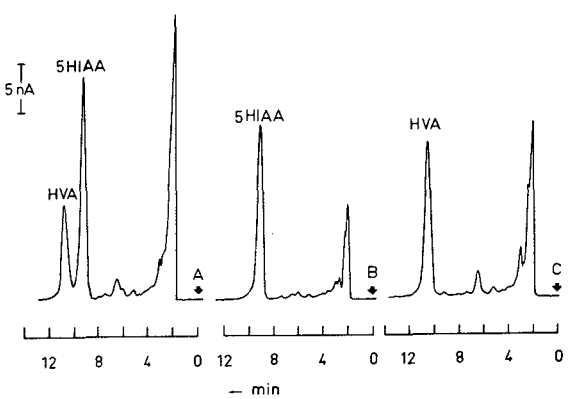

Fig. 2. Chromatograms of a CSF sample purified by two different methods. (1) HVA and 5-HIAA were collected in one fraction of 2.0 ml (A). (2) HVA and 5-HIAA were separated on Sephadex G-10, 5-HIAA was collected in a 2.5-ml fraction (B) and HVA was collected in a 1.1-ml fraction (C). See also Fig. 1.

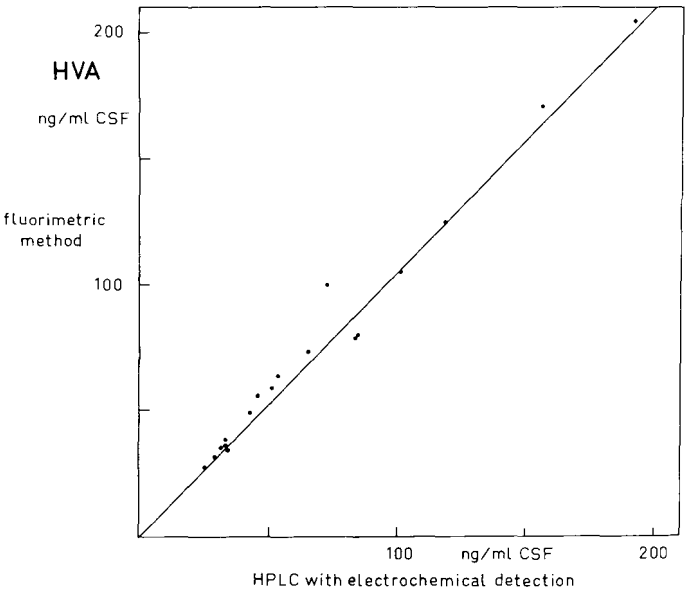

Fig. 3. Correlation between the automated fluorimetric assay and the HPLC method for HVA in CSF samples. The determinations were carried out on identical fractions obtained after purification on Sephadex G-10.

samples individually when oxidation is prevented [4]. The sample clean-up procedure, specific fluorophore formation and appropriate blank procedure resulted in an assay for HVA which is obviously not influenced by aspecific fluorescence or quenching. Muskiet et al. [8] have compared the fluorimetric HVA assay with a mass fragmentographic method and found that for HVA levels below 100 ng/ml the correlation coefficient was somewhat less satisfactory. The authors conclude that "when relatively large changes in the levels of HVA in CSF are to be measured, semiautomated fluorimetric assays may also be sufficiently reliable". The present results are slightly more positive about the automated fluorimetric HVA assay.

Fig. 4 shows the results of the 5-HIAA values obtained by the two methods. The two assays were carried out on identical CSF samples in different laboratories so, unlike in the HVA assay, variations in the isolation procedure are also included in the correlation coefficient. It is obvious that the correlation is poor (r = 0.773). Similar results have been obtained by Sjöquist and Johansson [3] for the comparison of a fluorimetric assay and a mass fragmentographic method. The fluorimetric determination used is based on a fluorophore formation between the indole derivative and *o*-phthaldialdehyde. This reagent is, however, not specific as it reacts with various primary amines and amino acids. Although it cannot be concluded from Fig. 4 which of the two methods is responsible for the poor correlation, we feel that the method based on an efficient chromatographic separation is very likely to be superior to the fluorimetric method. The fact that 5-HIAA values obtained with the fluorimetric method are somewhat higher than the results of the HPLC method

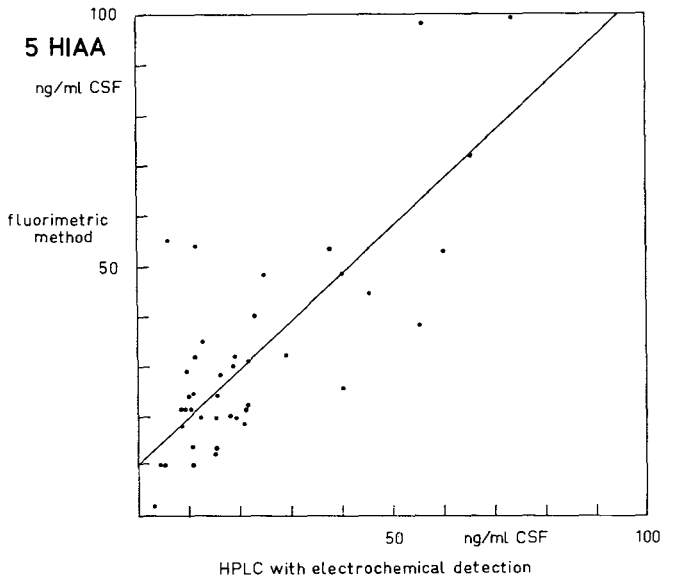

Fig. 4. Correlation between the automated fluorimetric assay and the HPLC method for 5-HIAA in CSF samples. The purification methods for both assays were carried out independently.

(*Y*-intercept = 10 ng/ml), suggests an aspecific fluorophore formation in the latter method.

DA, DOPAC and HVA in brain tissue

DA, DOPAC and HVA were determined in striata of rat brain. The HPLC assay and the fluorimetric assay were carried out on identical Sephadex fractions, which means that the correlation is only concerned with the detection procedure. Results are given in Figs. 5—7 and summarized in Table I.

Fig. 5 shows the correlation graph for DA. The HPLC method resulted in higher concentrations, and there was a considerable scatter of points around the calculated regression line. The fluorimetric assay for DA was based on fluorophore formation by reaction with ethylenediamine. The aspecific fluorescence of this method, which was estimated by recording the fluorescence of nervous tissue samples obtained from non-dopaminergic brain areas such as the cerebellum, is usually very low (less than 5% of the DA-induced fluorescence in the striatum [5]). Quenching of the fluorescence is a likely explanation for the lower DA concentrations found with the fluorimetric method. This is supported by the fact that the analytical recovery (300 ng of DA added to cerebellar tissue on different days) is more complete for the HPLC method (87.4 ± 6.2% (S.D.), n = 34) than for the fluorimetric method (73.4 ± 6.3% (S.D.), n = 31).

A good correlation was found for DOPAC and HVA for the two analytical methods (Figs. 6 and 7). Ethylenediamine is used to induce a DOPAC-specific fluorescence in a similar manner to the fluorimetric assay of DA. Unlike the DA assay there is no evidence for quenching. In comparison with DA, the

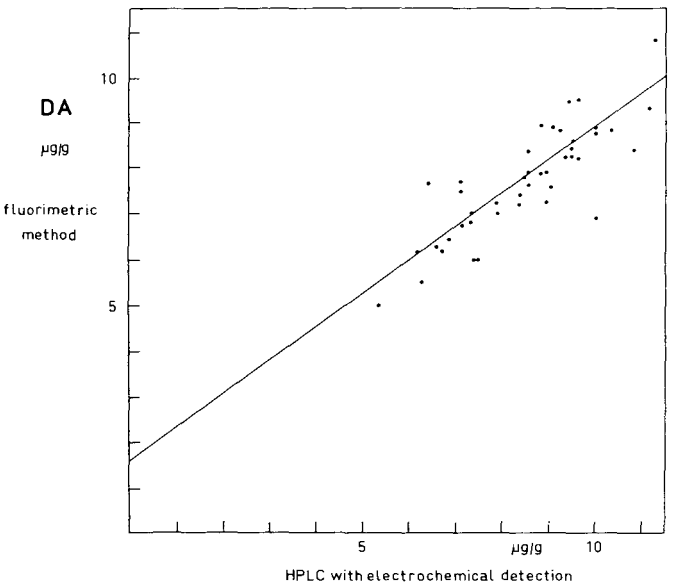

Fig. 5. Correlation between the automated fluorimetric method and the HPLC method for DA in striata of rats. The determinations were carried out on identical fractions obtained from the Sephadex G-10 procedure.

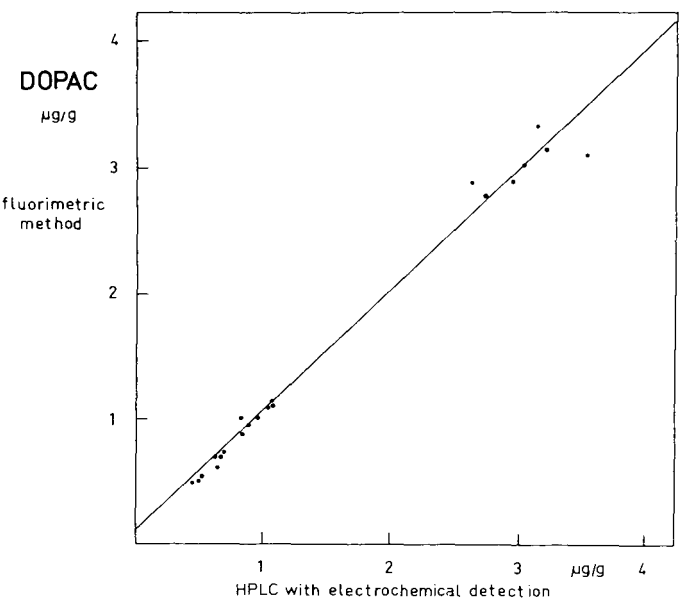

Fig. 6. Correlation between the automated fluorimetric method and the HPLC method for DOPAC in striata of rats. The determinations were carried out on identical fractions obtained from the Sephadex G-10 procedure. Seven samples were obtained from haloperidol-treated rats which causes an increase in DOPAC levels.

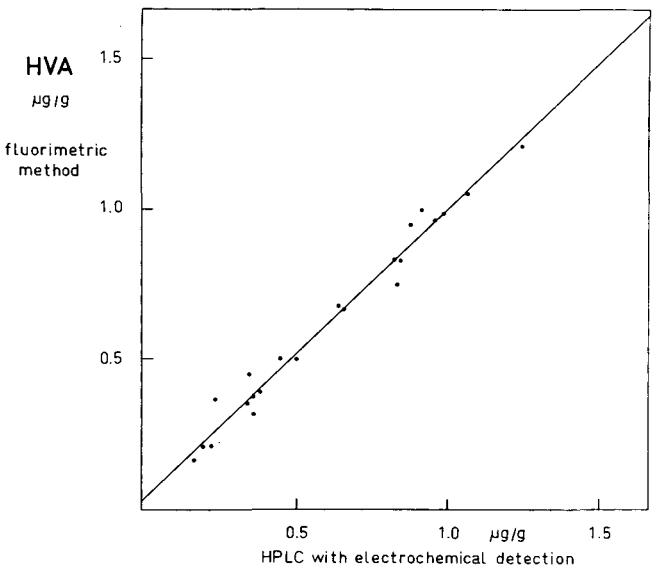

Fig. 7. Correlation between the automated fluorimetric assay and the HPLC method for HVA in striata of rats. The determinations were carried out on identical fractions obtained from the Sephadex G-10 procedure. Nine samples were obtained from haloperidol-treated rats which causes an increase in HVA levels.

TABLE I

SUMMARY OF THE CORRELATIONS FOUND FOR VARIOUS ASSAYS

Method	*r*	Slope	Intercept
HVA in CSF (Fig. 3)	0.990	1.066	0.45
5-HIAA in CSF including the Sephadex G-10 procedure (Fig. 4)	0.773	0.945	10.0
DA in striata (Fig. 5)	0.849	0.725	1.61
DOPAC in striata (Fig. 6)	0.993	0.973	0.058
HVA in striata (Fig. 7)	0.988	0.965	0.037

purification of DOPAC on Sephadex G-10 is probably more efficient in removing interfering compounds from the tissue samples. The analytical recoveries of DOPAC and HVA (30 ng added to cerebellar tissue) were monitored weekly in our laboratory, the results (± S.D.) were similar for the two methods. HVA (fluorimetric), 76.7 ± 9.3% (n = 24); and HVA (HPLC), 76.1 ± 7.5% (n = 25). DOPAC (fluorimetric), 82.2 ± 10.7% (n = 25); and DOPAC (HPLC), 81.4 ± 6.2% (n = 23).

CONCLUSIONS

In conclusion, semiautomated fluorimetric methods for DOPAC and HVA in brain tissue and HVA in CSF showed a good correlation with HPLC methods

based on amperometric detection. The fluorimetric determination of DA (ethylenediamine method) in striatal samples appeared somewhat less reliable than the HPLC method, probably due to fluorescence quenching. Although the fluorimetric assay of DA is useful for quantitation of DA in striatal samples, lower recoveries and more experimental variation are to be expected. The fluorimetric determination of 5-HIAA in CSF (*o*-phthaldialdehyde method) is not very reliable, as it correlated very poorly with the chromatographic method. Determinations of 5-HIAA in CSF have often been performed in biological psychiatric research [9, 10]. The present data imply that the results of these studies should be interpreted with care when fluorimetric methods are used.

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CHROMBIO. 1415

ASSAY OF FREE AND CONJUGATED CATECHOLAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid and simple method for the analysis of free and conjugated catecholamines in body tissues and fluids is described. The free catecholamines were isolated by standard alumina procedures before and after hydrolysis of the conjugated compounds to free compounds by heating the samples in perchloric acid. Free catecholamines were then separated by high-performance liquid chromatography and detected by electrochemical detection. Conjugated compound was the difference between the total and free amount in each sample. This method was utilized to measure free and conjugated norepinephrine, epinephrine, and dopamine in human urine and rat adrenal gland, and to measure free and conjugated dopamine in rat whole brain and kidney.

INTRODUCTION

Conjugated dopamine (DA) has been found in greater concentrations than free DA in human plasma [1,2] and in both human and monkey urine and cerebrospinal fluid [3,4]. Much of the conjugated DA is thought to occur as sulfate esters [1,5,6]. In the course of developing methods to determine the physiological role of these DA sulfate esters, we found it necessary to have a simple assay available for the analysis of free and conjugated DA in various body tissues and fluids. This report details a method developed for this purpose, as modified to measure free and conjugated norepinephrine (NE) and epinephrine (EPI) in addition to free and conjugated DA. The free catecholamines (CA) are isolated by standard alumina procedures before and after hydrolysis of the conjugated CA to free CA. Free CA are then separated by reversed-phase paired-ion high-performance liquid chromatography (HPLC) and detected by electrochemical detection. We report values for free and

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conjugated DA, NE, and EPI in human urine and rat adrenal glands. Free and conjugated DA levels are reported for rat kidney and whole brain.

METHODS

Chromatographic apparatus

Two different liquid chromatographs were used. Each system was equipped with a stainless-steel column (25 cm × 4 mm) prepacked with 5-μm octadecyl silica (Bioanalytical Systems), a guard column (2 cm × 4.5 mm) packed with 40-μm C_{18}/Corasil (Waters Assoc., Milford, MA, U.S.A.), and an electrochemical detector (Bioanalytical Systems) consisting of a thin-layer CP-O carbon-paste working electrode and an LC-4 or LC-4A controller. One liquid chromatograph consisted of the following components purchased from Bioanalytical Systems: Waters M-45 pump, Rheodyne Model 7125 injection valve equipped with a 20- or 200-μl loop, and a Houston Instruments B-5000 strip chart recorder. Occasionally, a Constametric I pump (Laboratory Data Control) was used. A column of air connected in parallel with the solvent flow path served as a pressure dampener. The other liquid chromatograph was completely automated and consisted of the following components purchased from Waters Assoc.: Waters M-45 pump, WISP Model 710-B automatic sample injector, Model 720 system controller, and Model 730 data module.

Chromatographic conditions

The mobile phase utilized was a modification of that described by Davis and Kissinger [7] and consisted of 0.075 *M* phosphate buffer, pH 2.8, containing EDTA (1 m*M*), sodium octyl sulfate (30 mg/l), and methanol (5%). The mobile phase was passed through a 0.45-μm filter, degassed by sonication, and stirred continuously during use. The flow-rate varied depending on chromatographic conditions, but it was usually in the range of 1.0—2.4 ml/min. A gradient of increasing flow-rate was sometimes used to decrease retention time of late-eluting compounds. Hydrodynamic voltammograms for the CA under the conditions described above showed oxidation potential plateaus ranging from 0.60 to 0.75 V vs. and Ag/AgCl reference electrode. Consequently, the working electrode was maintained at an oxidation potential of 0.70 V for the analyses.

Assay of samples

Free and total catecholamines (CA) were measured in replicate aliquots of tissue homogenate supernatants or urine by a modified alumina adsorption procedure. Dihydroxybenzylamine (DHBA) and/or deoxyepinephrine (DOE) were used as internal standards. An aliquot of urine or tissue supernatant containing EDTA, sodium metabisulfite, and an appropriate amount of internal standard was hydrolyzed in 0.4 *N* perchloric acid at 100°C for 120 min to convert conjugated CA to free CA. The hydrolysis step was omitted for assay of free CA. The free CA were then isolated by standard alumina procedures described previously [4]. The samples were dissolved in mobile phase and injected into the HPLC system described above. Injection volume ranged from 5 to 100 μl. The ratios of endogenous DA, NE, or EPI to internal standard were

determined in each sample from the peak heights. The amount of each CA in each sample was calculated by inverse linear regression analysis of a standard curve constructed by addition of known amounts of each CA to internal standard and carried through the alumina adsorption and hydrolysis procedures. Conjugated DA, NE, or EPI was the difference between total and free amounts in duplicate aliquots.

Data analysis

Free and total amounts or concentrations of each CA were compared by one-tailed Student's *t*-test for paired observations [8]. A p value less than or equal to 0.05 was considered to be statistically significant. Conjugated CA was the difference between the total and free in each sample.

Collection and treatment of samples

Human urine was collected and prepared as described previously [9]. Female Wistar rats weighing 190—210 g were fasted overnight and sacrificed by decapitation. Brains and peripheral tissues were rapidly removed, dissected on ice if appropriate, frozen on dry ice, weighed, and immersed in tubes containing 0.4 N cold perchloric acid containing 2.5% sodium metabisulfite and saturated with EDTA. The tubes were stored at —80°C until use. The tubes were later thawed before use and appropriate amounts of internal standards were added to each tube. Each sample was then homogenized for 15 sec in a Brinkmann Polytron Homogenizer (Model PT 10-35) and centrifuged at 4°C at approximately 12,000 g for 20 min. Aliquots of the supernatant were then assayed as described above. Urine was centrifuged at 4°C at approximately 12,000 g for 20 min, aliquoted into tubes containing internal standard, and assayed as described above.

Determination of optimal hydrolysis conditions

Aliquots of human urine were assayed for free and total CA as described above, except that each aliquot was heated in 0.4 N perchloric acid for times ranging from 30 to 240 min.

RESULTS

HPLC assay

Calibration curves for standards carried through the extraction and hydrolysis procedures were found to be linear throughout the ranges used for each assay. These varied from 5—100 pmoles (for DA in kidneys) to 5—100 nmoles (for NE and EPI in adrenals). The hydrolysis procedure had no effect on the peak heights of NE, EPI, DHBA, DA, or DOE standards. Reagent blanks carried through the alumina extraction, with or without hydrolysis, did not produce any peaks corresponding to any of the CA peaks measured. The minimum detectable amount of NE, EPI, and DA (giving a peak-to-noise ratio of two) was approximately 0.25 pmoles injected onto the column.

The percentage coefficient of variation (C.V.) between and within assays was calculated for a human urine sample. The within assay C.V. was 3.7% or less for each of the free CA, and 5.7% or less for each of the hydrolyzed CA.

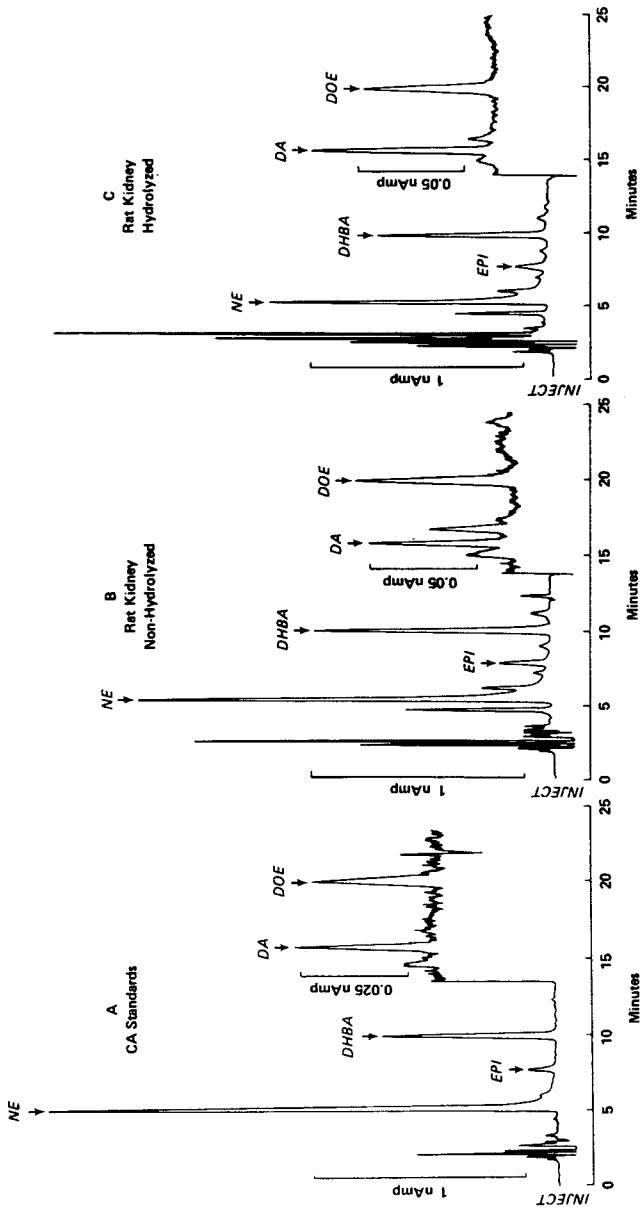

Fig. 1. Chromatograms of alumina extracts of catecholamine standard solution (A) and rat kidney homogenate supernatants before (B) and after (C) hydrolysis. There was no breakdown of any of the CA during the hydrolysis procedure. Note that total DA (hydrolyzed) is greater than free DA in this sample, indicating that substantial amounts of conjugated DA were present. HPLC conditions were as described in Methods. The flow-rate was held constant at 1.1 ml/min. Injection volume was 50 μl. The standard solution contained the following amounts of each CA per injection (assuming 100% recovery): NE, 200 pmoles; EPI, 10 pmoles; DHBA, 100 pmoles; DA, 5 pmoles, DOE, 20 pmoles.

The C.V. between assays was 8.8% or less for each of the free or hydrolyzed CA. Maximal recovery of total CA occurred in human urine after 120 min hydrolysis time. Consequently, 120 min was chosen as the hydrolysis time for all experiments.

Each of the CA of interest eluted from the HPLC column in a clearly defined peak. The capacity factors of NE, EPI, DHBA, DA, and DOE, plus those of other catechols which might interfere or be used as internal standards in these determinations, are given in Table I. None of the compounds injected resulted in interfering peaks in the analyses. Chromatograms of alumina extracts of CA standard solution and rat kidney supernatants before and after hydrolysis are shown in Fig. 1. The ratio of the DA peak height to either the DHBA or DOE internal standards is increased in the rat kidney after hydrolysis, indicating that substantial amounts of conjugated (total—free) DA are present.

TABLE I

CATECHOL RETENTION TIMES EXPRESSED AS CAPACITY FACTORS

n = 3 where replicates are indicated; n = 1 otherwise. 25 pmoles of each compound were injected. Capacity factor = (peak retention time — void volume time)/void volume time.

Compound	Capacity factor
Norepinephrine	1.60 ± 0.041
Epinephrine	2.91 ± 0.093
Dihydroxybenzylamine	4.05 ± 0.13
Dopamine	7.37 ± 0.29
Deoxyepinephrine	9.43 ± 0.36
3,4-Dihydroxyphenylglycol	1.19
Uric acid	1.34
3,4-Dihydroxymandelic acid	2.02
l-DOPA	3.66
3,4-Dihydroxyphenylacetic acid	10.5
Caffeic acid	11.3
α-Methyldopa	13.2
α-Methyldopamine	13.4
n-Acetyldopamine	14.1

Free and conjugated CA in rat tissue

The levels of free and conjugated NE, EPI, and DA in rat adrenal gland and free and conjugated DA in rat kidney and whole brain are shown in Fig. 2. Substantial amounts of conjugated DA were found in the kidney. The adrenal gland contained small but significant amounts of conjugated DA. Significant amounts of conjugated DA were not detected in the rat whole brain, nor were conjugated NE and EPI detected in the adrenal.

Because of the unexpected low levels or absence of conjugated DA in the rat adrenal gland and brain, several experiments were performed to verify these results. The assay was repeated six times on separate groups of four to eight rat adrenals, brains, and kidneys. The amount of conjugated DA detected

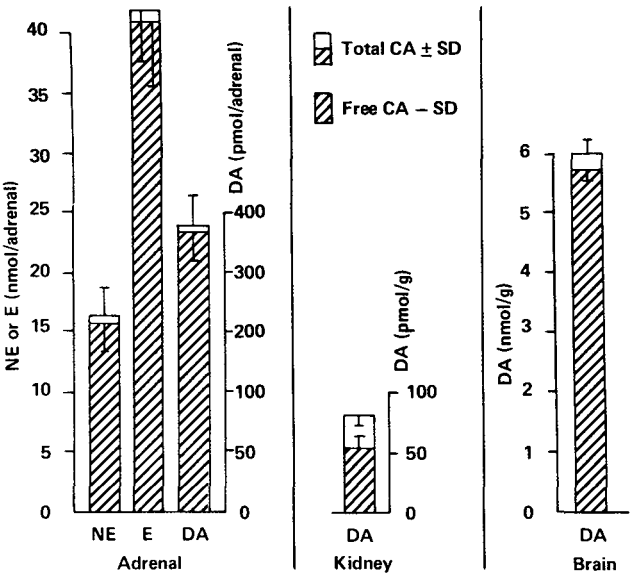

Fig. 2. Free and total catecholamines in rat tissue. Significant amounts of conjugated DA were found in the rat kidney but not in the whole brain. The adrenal contained small but significant amounts of conjugated DA, but not of conjugated NE or EPI. These results were verified in several separate experiments detailed in Results. n = 6 per group.

in the adrenals in these six repeated experiments ranged from 6.5 to 8.8% of total DA. Conjugated DA ranged from —4.5 to +3.4% of total DA present in rat brain. Repeated experiments on rat kidneys produced values for conjugated DA ranging from 26 to 34% of total DA.

The possibility that CA breakdown might be occurring during the hydrolysis procedure was considered and then eliminated by the fact that there were no differences in absolute peak heights of standards or sample CA peaks between hydrolyzed and non-hydrolyzed tubes. To test the hypothesis that incomplete hydrolysis might be occurring during the hydrolysis procedure, a mixture of dopamine-3-O-sulfate and dopamine-4-O-sulfate was added to rat adrenal glands. The adrenals were then assayed for free and conjugated DA, and complete hydrolysis of the dopamine sulfate to free DA was found to occur.

We also considered the possibility that premature hydrolysis might be occurring during the tissue disruption procedure in 0.4 N perchloric acid. To test this hypothesis, one of each pair of rat adrenals or kidneys was homogenized in 0.4 N perchloric acid (the routine procedure) and the other was homogenized in 1 N acetic acid. They were then assayed for free and conjugated CA as described in the methods section. If hydrolysis were occurring during homogenization in 0.4 N perchloric acid, then the free NE, EPI, or DA determined in the tissues homogenized in perchloric acid should be greater than that in the contralateral tissue homogenized in acetic acid. Results, shown in Table II, indicate that there were no differences in any of the free or total CA measured due to the homogenization medium.

One additional experiment was conducted to identify more conclusively the

TABLE II

EFFECT OF HOMOGENIZATION MEDIUM ON FREE AND TOTAL DOPAMINE IN RAT KIDNEYS AND ADRENALS

n = 6 per group; S.D. in parentheses.

	Kidney (pmoles/g)		Adrenal (pmoles/adrenal)	
	Perchloric acid	Acetic acid	Perchloric acid	Acetic acid
Free DA	52.4 (8.1)	55.6 (9.6)	159 (24)	157 (34)
Total DA	79.0 (8.9)	78.4 (7.8)	169 (31)	163 (32)
Percentage conjugated	34.3 (15)	29.5 (6.4)	5.32 (4.7)	4.31 (6.1)

peaks of interest in the chromatograms from rat tissues. Hydrodynamic voltammograms were generated over a portion of the useful oxidation potential range for standards of DA, NE, and EPI and compared with that portion of the chromatogram from rat adrenals thought to contain each of these CA. Since the curves for the standard and the compound from the adrenal were in good agreement (Fig. 3), we concluded that the peaks in the adrenals were correctly identified [10].

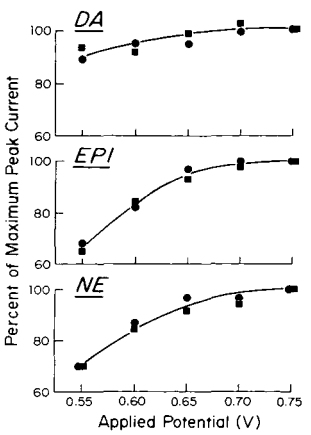

Fig. 3. Hydrodynamic voltammograms of NE, EPI, and DA in standard solutions (•) and rat adrenal gland (▪) alumina extracts. The close correlation in the responses to increasing oxidation potential strongly suggests that the peaks are correctly identified in the alumina extracts from the rat adrenal gland. HPLC conditions were as described in Methods.

Free and conjugated CA in human urine

Table III shows the mean daily urinary excretion of free and conjugated NE, EPI, and DA in the six subjects studied. Excretion of each of these CA in the conjugated form accounted for more than 50% of their total daily excretion. Conjugated DA accounted for 66.8 ± 7.7% of the total daily DA excretion as determined by HPLC in the present study. In an earlier study using

TABLE III

FREE AND CONJUGATED CATECHOLAMINES IN HUMAN URINE DETERMINED BY HPLC

n = 6, S.D. in parentheses.

	Excretion rate (nmoles/day)			Percentage conjugated
	Free	Conjugated	Total	
NE	653 (230)	727 (418)	1380 (623)	50.4 (7.4)
EPI	421 (139)	693 (594)	1114 (703)	55.9 (14)
DA	1161 (500)	2671 (1571)	3832 (2025)	66.8 (7.7)

a gas chromatographic—mass spectrometric method described elsewhere [4], we found that conjugated DA accounted for 61.5 ± 2.9% of the total daily DA excretion in four other normal human subjects (Elchisak and Ebert, unpublished). We have previously shown that dopamine-3-O-sulfate accounted for 73.1% of the conjugated DA excretion in four of these six subjects [9].

DISCUSSION

The HPLC assay for free and conjugated catecholamines reported in this paper has been in routine use in our laboratory for over one year. It is a rapid, sensitive, and relatively simple procedure for the analysis of free and conjugated catecholamines in most tissues and body fluids. The assay detailed here should be useful in future studies concerning the distribution of conjugated catecholamines in various body tissues and fluids. We are currently using this method to survey tissues and fluids of various species for the occurrence of conjugated DA. Since we are primarily interested in the distribution and functions of DA-3-O-sulfate and DA-4-O-sulfate in the body, we then examine those tissues and fluids in which conjugated DA occurs for the occurrence of the sulfated compounds. This procedure has been described elsewhere [9].

None of the possible interfering compounds listed in Table I, which might be carried through the alumina extraction procedure, actually interfered with the determination of the catecholamines of interest in the present study. However, the mobile phase must be carefully "fine tuned" to prevent dihydroxyphenylglycol, uric acid, and dihydroxymandelic acid from interfering with the NE peak. Varying amounts of methanol and sodium octyl sulfate can be added to the mobile phase, depending on column status, to eliminate interferences. We have most recently utilized a mobile phase containing 3% methanol and 40 mg/ml sodium octyl sulfate. Interference from uric acid can also be minimized, if necessary, by adding uricase to the sample mixture during the alumina adsorption step. This enzyme oxidizes uric acid to allantoin, a compound which is not electrochemically active and thus will not interfere with any of the catecholamine determinations. Interference from these compounds, in our hands, has only been a problem when an HPLC column which had been in constant use for approximately six months was used for the analyses.

The percentages of total NE, EPI, and DA excreted in human urine as the conjugated compound were found to be greater than 50% for each compound in the present study by the method described. These results are in good agreement with those found by other investigators using acid hydrolysis coupled with fluorometric [11,12], radioenzymatic [2,13], and HPLC [14] assay methods. These results clearly indicate that conjugation is a significant metabolic pathway for catecholamines in humans.

Significant amounts of conjugated DA in the whole rat brain were repeatedly not detected in the present study. To our knowledge, the occurrence of conjugated DA in whole rat brain has not previously been investigated. The levels of free DA in the whole rat brain found in the present investigation are in good agreement with those reported by other investigators using fluorometric [15], radioenymatic [16], and HPLC [17,18] assay methods. It is possible that conjugated DA could be present in discrete rat brain areas and not be detected in the whole brain because of effects of dilution. Recently, Buu et al. [19] have reported that DA-sulfate accounts for 31% of the total DA in rat hypothalamus, 12% in the hippocampus, and only 1% in the striatum. The distribution of conjugated DA, and specifically DA-sulfate, in the brains of various species should be further investigated. Correlation of DA-sulfate levels with the activity of the enzymes responsible for the formation of this compound and its possible in vivo hydrolysis to free DA should provide an insight into the function of this compound, if any, in the mammalian brain.

Small but significant amounts of conjugated DA were detected in the rat adrenal gland in the present study. Conjugated NE or EPI were not detected. To our knowledge, conjugation of CA has not been previously studied in rat adrenal. The effects of various stresses on the synthesis or utilization of conjugated DA remain to be evaluated. It is interesting to note, however, that administration of dexamethasone, a synthetic adrenocortical steroid, produced a five-fold increase in the activity of rat kidney phenolsulfotransferase [20], the enzyme responsible for the synthesis of DA-sulfate [21].

Conjugated DA accounted for approximately 30% of the total DA present in rat kidney in the present investigations. This is in contrast to the findings of Kuchel et al. [22], who reported that essentially all of the DA in rat kidney occurred as conjugated DA. It is difficult to reconcile these differences. Methodological differences between our assay and theirs cannot account for this discrepancy, since the results we obtained for human urine agree well with the results obtained by this group [2]. No details concerning the treatment of the rats were given by Kuchel's group, so discrepancies due to age, whether or not the animals were fasted, and the method of sacrifice cannot be compared. It is possible that one or more of these factors differed between the two studies, and that this might help account for the differences observed.

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URINARY 3-METHOXY-4-HYDROXYPHENYLGLYCOL DETERMINATION USING REVERSED-PHASE CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

A reversed-phase liquid chromatographic method with amperometric detection has been developed for the determination of urinary 3-methoxy-4-hydroxyphenylglycol. Before and after enzymatic deconjugation, it was purified by an extraction procedure and rapidly quantified under isocratic conditions. The 24-h excretion profile in normal human subjects (eight males and seven females) was determined; our results are consistent with those arrived at in a number of other studies. The present method is highly sensitive and selective; in addition, a good degree of precision is assured by use of 4-methoxy-3-hydroxyphenylglycol as internal standard.

INTRODUCTION

The determination of urinary 3-methoxy-4-hydroxyphenylglycol (MHPG) levels offers a valuable aid for the diagnosis of catecholamine-secreting tumors such as pheochromocytoma, neuroblastoma and ganglioneuroma [1—4].

In addition, the activity of the brain noradrenergic system has been related to a variety of pathological states such as depression, obesity, hypertension and mania [5—7]. A number of studies have determined that 20—50% of the MHPG excreted in urine originates in the central nervous system, while Maas et al. [8] recently estimated the average contribution by the brain to the total body production of MHPG to be 63% [8]. Hence, the measurement of MHPG concentrations in the urine may provide an index of the cerebral norepinephrine (NE) turnover and may prove helpful in the diagnosis of mental disease and in selection of an appropriate therapy.

MHPG is present in urine as a free metabolite (MHPG-free) or, more

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frequently, as the conjugate of sulfuric acid (MHPG-SO_4), which has been reported to be derived from the central NE metabolism [9], and as β-conjugate of glucuronic acid (MHPG-Glu); this latter seems to reflect the metabolism of systemic NE [10].

Various methods have been reported for the determination of this metabolite in urine. At the present time, the most commonly used techniques are gas chromatography with flame ionization detection [11] or electron-capture detection [12, 13], and gas chromatography—mass spectrometry [14]. Unfortunately these procedures require a pretreatment of the sample (e.g. derivatization) and call for equipment too sophisticated and too expensive for routine evaluation.

Reversed-phase liquid chromatography (RPLC) with electrochemical detection (ElCD) has recently been introduced and is an excellent analytical tool for the measurement of MHPG in urine. However, to ensure elimination of interferences, the most reliable procedures call for additional analytical steps, e.g. chemical conversion of the metabolite [15] or thin-layer chromatography for its purification [16].

This report presents a specific RPLC-ElCD method for determining free and conjugated urinary MHPG; it is based exclusively on extraction steps before chromatographic analysis. High precision was achieved by use of 4-methoxy-3-hydroxyphenylglycol (iso-MHPG) as internal standard.

MATERIALS AND METHODS

Apparatus and liquid chromatographic conditions

An LC-50 liquid chromatograph (Bioanalytical Systems, West Lafayette, IN, U.S.A.) equipped with a detector Model TL-3 packed with CP-O carbon paste (Bioanalytical Systems) was made use of for all determinations; the electrode potential was set at +0.80 V versus an Ag/AgCl reference electrode. The chromatographic column was a prepacked μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm average particle size) obtained from Waters Assoc. (Milford, MA, U.S.A.).

The mobile phase, 0.009 *M* citric acid and 0.089 *M* sodium acetate buffered to pH 5.1 containing 10% methanol, was degassed by filtration under vacuum through a Millipore 0.2-μm membrane and delivered at a flow-rate of 1.0 ml/min.

Reagents

All reagents used were of the highest purity (A.C.S. certified grade). Arylsulfatase (type VI from *Aerobacter aerogenes*, 31.2 units/ml), β-glucuronidase/arylsulfatase (glusulase, from *Helix pomatia*, containing 19,500 units/g sulfatase and 420,000 Fishman units/g β-glucuronidase) and MHPG piperazine salt were obtained from Sigma (St. Louis, MO, U.S.A.); iso-MHPG was from Paesel (Frankfurt, G.F.R.)

Solutions of standards were prepared in redistilled deionized water and kept frozen until used.

Procedure

Twenty-four-hour urine samples from laboratory personnel or student volunteers (eight men and seven women) were collected in glass bottles which contained 0.5 mg of sodium metabisulfite per ml of urine and maintained at 4°C. After collection, urine volume was recorded and creatinine was measured colorimetrically using a modification of Jaffe's method [17].

An aliquot of 20 ml was stored at −20°C until the time of assay. For determining MHPG, three samples were prepared as follows. Sample 1 (MHPG-free): 0.5 ml of urine plus 0.2 ml of 1.0 *M* sodium acetate buffer, pH 6.5 plus 0.1 ml of 2% EDTA solution. Sample 2 (MHPG-free + MHPG-SO_4): the same as sample 1 plus 25 μl of arylsulfatase type VI. The pH was adjusted to 7.1. Sample 3 (MHPG total): the same as sample 1 plus 10 mg of glusulase. The pH was adjusted to 5.2. These enzyme amounts have been used because further addition of glusulase or sulfatase did not increase the estimates of MHPG. To each sample 100 μl of a 20 ng/μl iso-MHPG solution were added and samples were incubated at 37°C for 20 h [15].

After cooling and addition of 0.7 ml of 1.0 *M* sodium acetate buffer, the pH was adjusted to 6.5 and each sample, saturated with sodium chloride, was extracted three times with 2 ml of ethyl acetate. The ethyl acetate pool was re-extracted two times with 0.5 ml of 0.1 *M* H_3BO_3. The boric acid pool was washed twice with 2 ml of diethyl ether, its pH adjusted to 8.7, and then extracted twice with 2 ml of ethyl acetate.

The ethyl acetate extracts were evaporated to dryness under reduced pressure using a rotavapor; 20 μl of residue, reconstituted with 0.5 ml of glass-distilled water, were injected into the RPLC system.

To determine the MHPG content of the original samples, a urine pool was made from the samples to be analysed. Different amounts of pure MHPG (125, 250, 500, 1000 and 2000 ng per 100 μl) and 100 μl of a 20 ng/μl iso-MHPG solution were added to each 0.5-ml sample of the pool; these were hydrolysed with arylsulfatase and subjected to the whole procedure. A standard curve was prepared by plotting the MHPG/iso-MHPG peak height ratio versus the MHPG added. A linear regression analysis was performed to determine the best linear graph. The equation for the standard curve was $Y = 0.45X + 0.85$ ($r = 0.994$). The slope of the linear regression line was found to be not significantly different when the standard curve was prepared from samples of urine, whether these were untreated or hydrolysed with glusulase.

The concentration of MHPG in the unknown samples was calculated according to the following equation:

$$\text{MHPG } (\mu\text{g/ml}) = \frac{\text{MHPG peak height}}{\text{Iso-MHPG peak height}} \times \frac{1}{\text{Slope of standard curve}}$$

RESULTS AND DISCUSSION

The major difficulty in the determination of MHPG in urine is its separation from several substances usually extracted by organic solvents at neutral pH; nor has the problem been fully solved by HPLC with ElCD, a technique increa-

singly applied to the evaluation of catecholamines and their metabolites in biological fluids. Despite the high resolving power of HPLC and of the selectivity of the electrochemical detector, MHPG still requires at least partial purification before chromatographic analysis. To achieve this end, in two recently published methods, after extraction into ethyl acetate, MHPG was oxidized to vanillin with periodate and then reduced to vanillyl alcohol [15] or isolated by thin-layer chromatography [16].

We obtained a satisfactory degree of purification by an extraction procedure where final washing with diethyl ether and MHPG reextraction from the borate buffer at pH 8.7 by ethyl acetate were important steps towards obtaining chromatograms with fewer interfering peaks and a straight baseline.

The precision of this method was increased by use of iso-MHPG as internal standard, iso-MHPG being a compound with extraction, chromatographic and detection characteristics similar to those of MHPG.

The presence in man of this compound, a possible metabolite of 4-O-methylation of norepinephrine, has been investigated by Mathieu et al. [18] and Muskiet et al. [19] by means of gas chromatography with electron-capture detection and mass fragmentography.

For normal subjects iso-MHPG was found to be present, though at an extremely low level (mean percentage of iso-MHPG relative to MHPG = 0.77), only by Muskiet et al., whereas Mathieu et al. did not detect it at all. Furthermore, the percentage of iso-MHPG relative to MHPG was in the range determined for normal subjects even in specimens of urine taken from patients with neural crest tumors (neuroblastoma and pheochromocytoma) or from patients treated with dopamine or catecholamine precursors (L-DOPA). Hence iso-MHPG should be regarded as generally not produced, or as produced only in negligible quantities; as a result it has been used as internal standard.

The potential at which the maximum oxidation current is generated was established for iso-MHPG at between + 0.7 and + 1.0 V. Given that the potential of + 0.8 V allows a highly sensitive analysis of MHPG without loss of selectivity, this potential was adopted for the protocol procedure.

Some authors [16] report the possible presence of contaminating peaks in ethyl acetate and small amounts of MHPG in commercially available enzyme preparations. In order to check this a reagent blank was run, and no measurable peaks were detected in the chromatograms.

In Fig. 1 typical chromatograms of a urine sample hydrolyzed with glusulase are shown. A peak whose retention time corresponds to that of MHPG was observed, while no peaks were detected which would correspond to that of iso-MHPG. The identity of this peak was further confirmed by the addition of authentic MHPG, and also on the basis of ratios of responses at several oxidation potentials against the reference standard.

In these preliminary experiments a mobile phase without methanol was used; to shorten the chromatographic analysis time, and in view of the very clear separation of MHPG and iso-MHPG from interfering peaks, in the protocol procedure 10% methanol was added. The retention times of two compounds were then decreased to 7 min and 10 min without loss of resolution (Fig. 2).

The precision of the assay was evaluated by analysing samples of a urine

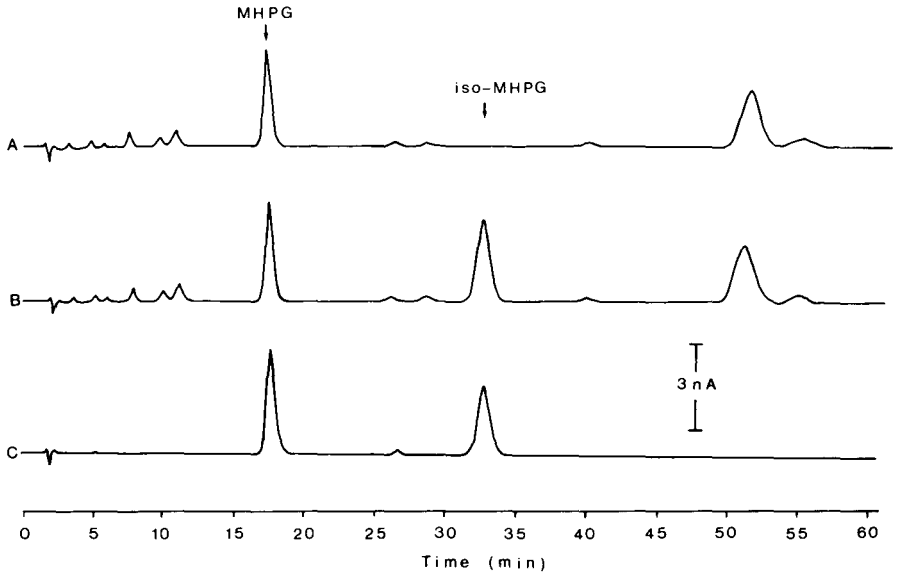

Fig. 1. Chromatogram of human urine. Aliquots of a urine sample were hydrolysed with glusulase and processed as described in the text. (A) Urine. (B) Urine with iso-MHPG added. (C) MHPG and iso-MHPG standards. Chromatographic conditions: C_{18} reversed-phase column; 0.009 *M* citric acid and 0.089 *M* sodium acetate buffer (pH 5.1); flow-rate 1.0 ml/min; electrochemical detector +0.8 V; room temperature.

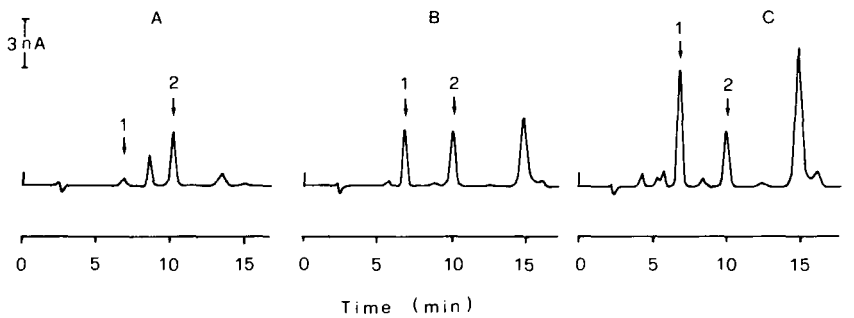

Fig. 2. Typical chromatograms of MHPG extracted from: (A) untreated urine (MHPG-free, 0.19 ng/μl); (B) urine hydrolyzed with arylsulfatase (MHPG-SO_4 + MHPG-free, 1.61 ng/μl); and (C) urine hydrolyzed with glusulase (MHPG-total, 3.46 ng/μl). Chromatographic conditions: as in Fig. 1 with a mobile phase containing 10% (v/v) methanol. Peaks: 1 = MHPG, 2 = iso-MHPG.

specimen either untreated or hydrolysed with glusulase. The within-run precision of quantitative results gave coefficients of variation of 1.7% and 1.9%, respectively; day-to-day precision evaluations yielded coefficients of variation of 2.6% and 3.0% ($n=10$).

The values of 24-h urinary MHPG-free, MHPG-SO_4, MHPG-Glu and MHPG total excretion are shown in Table I and are consistent with recently published data [15, 16]. Although the excretion of total and conjugated MHPG was lower

TABLE I

24 h URINARY MHPG EXCRETION IN NORMAL HUMAN SUBJECTS

Data are given as mean ± S.D. M = males (n=8), F = females (n=7).

		mg MHPG per 24 h	μg MHPG per mg creatinine
MHPG-free	M	0.112 ± 0.03	0.058 ± 0.02
	F	0.140 ± 0.12*	0.120 ± 0.09*
MHPG-SO_4	M	1.340 ± 0.30	0.700 ± 0.22
	F	0.809 ± 0.31	0.696 ± 0.28
MHPG-Glu**	M	1.470 ± 0.43	0.783 ± 0.34
	F	0.751 ± 0.31	0.625 ± 0.27
MHPG total	M	2.930 ± 0.73	1.540 ± 0.57
	F	1.702 ± 0.50	1.440 ± 0.40

*F significant at 1% level (F vs. M).
**As the difference of the MHPG total – (MHPG-free + MHPG-SO_4).

in women than in men, no significant difference was found when the values were expressed as mg MHPG excreted per g creatinine; free MHPG excretion alone was significantly higher in women. In addition, our results confirm that MHPG-SO_4 is less than, or equal to, MHPG-Glu in 24-h human urine samples [6, 14, 15].

In conclusion, the present method is very reproducible, and selective and sensitive enough for the determination of low concentrations of urinary free MHPG; in addition, it appears to be simpler than the existing HPLC procedures.

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SIMULTANEOUS DETERMINATION OF FREE 3-METHOXY-4-HYDROXY-MANDELIC ACID AND FREE 3-METHOXY-4-HYDROXYPHENYL-ETHYLENEGLYCOL IN PLASMA BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A new assay method is described for the simultaneous determination of free 3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenylethyleneglycol in plasma utilizing separation and purification by Bio-Gel P-10 followed by high-performance liquid chromatography with electrochemical detection. This technique is sensitive and reliable, and offers an inexpensive and practical alternative to gas chromatographic—mass fragmentographic methods for the monitoring of plasma levels of these catecholamine metabolites in the study of selective metabolic pathways of endogenous norepinephrine originating in the peripheral and the central nervous systems.

INTRODUCTION

Determination of the changes in the turnover of the monoamine transmitters and their metabolites has indicated their importance in the normal and pathological functioning of the brain. For instance, variations of urinary 3-methoxy-4-hydroxymandelic acid (VMA) and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) have been associated with depressive disorders [1—3] or states of stress and anxiety [4, 5].

The determination of the monoamine metabolites in physiological samples has mostly been performed by gas chromatography [6, 7] or by a gas chromatographic—mass fragmentographic procedure [8—11]. These latter techniques, while offering excellent selectivity and high sensitivity are either slow or very expensive to employ on a routine basis. Moreover, they require a derivatization step before chromatography can be carried out.

Reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection has been shown to provide high selectivity and sensitivity for the analysis of monoamine metabolites in urine [12—14], in cerebrospinal

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fluid [15] and brain tissue [16—18]. These procedures cannot however be applied to plasma samples since a low plasma concentration of these metabolites and the presence of electroactive compounds carried by proteins and lipoproteins generate peaks which hamper greatly the resolution of the chromatogram.

The aim of this article is to describe a simple, fast and sensitive method for the plasma determination of catecholamine metabolites using molecular sieve chromatography as a clean-up procedure followed by HPLC analysis with electrochemical detection.

MATERIALS

Solvents and chemicals

Methanol, HPLC grade, was purchased from Fisher Scientific (Montreal, Canada). All other reagent grade chemicals were obtained from Baker (Canlab, Montreal, Canada). The standards, *dl*-VMA and MHPG piperazine salt, were purchased from Sigma (St. Louis, MO, U.S.A.); normetanephrine (NMN), 3,4-dihydroxyphenylethyleneglycol (DOPEG), 3,4-dihydroxy-*d*-mandelic acid (DOMA) and 3,4-dihydroxyphenylacetic acid (DOPAC) from Calbiochem (La Jolla, CA, U.S.A.). The Bio-Gel P-10 (100—200 mesh) was obtained from Bio-Rad (Mississauga, Canada). The water used throughout the experiment was deionized and glass double-distilled.

Instrumentation for extraction and evaporation

The equipment used in the extraction procedure was the Evapo Mix and the Vortex evaporator (all from Buchler, Fort Lee, NJ, U.S.A.), connected to a vacuum pump for the organic solvent evaporation.

METHODS

Preparation of the gel column

Dry Bio-Gel P-10 powder (1 g) was suspended in 50 ml of distilled water by gentle stirring and allowed to swell for at least 30 min. It was rinsed and decanted twice with distilled water, then equilibrated by replacing the water with the eluting buffer, decanting and renewing this buffer four times. The eluting buffer was 0.05 *M* sodium phosphate, pH 6, containing $2.7 \cdot 10^{-3}$ *M* EDTA and $6.3 \cdot 10^{-5}$ *M* ascorbic acid.

The barrel of a 10-ml disposable syringe (Beckton-Dickinson, Mississauga, Canada) was used as a column. For this purpose, a cotton-wool plug was placed at the bottom. In order to standardize the chromatographic procedure, a two-channel peristaltic pump (Buchler) was used. The outlet of the column was linked to the fraction collector by a catheter and a tubing connector passing through one of the channels of the pump. An identical tubing system going through the other pump channel provided the connection between the buffer reservoir and the upper part of the column. These instrumental conditions ensured reproducibility of the flow-rate and flexibility in the operation. The entire system can, in effect, be shut off and reopened at any time and still retain the same characteristics.

The syringe was partly filled with buffer and the gel slurry was added, stirring constantly, then allowed to pack under gravity. The final volume of the gel bed was 5 ml. This and the following operations were all carried out at room temperature.

The characteristic parameters of the elution diagram were established using a 2 mg/ml solution of Dextran Blue (Pharmacia, Uppsala, Sweden) containing 100 ng/ml each of VMA and MHPG. An LKB (Bromma, Sweden) Model 7000 fraction collector with disposable styrene tubes (12 × 75 mm) was used for collecting the eluate.

Plasma sample preparation

Clean-up step by gel filtration. The liquid above the gel bed was drained. When the surface of the gel bed was almost dry, the flow was stopped and 1 ml of plasma mixed with 10 μl of internal standard solution containing 8 ng of hydroquinone (HQ) was carefully applied along the walls in order to avoid disturbances in the gel bed. As soon as the sample was applied, the flow was resumed and the sample allowed to penetrate the gel until the surface was again almost dry. Then, 1 ml of eluting buffer was applied, as described above, in order to rinse the walls of the column and the gel surface and the draining procedure was repeated. A final aliquot of 1 ml of buffer was applied and the column connected to the reservoir of eluting buffer. The elution was carried out at a flow-rate of 30 ml/cm^2/h. Fractions of 0.5 ml were collected from the moment that the plasma entered the gel bed. The first eight tubes, corresponding to a volume of 4 ml, containing proteins and high-molecular-weight substances were discarded. The following seven tubes, for a total of 3.5 ml, were collected and their contents pooled for the subsequent analytical step. The gel column was rinsed with 2 ml of buffer before the application of the next sample.

Extraction. The 3.5 ml of pooled eluate were put into a 20-ml screw-cap test tube and 2 g of sodium chloride were added. The pH was adjusted to 3 with phosphoric acid, 30% (v/v). A volume of 10 ml of ethyl acetate was then added. The tube was shaken for 5 min on the Evapo Mix and centrifuged for 5 min at 2000 *g*. The organic upper layer was collected and the aqueous phase was then reextracted twice with 5 ml of ethyl acetate. The three ethyl acetate extracts were pooled and evaporated to dryness under vacuum at 30°C on the Vortex evaporator. The tube was then rinsed with 1.5 ml of methanol, which were evaporated to dryness. The residue was redissolved in 150 μl of the mobile phase and 100 μl were injected onto the HPLC column.

HPLC instrumentation

The HPLC system consisted of a Model 6000 A solvent delivery pump with a U6K injector (all from Waters Assoc., Milford, MA, U.S.A.), and an ODS reversed stationary phase column (Spherisorb 5 μm, 250 × 3 mm, Brownlee, Santa Clara, CA, U.S.A.) protected by a MPLC guard column (Brownlee). The detection system consisted of a thin-layer flow-through electrochemical cell with glassy carbon as the working electrode, a silver/silver chloride reference electrode (Bioanalytical Systems, W. Lafayette, IN, U.S.A., Models TL 5 and LC 4A), and a recorder—integrator (Data Module 730, Waters Assoc.).

The glassy carbon electrode was repolished once a week or as required, using the polishing kit supplied with the detector.

HPLC conditions

The solvent system consisted of a 97:3 (v/v) mixture of 0.07 *M* sodium phosphate buffer and methanol containing sodium EDTA (0.01%). The pH was adjusted to 2.5 with phosphoric acid. This mobile phase was filtered through 0.45-μm type HA Millipore filters and degassed under vacuum. The flow-rate was set at 1.2 ml/min. The effluent was passed through the detector cell and monitored at a potential of +0.8 V versus the silver/silver chloride reference electrode. The chart speed of the recorder was set at 0.25 cm/min.

Standard solutions of HQ, VMA and MHPG

Stock solutions of HQ, VMA and MHPG were prepared at concentrations of 100 μg/ml in methanol and stored at −40°C. Working standard solutions were obtained by appropriate dilution of the stock solutions with 0.05 *M* citric acid.

Preparation of spiked plasma samples

One volume of pooled human plasma was dialysed for 24 h against 200 volumes of an 0.15 *M* sodium phosphate buffer pH 7.4 for elimination of endogenous VMA and MHPG. The dialysed plasma was then spiked with known concentrations of VMA and MHPG ranging from 2 to 32 ng/ml and with HQ (8 ng/ml) as internal standard.

Calculations

All measurements were done by estimation of peak heights. Four calibration standards were used with each set of unknown samples to determine the concentrations of VMA and MHPG. The calibration standards were prepared from the pooled dialysed plasma spiked with a known quantity of VMA and MHPG (4—8 ng/ml) and internal standard. The calibration standards were run through the entire procedure along with other samples to be analysed. The ratio of the peak height (PHR) for each metabolite to that of the internal standard was used to calculate the concentration according to the formula:

$$\text{Concentration sample} = \frac{\text{PHR}_{\text{sample}}}{\text{PHR}_{\text{plasma standard}}} \times \text{concentration}_{\text{plasma standard}}$$

RESULTS AND DISCUSSION

Clean-up procedure

A molecular sieve was selected for the elimination of proteins from plasma prior to chromatography; the principle was identical to the desalting of a protein solution. The current methods for plasma deproteinization by perchloric acid, ethanol or by ultrafiltration did not give satisfactory results due to the presence of interfering peaks on the chromatograms, thus resulting in poor resolution. The experimental conditions were chosen based on several

criteria: a satisfactory separation of proteins from the small molecular size compounds enabling a chromatogram, devoid of interfering peaks, to be obtained together with recovery of the metabolites in a volume of eluate suitable for extraction with small volumes of solvent. These objectives were met by using a short and wide gel column providing a high flow-rate. The size of the sample, which was fairly large compared to the total bed volume, nevertheless remained compatible with the characteristics of the column. Indeed, the parameters, as determined for our system on the elution diagram (see Fig. 1) gave a separation volume of 3 ml: thus, theoretically, a 3-ml sample could be applied. Although sample volumes as high as 30% of the total bed volume which would represent in our case 1.5 ml [19], could be used, we have deliberately kept the volume of the applied sample to 1 ml in order to minimize the risk of cross contamination by overlapping peaks. The polyacrylamide matrix, Bio-Gel P-10, proved to be satisfactory for the deproteinization procedure, as it is easy to handle and re-usable for months without deterioration once the volume characteristics of the column have been established.

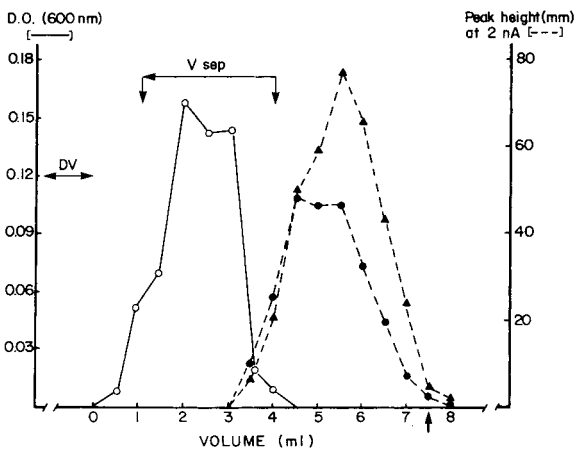

Fig. 1. Elution profile of a solution of Dextran Blue, VMA and MHPG. The sample volume applied is 1 ml. The gel bed dimensions are 3 × 1.46 cm. V sep = V_e (MHPG or VMA) — V_e (Dextran Blue). V_e is the volume eluted from the start of the sample application on the column to the inflexion point (↓) of the elution peak. Dead volume (DV) refers to the volume of the tubing system. ○, Dextran Blue; ●, VMA; ▲, MHPG.

HPLC analysis

Under the conditions described, HQ (internal standard), VMA and MHPG gave sharp, well separated peaks with retention times of 9, 10.3 and 14.8 min, respectively. The chromatograms of aqueous standards, dialysed plasma blanks, dialysed plasma spiked with HQ, VMA and MHPG, and plasma sample from one subject are illustrated in Fig. 2A, B, C and D. Under these chromatographic conditions, no interference with the other naturally occurring acidic and neutral electroactive endogenous compounds were observed, as shown in Fig. 2E.

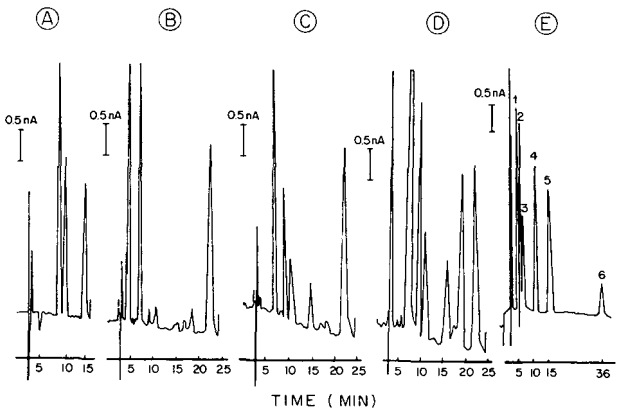

Fig. 2. Chromatography of VMA and MHPG. (A) Chromatogram of aqueous standards (5 ng of HQ, VMA, MHPG, in their order of elution); (B) plasma blank (dialysed plasma); (C) plasma blank spiked with 8 ng of HQ (internal standard) and 4 ng each of VMA and MHPG; (D) plasma sample from one subject (the estimated concentrations for VMA and MHPG are 4.12 and 4.07 ng, respectively); (E) separation of a synthetic mixture containing 5 ng each of the following: (1) 3,4-dihydroxymandelic acid; (2) 3,4-dihydroxyphenylglycol; (3) normetanephrine; (4) VMA; (5) MHPG; (6) 3,4-dihydroxyphenylacetic acid. Chromatographic conditions: 250 × 3 mm column of Spherisorb-ODS, 5 μm; mobile phase, 0.07 *M* phosphate buffer—methanol (97:3, v/v) containing 0.01% sodium EDTA; flow-rate 1.2 ml/min; detector, 0.8 V vs. the Ag/AgCl reference electrode.

Recovery

Known amounts of VMA and MHPG were added to the dialysed plasma samples and were put through the whole analytical procedure. The peak heights of VMA and MHPG from these samples were compared to those of aqueous standard solutions of these metabolites, at known concentrations, to determine the absolute percent recovery. The data are given in Table I. The average percent recovery is 59.3% and 52.7% for VMA and MHPG, respectively.

To investigate the influence of the pH of the eluting buffer with which the ethyl acetate extraction was performed, aliquots of 3.5 ml were spiked

TABLE I

ABSOLUTE RECOVERY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF VMA AND MHPG IN PLASMA

Concentration spiked into plasma* (ng/ml)	VMA		MHPG	
	Absolute recovery (%)	Relative standard deviation (%) (n = 3)	Absolute recovery (%)	Relative standard deviation (%) (n = 3)
2	63.1	6.0	57.4	5.2
4	59.5	5.1	49.1	11.4
8	51.6	7.5	55.8	7.7
16	59.6	6.7	51.7	5.2
32	62.8	8.5	49.5	6.3

*Concentrations were identical for VMA and MHPG.

TABLE II

INFLUENCE OF pH ON THE RECOVERY OF VMA, MHPG AND HQ FOLLOWING THE ETHYL ACETATE EXTRACTION

Results represent the absolute percent recovery by comparing the peak heights of VMA, MHPG and HQ obtained from spiked samples [2] to that of aqueous standard solutions.

pH of buffer	VMA	MHPG	HQ
1.5	80.7%	38.9%	50.5%
3	59.5%	58.5%	43.5%
5	37.9%	54.6%	9.0%

with 5 ng each of VMA, MHPG and HQ and the pH varied. The results are shown in Table II. The VMA recovery is lower at pH 3 and pH 5 where this acidic metabolite is partially ionized. As expected, the recovery of the neutral metabolite MHPG is not affected between pH 3 and pH 5. Nevertheless, a lower recovery has been noted at pH 1.5. The recovery of the internal standard is 43.5%. The use of an antioxidant such as ascorbic acid at $6.3 \cdot 10^{-5}$ *M* in the eluting buffer is necessary for constant recovery of VMA, MHPG and HQ. Other antioxidants, dithiothreitol and sodium bisulfite were tested. In our experimental conditions, dithiothreitol is not useful, since it produces a peak interfering with MHPG. Important variations in the results have been noted when using sodium bisulfite at $5.3 \cdot 10^{-3}$ *M* in the eluting buffer.

Linearity of response and detection limits

For calibration purposes, the standard curves for VMA and MHPG, added to dialysed plasma in concentrations ranging from 2 to 32 ng/ml, were prepared by plotting concentration against peak height ratio. Least squares linear regression analysis was used to determine the slope, y intercept and correlation coefficient. For VMA, $y = 0.024x + 0.003$ ($r = 0.996$); for MHPG, $y = 0.028x + 0.016$ ($r = 0.998$) where y is peak height ratio and x is the concentration in ng. Under these conditions, good linearity is obtained for both metabolites. In other experiments, a linear relationship was also obtained for these substances at concentrations up to 100 ng/ml.

The detection limit for both metabolites in spiked plasma samples, based on a signal-to-noise ratio of 2, was found to be 2 ng/ml for VMA and 1.2 ng/ml for MHPG.

Reliability and accuracy

The reliability of the assay was tested with three plasma samples for each concentration of 2, 4, 8, 16 and 32 ng/ml of both metabolites. The relative standard deviations are given in Table I.

In order to check the accuracy of the method, a technician to whom the exact concentration was unknown, did triplicate determinations of plasma samples spiked with VMA, MHPG (3 ng/ml) and with HQ (8 ng/ml) as an internal standard. The values obtained were 2.8 ± 0.23 ng/ml (mean ± S.D.) for VMA and 2.8 ± 0.28 ng/ml for MHPG.

Method validation

Using vacuum blood collection tubes containing EDTA as an anticoagulant, blood samples (7—8 ml) were collected from the antecubital vein of three volunteers after an overnight fast. Plasma was removed immediately after centrifugation and stored at —40°C. The results of triplicate determinations of free VMA and MHPG present in these samples are given in Table III. Notable individual differences are observed in the plasma level of MHPG. Our findings confirm the results reported by Dekirmenjian and Maas [7]. The MHPG average concentration in our study is almost identical to that reported by Takahashi et al. [10] with the gas chromatographic—mass fragmentographic procedure. Nevertheless, plasma levels of free VMA from our determinations are lower [9, 10].

TABLE III

MEAN PLASMA FREE VMA AND MHPG IN VOLUNTEERS

Experiments were done in triplicate.

Subject	VMA (ng/ml)	MHPG (ng/ml)
1	4.77 ± 0.14	4.87 ± 0.32
2	4.27 ± 0.007	5.85 ± 0.7
3	4.58 ± 0.225	3.15 ± 0.25

In conclusion, liquid chromatographic analysis coupled with sensitive electrochemical detection as described in this paper, combined with a simple clean-up procedure using a polyacrylamide gel provides an inexpensive method to determine the profile of endogenous catecholamine metabolites. This method also offers an attractive possibility for monitoring plasma levels of VMA and MHPG in patients with psychiatric and metabolic disorders.

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CHROMBIO. 1419

PROFILING OF URINARY MEDIUM-SIZED PEPTIDES IN NORMAL AND UREMIC URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

This report describes the profiling of medium-sized peptides in both normal and uremic urine by ion-pair reversed-phase high-performance liquid chromatography using an acetonitrile—heptafluorobutyric acid solvent system as eluent. Several medium-sized peptide peaks could be detected in both normal and uremic urine at low picomole level by using post-column fluorescence derivatization with fluorescamine. Contrary to expectation, uremic urine contained slightly larger amounts of medium-sized peptides compared with normal urine.

INTRODUCTION

In recent years, the so-called "middle molecules" (MMs) in the molecular weight range of 300—5000 as postulated in the middle molecule hypothesis [1], which would normally be removed by the kidneys, have been considered to play a major role in uremic toxicity [2]. Many authors have reported that MMs are peptidic substances [2—5].

High-performance liquid chromatography (HPLC) is an emerging new technology that is of value in the analysis and separation of peptides [6—8]. The excellent resolving power of HPLC is especially advantageous for the analysis and separation of peptides existing in urine that are present in trace amounts in complex mixtures.

It seems highly probable that some of the MMs are excreted in the urine. We have therefore attempted to profile the medium-sized peptides existing in both normal and uremic urine by HPLC.

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EXPERIMENTAL

Urine samples

The normal urine samples were obtained from three healthy subjects. The uremic urine samples were obtained from three dialysis patients who excreted about 400—900 ml of urine per day. Freshly voided urine was collected and stored frozen at −60°C until use.

Apparatus and chemicals

A Shimadzu Model LC-3A HPLC system (Shimadzu, Kyoto, Japan) was used, which included a Model SIL-1A injector, a Model SGR-1A step gradient former, a Model GRE-2B linear gradient former, a Model CRD-5A chemical reaction detector, a Model SPD -2A variable-wavelength UV detector equipped with an 8-μl flow-cell, and a Model RF-500LC spectrofluorometer equipped with a 12-μl flow-cell. Sources of polyamines and standard peptides used have been given previously [9,10]. All other reagents were obtained from Nakarai (Kyoto, Japan) and were of analytical or HPLC grade. All glassware used was siliconized.

Sample treatment

The normal urine samples were filtered through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and the uremic urine samples were filtered through a Centriflo CF-50A (Amicon, Lexington, MA, U.S.A.) which has a nominal molecular weight cut-off of about 50,000. The peptide condensation, desalting and the separation of peptides from amino acids were performed by the method of Bohlen et al. [11] with some modifications. The filtrates of urine were pumped through a LiChroprep RP-18 (Merck, Darmstadt, G.F.R.) column, 10 × 0.8 cm, at a flow-rate of 2.0 ml/min. The column was washed with trifluoroacetic acid (TFA) -water (1:99, v/v), after which the column was eluted with *n*-propanol—TFA -water (60:1:39) at a flow-rate of 2.0 ml/min. The column effluent was monitored by UV spectrophotometry at 210 nm. The eluted fraction from the column was collected and lyophilized (cross-hatched area, Fig. 1).

Ion-pair reversed-phase HPLC

The lyophilized materials (see above) were redissolved in acetonitrile—heptafluorobutyric acid (HFBA)—water (10:0.1:89.9) and injected onto a LiChrosorb RP-18 (5-μm, Merck) column, 25 × 0.46 cm. The elution was carried out with acetonitrile -HFBA—water (10:0.1:89.9) isocratically for 30 min followed by a linear acetonitrile gradient of 0.4%/min at a flow-rate of 1.5 ml/min. The column effluent was monitored by UV spectrophotometry at 210 nm or by post-column fluorescence derivatization with fluorescamine [12]. At full-scale sensitivity, about 5—10 pmol of peptides could be detected by the fluorescamine method. All chromatograms were run at room temperature.

Molecular weight distribution

The molecular weight distribution was estimated by high-performance gel

chromatography reported earlier [9,10] with the use of a TSK-GEL 2000SW column (60 × 0.75 cm; Toyo Soda, Tokyo, Japan). The elution was done with 0.05 *M* sodium phosphate buffer, pH 7.2, containing 0.3% (w/v) sodium dodecyl sulfate at a flow-rate of 0.3 ml/min. The column effluent was monitored by UV spectrophotometry at 210 nm.

RESULTS

A typical chromatogram of normal urine samples on a LiChroprep RP-18 column is shown in Fig. 1. The fraction of the cross-hatched area was collected and subjected to ion-pair reversed-phase HPLC.

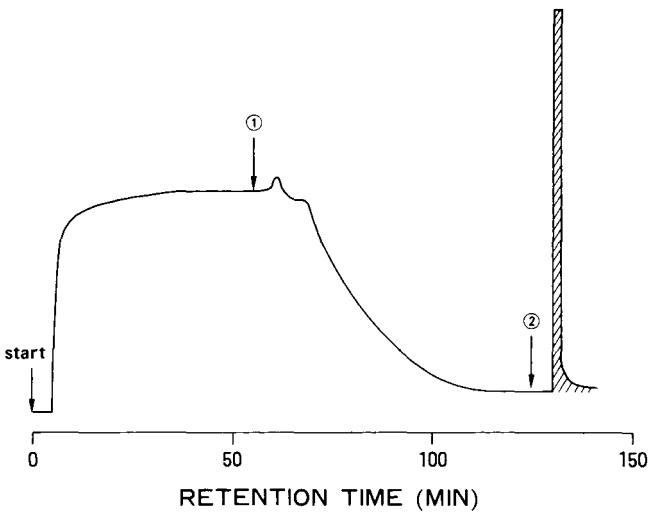

Fig. 1. Peptide condensation, desalting and amino acid removal. Arrows 1 and 2 indicate the beginning of the elution with TFA—water (1:99) and with *n*-propanol—TFA—water (60:1:39), respectively. Detection: 1.28 a.u.f.s. at 210 nm.

The retention times of standard samples chromatographed on a LiChrosorb RP-18 column are tabulated in Table I. The reproducibility of the results was better than ± 2.0% (relative standard deviation). The sample volume of injection, up to 1000 μl, had no significant effect on the result. The medium-sized peptides with molecular weights above 500 showed suitable retention times and were well separated from polyamines. The elution order of peptides seems to follow the molecular weight and hydrophobicity.

Typical elution profiles of normal urine samples, representing original urine volumes of 10 and 50 ml, are shown in Figs. 2 and 3, respectively. Fig. 2 shows that many fluorescamine-positive peaks could be well separated. The UV absorbance profile showed a similar result to that of the fluorescamine method.

The molecular weight distribution of fractions A and B (see Fig. 3) was estimated by high-performance gel chromatography. The results revealed that both fractions contained a large amount of medium-sized substances (Fig. 4).

TABLE I

RETENTION TIME AND MOLECULAR WEIGHT OF STANDARD SAMPLES CHROMATOGRAPHED ON A LICHROSORB RP-18 COLUMN

Sample	Retention time (min)	Molecular weight
Spermidine	54	145
Spermine	50	202
Leu-enkephalin	81	556
Met-enkephalin	73	574
Vasopressin	73	1084
Angiotensin I	112	1297
Angiotensin II	100	1046
Dynorphin	118	1724
Glucagon	119	3485

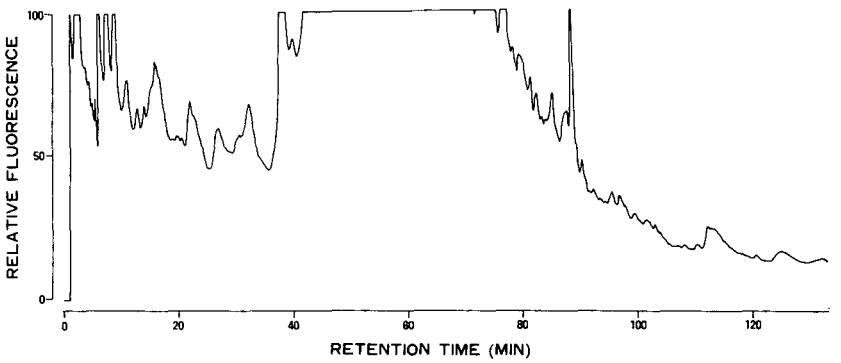

Fig. 2. Typical elution profile of normal sample, representing an original urine volume of 10 ml, chromatographed on a LiChrosorb RP-18 column. The sensitivity setting of the fluorometer was an eight-fold attenuation of the full-scale sensitivity.

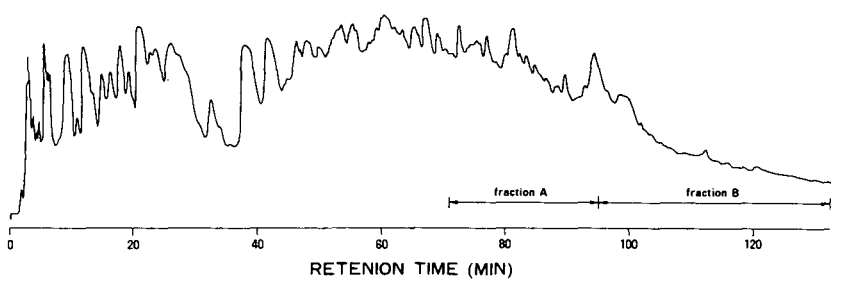

Fig. 3. Typical UV-absorbance profile of a normal sample, representing an original urine volume of 50 ml, chromatographed on a LiChrosorb RP-18 column. Detection: 1.28 a.u.f.s. at 210 nm.

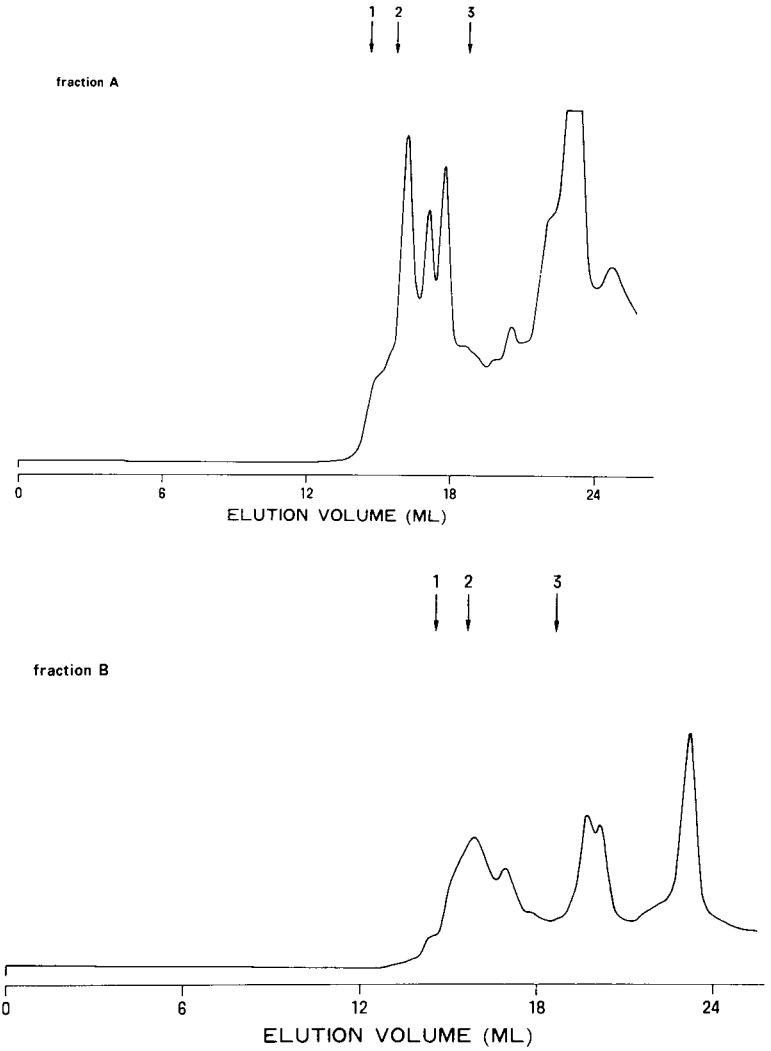

Fig. 4. The estimation of molecular weight distribution by high-performance gel chromatography. Fractions A and B (Fig. 3), representing an original urine volume of 20 ml, were used for samples. Arrows 1, 2 and 3 indicate the elution volumes of insulin, oxytocin and Leu-enkephalin, respectively. Detection: 0.64 a.u.f.s. at 210 nm.

A typical elution profile of uremic samples is shown in Fig. 5. The concentrations of medium-sized substances having a retention time over 80 min were slightly higher than those of the normal samples. However, the sample obtained from a nephrotic uremic patient showed a different elution profile (Fig. 6). No peptide peak unique to uremic or normal urines could be detected.

DISCUSSION

Until now, the separation of peptides from amino acids has been a difficult

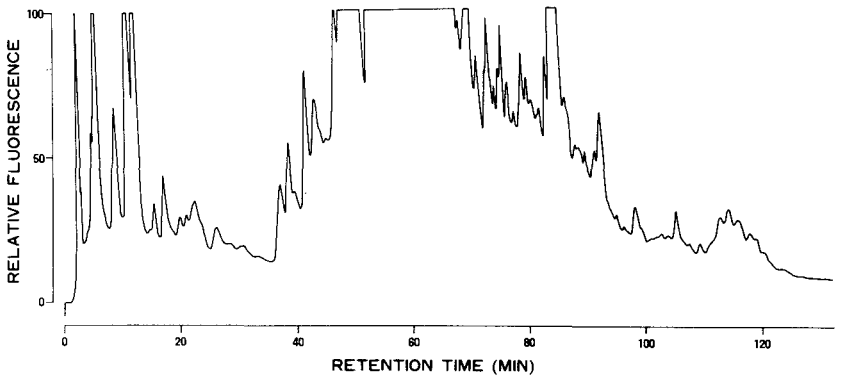

Fig. 5. Typical elution profile of a uremic sample, representing an original urine volume of 10 ml. The conditions were the same as in Fig. 2.

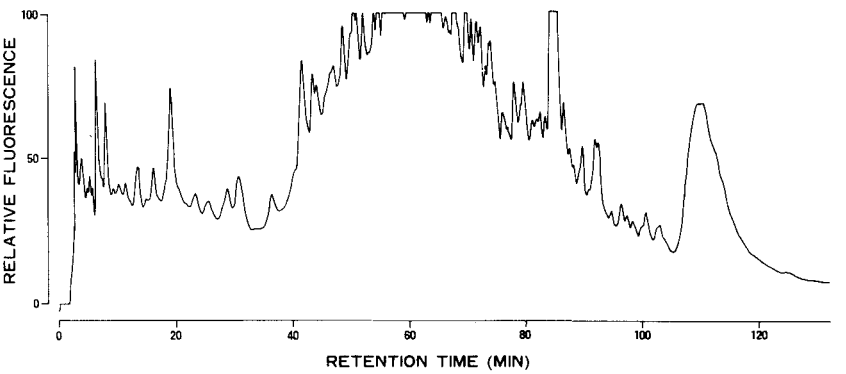

Fig. 6. Elution profile of a uremic sample, representing an original urine volume of 10 ml, obtained from a nephrotic uremic patient. The conditions were the same as in Fig. 2.

task despite a number of specific techniques that have been introduced (for example, see ref. 13). The technique of batch adsorption of peptides in a large amount of body fluids to octadecasilyl-silica particles is a very efficient first step in the concentration, desalting and separation from amino acids [11, 14, 15]. The acetonitrile—HFBA solvent system is excellent for the elution of peptides from a reversed-phase column [16, 17]. This system is volatile and allows detection of peptides at wavelengths in the range 200—220 nm.

Fluorescamine is a selective reagent for substances containing primary amino groups such as proteins, peptides and amino acids [18]. In addition, as little as 10 pmol of peptides can be easily detected.

The retention time, fluorescamine reactivity, UV absorbance characteristics and molecular weight distribution strongly indicated that most peaks existing in fractions A and B were peptidic substances. It is likely that a large number of urine samples will reveal many more medium-sized peptide peaks.

Contrary to expectation [9], the urine samples obtained from dialyzed patients whose creatinine clearance was less than 3 ml/min contained slightly

larger amounts of peptides compared with normal samples. Many peptides and low molecular weight proteins such as lysozyme, β_2-microglobulin and various peptide hormones are freely filtered through the glomeruli and removed from the luminal fluid by proximal endocytosis or luminal hydrolyzation and subsequent reabsorption [19, 20]. Therefore, it seems highly probable that the peptides in uremic urines are due to tubular dysfunction in a diseased kidney. That is to say, the peptides filtered through the glomeruli are very scarce in uremia, but most of these peptides are excreted in the urine without reabsorption and degradation. These results are consistent with the important role of residual renal function in the elimination of MMs [21]. The urine sample obtained from a nephrotic uremic patient contained a large medium-sized peptide peak, but the significance of this peak could not be elucidated in this study. This requires further study.

Further characterization, especially as to toxicity, of medium-sized peptides existing in normal and uremic urine is now in progress in our laboratory.

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CHROMBIO. 1441

STUDY OF LEUCINE-ENKEPHALIN IN RAT BRAIN BY A NEW HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

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SUMMARY

A specific method is presented for the assay of leucine-enkephalin (Leu-Enk) and its metabolites by reversed-phase high-performance liquid chromatography on a μBondapak C_{18} column with a mobile phase of methanol—water and 0.005 *M* tetrabutylammonium phosphate. The four substances are resolved by a linear program from 8 to 70% methanol in 25 min. Detection is achieved by monitoring the absorbance at 280 nm. Time of analysis can be reduced by means of a new high-speed liquid chromatography package.

This method allows the study of the effect of Phe-Ala on the cerebral metabolism of Leu-Enk. For this purpose, membrane preparations from rat striatum were incubated in the presence of [^{3}H]Leu-Enk with different concentrations of Phe-Ala from 10^{-7} to 10^{-3} *M* during 1 h at 37°C. Collected eluates were counted by liquid scintillation. The results suggest the presence of three membrane enzymes which generate the three metabolites, Tyr, Tyr-Gly-Gly and Tyr-Gly, in order of abundance. Maximum inhibition of [^{3}H]Leu-Enk degradation is obtained at a concentration of 10^{-3} *M* Phe-Ala.

INTRODUCTION

In 1975, Hughes et al. [1] isolated from pig brain two peptides with morphine-like activity at the opiate receptor level. Both of their structures corresponded to related pentapeptides differing only in the C-terminal amino acid and were accordingly named methionine- and leucine-enkephalin. Subsequently, Larsson et al. [2] demonstrated that these two peptides are localized in separate neurones in brain and intestine. Also, different studies carried out at the biological and pharmacological level suggest the occurrence of multiple types of opiate receptors: the so-called mu receptors which correspond preferentially to morphine whereas the delta receptors show a higher

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affinity for certain enkephalin derivatives, such as (D-Ala^2-D-Leu^5)-enkephalin [3]. Their different localization in various brain structures, all of them related to the cerebral cortex and limbic system, point to the possible mediation of analgesic actions by the mu receptors and emotional behaviour by the delta receptors. On the other hand, these and related peptides appear to act as neurotransmitters, being localized in specific neuronal populations where they modify neuronal activity [4] through their release upon membrane depolarization.

The identification of a mechanism responsible for the specific enzymatic inactivation of these putative neurotransmitters would provide a better knowledge of their function, especially since no specific uptake system has been found to terminate their interaction with the opiate receptors. Along these lines, it is known that both Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) can be variously cleaved by different enzymes. Soluble and membrane-bound aminopeptidases cleave the Tyr-Gly bond, carboxypeptidases work on the Phe-Met or Phe-Leu bonds whereas membrane-bound dipeptidylcarboxypeptidases, enkephalinase A [5] or ACE [6] cleave the Gly-Phe bond and a dipeptidylaminopeptidase (enkephalinase B) cleaves the Gly-Gly bond. Very recently, two membrane-bound aminopeptidases have been solubilized and characterized in rat brain [7].

Nevertheless, it is still not clear which of these enzymes, if any, may specifically inactivate both of these neuropeptides at the synaptic level. For this purpose, it would be helpful to be able to study in some detail the resulting metabolic breakdown products of the enkephalins and corresponding turnover, as well as the effects of different drugs on the activity of the responsible enzymes. This requires an analytical approach based on the isolation and unequivocal characterization of the precursor peptide and its metabolites. In this regard, high-performance liquid chromatography (HPLC) may be specially suited. The separation and analysis of a variety of neuropeptides by HPLC on reversed-phase columns has been described [8, 9]. However, no comparable work has been done on the application of these techniques to neuropeptide turnover studies, which to date have been approached through the more restrictive use of thin-layer chromatography (TLC) [10], and incomplete HPLC separation [7].

This work addresses the practical problems related to the separation of Leu-enkephalin from its metabolites; tyrosine (Tyr), tyrosine-glycine (Tyr-Gly) and tyrosine-glycine-glycine (Tyr-Gly-Gly). Due to their structural similitude, the di- and tripeptide are especially difficult to resolve chromatographically and, in fact, their separation has not been described in the literature. The significance of the separation of the parent neuropeptide and its metabolites would lie in the possibility of having access to a practical means of evaluating which of the enzymes that reportedly act on these peptides may do so specifically at the synaptic level.

The HPLC system described has been initially applied to the study of Leu-enkephalin degradation in the presence of different concentrations of the dipeptide Phe-Ala, a potent enkephalinase inhibitor.

EXPERIMENTAL

Chemicals

Tyrosine, tyrosine-glycine, tyrosine-glycine-glycine as well as leucine-enkephalin and phenylalanine-alanine were purchased from Sigma (St. Louis, MO, U.S.A.). The methanol and Pic A (commercial 0.005 *M* tetrabutylammonium phosphate solution for paired ion chromatography) were from Scharlau (Ferosa, Barcelona, Spain) and Waters Assoc. (Milford, MA, U.S.A.), respectively.

Instrumental conditions for HPLC

Leu-enkephalin was resolved from its metabolites in two different HPLC systems.

The first separations were carried out on a reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm particle size) mounted on a Model 6000 A solvent delivery system, with Model U6K universal injector and a Model 660 solvent programmer, all of them from Waters Assoc. Elution was monitored with a UV absorbance detector Model 400, also from Waters, operated at 280 nm.

Results were subsequently confirmed on a Perkin-Elmer Model 3B liquid chromatograph equipped with an LC-85 UV detector with a 2.3-μl flow-cell for high-speed HPLC [11]. Elution was also monitored at 280 nm. In this case, the column was an RP-18 (10 cm × 4.6 mm I.D., 3 μm particle size).

Chromatographic conditions

In the usual HPLC system, the four components are completely resolved by means of a 25-min linear gradient from 8 to 70% methanol. Flow-rate was adjusted to 1.5 ml/min.

In the high-speed HPLC system equipped with the 3-μm particle column, the low-strength eluent was prepared by adding 10% methanol to the 0.0005 *M* solution of tetrabutylammonium phosphate. The high-strength eluent was methanol. In this case a 4-min linear program was run from 15 to 50% of high-strength eluent at a flow-rate of 1.5 ml/min.

Preparation of the sample for HPLC

A membrane preparation of rat striatum was obtained according to the following scheme [7]. All striata of Sprague—Dawley rats were homogenized in 10 volumes of 50 m*M* Tris—HCl pH 7.4. The homogenates were centrifuged at 19,000 *g* for 20 min. The pellet was repeatedly washed with Tris—HCl buffer, resuspended and homogenized in a glass—glass potter.

The sample was centrifuged again in identical conditions as before and the pellet resuspended in 2 × 500 μl of Tris buffer and then homogenized using the same potter.

Volumes of 200 μl of the final homogenate were incubated at 37°C for 60 min in the presence of [*Tyr*-^{3}H] leucine-enkephalin and varying concentrations of Phe-Ala (10^{-7}—10^{-3} *M*).

Incubation is interrupted with 1 *M* hydrochloric acid and after centrifuga-

tion the supernatant is ready for direct injection in the HPLC system. The collected eluate fractions corresponding to the retention volumes of the peptide and the three metabolites are counted in a liquid scintillator.

RESULTS AND DISCUSSION

Fig. 1 illustrates the HPLC profile obtained using a conventional reversed-phase 10-μm particle size column. As shown, the separation of a mixture of Tyr, Tyr-Gly, Tyr-Gly-Gly and Leu-Enk is excellent but it takes more than 25 min. This, in practice, could become a disadvantage in the assay of a large series of biological samples since to the approximately 27 min of the separation one has to add the time needed for the reversed program and re-equilibration to the initial conditions, estimated at an extra 15 min per run, so that the total time comes to 42 min per injection. On the other hand, the collection of fractions for liquid scintillation counting can be substantially speeded up by resorting to a double injection of each sample, so that in the second injection Leu-enkephalin is eluted much earlier under stronger eluent conditions.

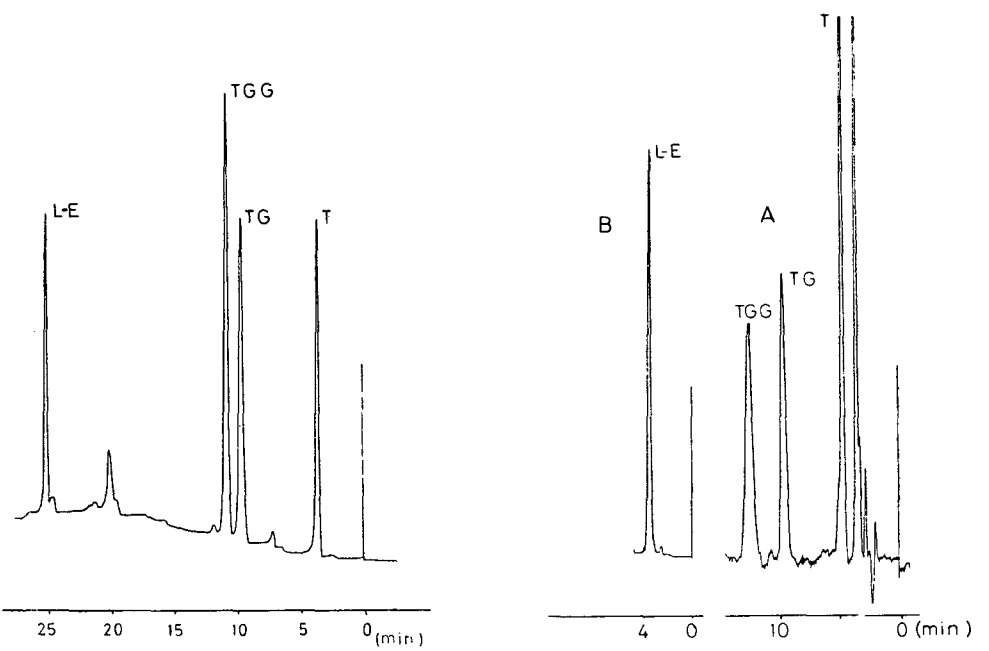

Fig. 1. HPLC profile from a sample containing known amounts of Tyr (T), Tyr-Gly (TG), Tyr-Gly-Gly (TGG) and Leu-Enk (L-E) standards. Column: μBondapak C_{18}. Linear program: 8% B to 70% B in 25 min. Flow-rate adjusted to 1.5 ml/min. Solvent A: Pic A/water. Solvent B: methanol.

Fig. 2. (A) HPLC profile from a biological sample containing added amounts of Tyr (T), Tyr-Gly (TG) and Tyr-Gly-Gly (TGG) standards. Column: μBondapak C_{18}. Isocratic conditions: Pic A/water—methanol (90:10). Flow-rate adjusted to 1.5 ml/min. (B) HPLC profile from a sample of authentic Leu-Enk (L-E). Column: μBondapak C_{18}. Isocratic conditions: Pic A/water—methanol (35:65). Flow-rate adjusted to 1.5 ml/min.

For instance, Fig. 2A shows the separation achieved injecting a biological sample spiked with Tyr, Tyr-Gly and Tyr-Gly-Gly. The eluent in this case is a mixture of Pic A with 10% methanol. Fig. 2B, on the other hand, shows the elution profile of a standard of Leu-enkephalin. The elution time has been shortened to less than 4 min by increasing the proportion of the methanol to 65%. Under these isocratic conditions (Fig. 2) the two assays can be carried out in a total combined time of 20 min, thus cutting in half the time required for the complete separation in a single injection under gradient conditions.

Consequently, a suitable scheme for the work-up of biological samples would be to divide the samples into two batches, collecting the metabolites in a first series of injections and the undegraded peptide in a second series of injections of the same samples.

The chromatographic reproducibility of retention volumes for Tyr, Tyr-Gly, Tyr-Gly-Gly and Leu-Enk, which is of importance for the appropriate collection of the corresponding eluate fractions, was calculated as 4.89 ± 0.07 ml, 13.87 ± 0.09 ml, 16.71 ± 0.21 ml and 4.57 ± 0.16 ml ($\bar{x} \pm s$, n=16), respectively.

The HPLC procedure herein described was applied to the study of the catabolism of Leu-enkephalin in rat brain in the presence of variable concentrations of Phe-Ala (10^{-7}—10^{-3} M). For this purpose, rat striatum membrane preparations were incubated with [*Tyr*-^{3}H] Leu-enkephalin and Phe-Ala. The incubates were directly injected in the liquid chromatograph and the eluent fractions corresponding to the parent peptide and metabolites were collected for radioactivity counting. The results thus obtained demonstrate the formation of three metabolites: Tyr, Tyr-Gly and Tyr-Gly-Gly. No radioactivity response could be obtained for the tetrapeptidic metabolite Tyr-Gly-Gly-Phe. Although the abundance of the three metabolites relative to the parent enkephalin varies somewhat according to the concentration of Phe-Ala, their ranking in terms of predominance does not change, as illustrated in Fig. 3.

Tyrosine, the major metabolite, invariably shows relative abundances greater than 50% in all cases, followed by the tripeptide Tyr-Gly-Gly with relative abundances of the order of 9—15%. In contrast, the dipeptide Tyr-Gly does not amount to more than 6% of the total radioactivity. Also, depending on the concentration of Phe-Ala, the recovery of unchanged Leu-enkephalin stays within 11—27%.

Another interesting observation is that these variations are not uniform across the whole concentration range of Phe-Ala. For instance, Leu-enkephalin degradation to Tyr increases when the concentration of Phe-Ala is increased from 10^{-7} M to 10^{-6} M, whereas the change from 10^{-4} M to 10^{-3} M results in less degradation with increased formation of the tripeptide. This is illustrated in Fig. 3, which depicts the individual variations of the relative amounts of each compound.

The assay of these enzymatic activities in four replicate samples run for each one of the inhibitor (Phe-Ala) concentrations shown in Fig. 3, gave coefficients of variation for the various metabolites of the order of 3—15%, reflecting an adequate intra-assay variability for biological applications.

An interesting alternative for the more rapid separation of these compounds would lie in the possibilities afforded by the modern technique of high-speed

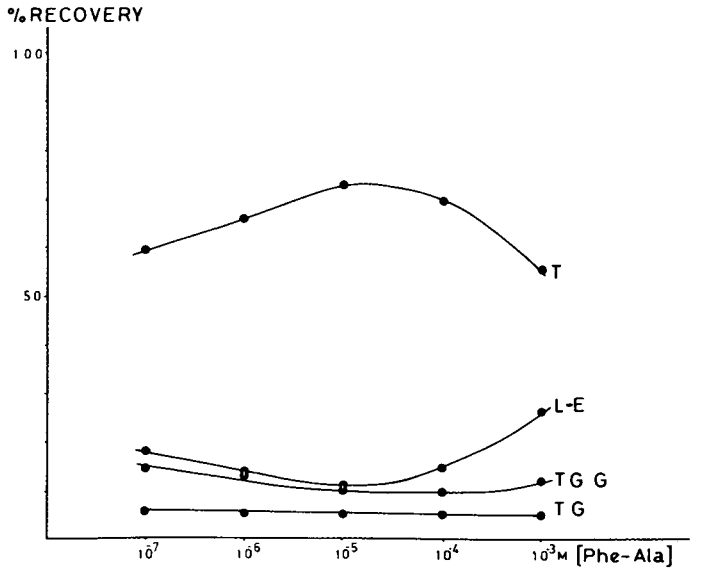

Fig. 3. Percentage recovery of Tyr (T), Tyr-Gly (TG), Tyr-Gly-Gly (TGG) and Leu-Enk (L-E) in a rat brain membrane preparation from striatum incubated with different concentrations of an enkephalinase inhibitor (Phe-Ala).

liquid column chromatography [11] carried out on specially designed instrumentation and columns of conventional diameter packed with 3-μm particles.

The results obtained by application of this new methodology to the problem posed by the separation of these four compounds are illustrated by the profile of Fig. 4. Note the substantial reduction in total analysis time, which is now of the order of 6 min plus 1 min re-equilibration of the system to initial conditions, compared to the 42 min in the conventional system; that is, a reduction of time by a factor of seven. In this manner we can inject, separate and collect the eluates corresponding to these peptides in only 7 min.

Nevertheless, the technique at present is still not free from some practical limitations like the restricted capacity of these small columns. On the other hand, this type of chromatography requires special instrumentation regarding the detector and connecting tubes in order to reduce extra column bandwidth to a level compatible with the very high efficiency of the 3—5-μm particles used to pack the columns.

The data herein presented demonstrate the convenience and speed of HPLC for the separation of Leu-enkephalin and its metabolites in biological samples. The method simplifies significantly the necessary purification process, being more specific, rapid and reliable than conventional TLC methods. Also, the individual fractions are more readily collected and counted, although presumably a radioactivity counter for TLC could also be used. Nevertheless, the speed of the HPLC method facilitates the assay of a large number of biological samples which would allow an extensive study of this particular topic in the near future.

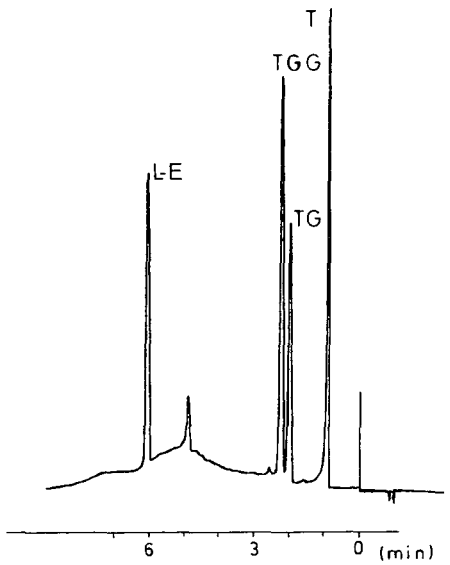

Fig. 4. HPLC profile from a sample containing Tyr (T), Tyr-Gly (TG), Tyr-Gly-Gly (TGG) and Leu-Enk (L-E) standards. Column: RP-18 (3-μm particle size). Linear program: 15% B to 50% B in 4 min. Flow-rate adjusted to 1.5 ml/min. Solvent A; Pic A/water—methanol (90:10). Solvent B: methanol.

The first results obtained in this fashion seem to confirm the absence of the tetrapeptide that would be generated by the action of a carboxypeptidase, demonstrating that the major metabolites present in these incubates are Tyr and Tyr-Gly-Gly [12]. However, the relative abundance of these two metabolites is appreciably modified by the concentration of the added Phe-Ala.

The dipeptide Tyr-Gly is always a minor component. Also, the observed predominance of Tyr indicates a greater activity of aminopeptidase compared with enkephalinase A [13].

Finally, the method is equally applicable to the study of the metabolism of Met-enkephalin since the metabolites in this case would be identical.

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CHROMBIO. 1456

METHOD FOR DETERMINATION OF ANGIOTENSIN-CONVERTING ENZYME ACTIVITY IN BLOOD AND TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simplified method for an angiotensin-converting enzyme activity assay in biological samples was developed. Samples were incubated with hippurylhistidylleucine, an artificial substrate of angiotensin-converting enzyme. The reaction was terminated by the addition of metaphosphoric acid and liberated hippuric acid in the supernatant was quantitated directly by reversed-phase high-performance liquid chromatography. Tissues were homogenized in the presence of Nonidet-P40, a detergent, and the resulting supernatant was used for the assay of tissue angiotensin-converting enzyme activity by high-performance liquid chromatography. The present procedure made it possible to determine angiotensin-converting enzyme activity in whole blood and the total activity in tissues. A comparative study of angiotensin-converting enzyme activity in plasma, kidney and lung of five experimental animals showed a high degree of variation from species to species.

INTRODUCTION

Angiotensin-converting enzyme (E.C. 3.4.15.1, dipeptidyldipeptidase) (ACE) hydrolyzes angiotensin I to give the potent vasodepressor angiotensin II. The enzyme also degrades the vasodepressor peptide bradykinin [1]. ACE was found in various tissues including lung, kidney, serum, brain and testicles [2]. Elevated activity of ACE was demonstrated in serum of patients with sarcoidosis [3] and Gaucher's disease [4]. Recently, a competitive inhibitor of ACE has been shown to be a antihypertensive drug [5, 6] and plasma ACE activity might be a useful guide to plasma level of ACE inhibitor after its administration [7]. With these current findings, a convenient and exact method for the determination of ACE is desired. For the determination of ACE, numerous compounds have been used as substrate. With physiological

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substrate, the conversion of angiotensin I to angiotensin II has been followed by radioimmunoassay [8], contractile response [9], and the determination of released histidylleucine (His-Leu) with fluorimetric coupling agents [10—13]. However, these methods are still subject to errors due to the hydrolysis of both angiotensin I and His-Leu by the other peptidases when crude tissue preparation was used as the enzyme source of ACE.

Another approach is a method using artificial substrate, usually a tripeptide with a blocked amino-terminal. Hippurylhistidylleucine (Hip-His-Leu) may be the most widely used substrate among artificial substrates. The procedure is based on the estimation of His-Leu or hippuric acid liberated from the substrate. One of the products, His-Leu, can be measured fluorimetrically after reaction with *o*-phthaldialdehyde [11—13] or fluorescamine [10]. Although these methods have been applied to the assay of ACE activity in serum [13] and brain [14], they have the disadvantage that a part of His-Leu undergoes hydrolysis during incubation [11, 14, 15]. The other product, hippuric acid, can be determined spectrophotometrically at 228 nm after its extraction [16]. However, this method is somewhat tedious; for the crude tissue preparations, problems are encountered with high background absorbance caused by the substances which are extracted into ethyl acetate together with hippuric acid. Furthermore, lipemic sera result in a turbid sample solution [11, 17]. To avoid these problems, a modification procedure using a radioactive substrate of Hip-His-Leu was recently reported [17].

In this paper, a simple and widely applicable method for the determination of ACE in biological sample is presented by the high-performance liquid chromatographic (HPLC) determination of hippuric acid liberated from Hip-His-Leu.

EXPERIMENTAL

Animals and materials

Unless otherwise stated, male Wistar rats weighing about 200 g were used. Hip-His-Leu was purchased from the Institute for Protein Research (Osaka, Japan). Nonidet-P40 was obtained from Shell Chemicals (Manchester, Great Britain). All other chemicals were commercially available of reagent grade.

Enzyme preparations

Blood was collected from the abdominal aorta of rat anesthesized with diethyl ether and put into a heparinized test tube. Tissue samples were prepared as follows. Lung and kidney were removed immediately after sacrifice and rinsed gently with chilled saline. They were chopped into small pieces and suspended in 5 volumes of chilled Tris—HCl buffer (pH 7.8) containing 30 m*M* KCl, 5 m*M* magnesium acetate, 0.25 *M* sucrose and Nonidet-P40. The suspension was homogenized and centrifuged at 20,000 *g* for 20 min at 4°C. The resultant supernatant was used as the enzyme preparation.

Standard assay method of ACE activity

A 50-μl aliquot of blood or diluted enzyme preparation was incubated in a total volume of 250 μl of 100 m*M* phosphate buffer (pH 8.3) containing

300 m*M* NaCl and 5 m*M* Hip-His-Leu for 30 min at 37°C. The reaction was terminated by adding 0.75 ml of 3% metaphosphoric acid and the mixture was centrifuged for 5 min. A 20-μl aliquot of the resultant supernatant was injected into the chromatograph. Control incubation was also carried out in the absence of Hip-His-Leu or enzyme preparation. One unit of activity is defined as the amount of enzyme catalyzing the release of 1 μmol of hippuric acid from Hip-His-Leu per minute at 37°C [16].

High-performance liquid chromatography

The following instruments were obtained from Shimadzu Seisakusyo (Kyoto, Japan): an LC-3A pump, a spectrophotometric detector SPD-2A, and a Chromatopac C-R1A recorder. Injection was achieved using a Rheodyne Model 1725 injector valve fitted with a 20-μl loop. The analytical column was 25 × 0.4 cm I.D. packed with 7.5 μm Nucleosil 7 C_{18}, Macherey-Nagel & Co. (Düren, G.F.R.). The mobile phase consisted of methanol—10 m*M* KH_2PO_4 (1:1) and adjusted to pH 3.0 with phosphoric acid (flow-rate 1.0 ml/min). Quantitation was done by using peak heights at 228 nm.

RESULTS

Determination of hippuric acid

Fig. 1A shows the chromatographic separation of 20 μl of standard mixture of 135 μ*M* hippuric acid and 5 m*M* Hip-His-Leu. Retention times were 3.5 min for the former and 5.2 min for the latter. Under the same conditions, His-Leu and benzoic acid were eluted at 2.0 min and 7.3 min, respectively. These indicated that hippuric acid could be clearly separated from the other hydrolyzed products of Hip-His-Leu.

Recovery of hippuric acid was examined by adding several protein precipitants such as metaphosphoric acid, perchloric acid, ethanol, trichloroacetic acid, and sulfosalicyclic acid. Among them, metaphosphoric acid gave a sharp peak (Fig. 1A) and good recovery (Table I) of hippuric acid. Hippuric acid was stable during the incubation for 30 min at 37°C.

Typical chromatograms obtained in the present study were shown in Fig. 1B—E where the upper diagrams showed the formation of hippuric acid after incubation of biological samples with Hip-His-Leu. No endogenous interfering substance with the determination of hippuric acid was detected even in the sample containing Nonidet-P40, a detergent used for solubilizing tissue ACE (lower diagrams).

Measurement of ACE activity in blood and tissues

As shown in Fig. 2, hippuric acid formation by serum and whole blood increased linearly with incubation time up to 60 min and with a sample volume of up to 100 μl.

ACE activity in whole blood, serum and plasma of rats measured by the present method is shown in Table II. No hemolysis was noticed during the course of incubation of whole blood with Hip-His-Leu. No difference in activity was observed between serum and plasma. The activity in whole blood was nearly equal to the predicted value both from the activity in plasma and the

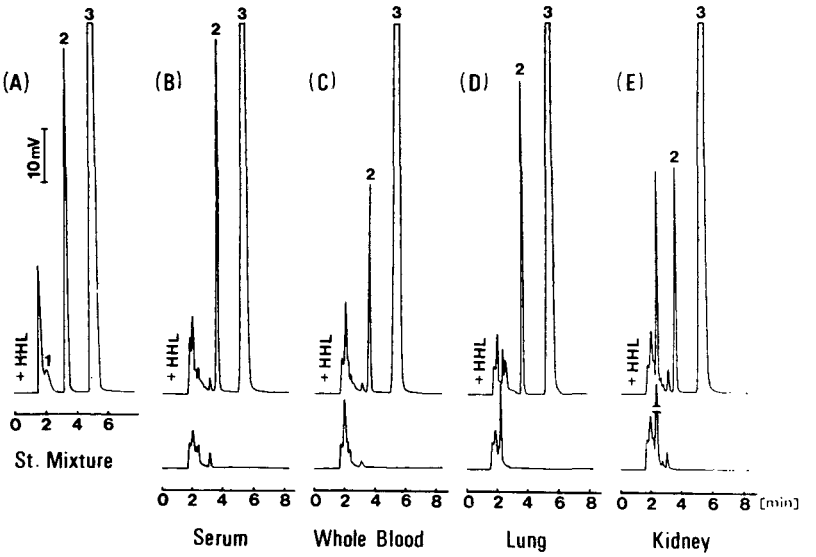

Fig. 1. Chromatograms obtained from various samples incubated with (upper diagram) or without (lower diagram) Hip-His-Leu (HHL). (A) Standard mixture of 2.7 nmol His-Leu, 2.7 nmol hippuric acid and 100 nmol Hip-His-Leu. (B) A 50-μl aliquot of serum or (C) whole blood was incubated with (upper diagram) or without (lower diagram) 5 m*M* Hip-His-Leu according to the standard ACE assay method as described in Experimental. After 30 min, 0.75 ml of 3% metaphosphoric acid was added and centrifuged. (D) Lung or (E) kidney was homogenized in 5 volumes of chilled Tris—HCl buffer containing 0.5% Nonidet-P40, and centrifuged. The supernatant was incubated with (upper diagram) or without (lower diagram) 5 m*M* Hip-His-Leu. In the case of lung, the supernatant was diluted 20 times with the buffer prior to incubation with Hip-His-Leu. Analytical conditions as described in Experimental. Peaks: 1 = His-Leu, 2 = hippuric acid, 3 = Hip-His-Leu.

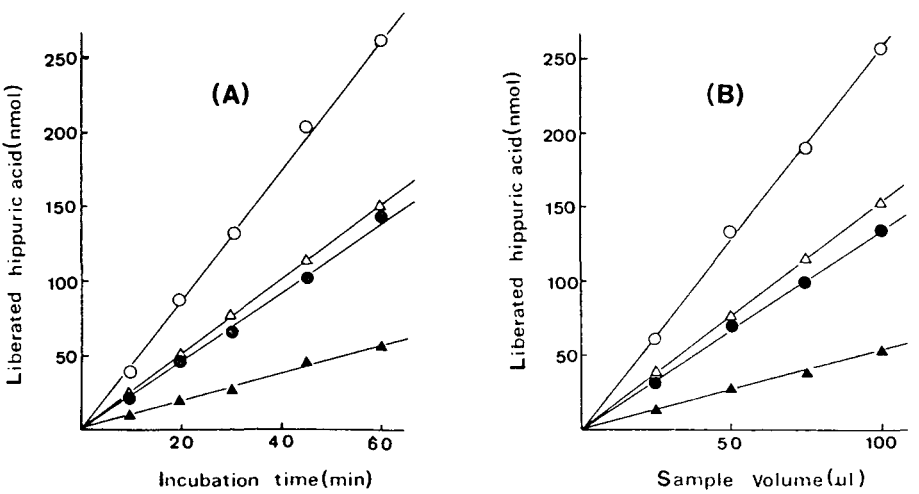

Fig. 2. Dependence of incubation time (A) and sample volume (B) of hippuric acid formation from Hip-His-Leu catalyzed by ACE in serum (○), whole blood (●), lung (△) and kidney (▲). (A) A 50-μl aliquot of sample was used; (B) incubation time was 30 min. Each point represents the mean of three determinations.

TABLE I

PERCENT RECOVERY OF HIPPURIC ACID WITH OR WITHOUT INCUBATION

To a 200-μl aliquot of 125 m*M* phosphate buffer (pH 8.3) containing hippuric acid were added 50 μl of blood or tissue sample, and then 0.75 ml of 3% metaphosphoric acid was added before or after incubation for 30 min. Hippuric acid in the supernatant was determined by chromatography under the conditions given in Fig. 1.

	Hippuric acid added (nmol)	Recovery (%)*	
		Before incubation	After incubation
Serum	75	99	102
	150	99	99
Whole blood	75	100	101
	150	97	100
Lung**	75	99	103
	150	97	98
Kidney**	75	102	103
	150	103	101

*Each value represents the mean of three determinations.
**Tissue samples of lung and kidney were prepared as described in the legend to Fig. 1.

TABLE II

ACE ACTIVITY OF SERUM, PLASMA AND WHOLE BLOOD IN RATS

Each value represents the mean of three determinations.

Rat No.	Hematocrit (%)	Liberated hippuric acid (nmol/min/ml)			
		Serum	Plasma	Whole blood*	Whole blood**
1	42	76.4	76.9	42.4	46.2
2	42	97.4	86.8	45.1	49.5
3	43	100.6	96.3	52.7	54.9
4	44	90.3	88.8	42.1	49.7
5	42	92.1	94.2	48.6	54.6

*Observed value.
**Calculated value from the activity in plasma and the estimated hematocrit.

estimated hematocrit, indicating that ACE in whole blood originates from plasma ACE.

As shown in Table III, addition of Nonidet-P40 to the homogenization medium of lung and kidney of rats resulted in a solubilization of most of the ACE activity in these tissues. Hippuric acid formation by the resulting supernatants increased linearly with both incubation time and volume of the sample (Fig. 2).

Variation of ACE activity in animals

Table IV shows ACE activity of serum, lung and kidney obtained from various species including rat, rabbit, mouse, guinea pig, and dog. The value obtained from rat lung was approximately similar to that reported by Lazo and Quinn [18] using 1-O-*n*-octyl-β-glucopyranoside as detergent. For serum ACE, the highest value was observed in the guinea pig. The decreasing order of variation was guinea pig > mouse > rat = rabbit > human > dog. On the other hand, in the lungs of animals studied, the lowest and highest ACE activities were found in the guinea pig and mouse, respectively. In kidney, high activity was found in the rabbit and mouse.

TABLE III

EFFECT OF 0.5% NONIDET-P40 ON THE EXTRACTION OF ACE FROM TISSUES

Rat lung and kidney were homogenized in 5 volumes of chilled Tris—HCl buffer (pH 8.3) with or without 0.5% Nonidet-P40 and centrifuged.

Tissue	ACE activity* (%)			
	Without Nonidet-P40		With Nonidet-P40	
	Supernatant	Pellet	Supernatant	Pellet
Lung	26.8	73.2	98.7	1.3
Kidney	16.9	83.1	90.0	10.0

*ACE activity in the supernatant and pellet fraction was expressed as percent against the sum of the values in the supernatant and pellet fractions. Each value represents the mean of three determinations.

TABLE IV

ACE ACTIVITY IN SERUM, LUNG AND KIDNEY OBTAINED FROM VARIOUS SPECIES

Values are expressed as mean ± S.D. obtained from five men and five animals.

Animal	Age or body weight	ACE activity		
		Serum (U/ml)	Lung (U/g wet wt.)	Kidney (U/g wet wt.)
Human	25—40 years	0.028 ± 0.006	—	—
Dog (Beagle ♂)	7.5 months	0.005 ± 0.001	—	—
Rabbit (albino ♂)	3.5 months	0.077 ± 0.019	7.19 ± 1.76	4.76 ± 1.82
Rat (Wistar ♂)	7.5 weeks	0.090 ± 0.021	6.02 ± 0.57	0.13 ± 0.03
Mouse (ICR ♂)	7.0 weeks	0.368 ± 0.043	12.04 ± 0.94	5.29 ± 0.29
Guinea pig (Hartler ♂)	270—310 g	1.176 ± 0.178	0.84 ± 0.44	0.14 ± 0.06

DISCUSSION

The use of HPLC for the assay of serum ACE has been reported by Chiknas [19] and Nagamatsu et al. [20], both using Hip-His-Leu as substrate. The former assay is based on the ethyl acetate extraction of hippuric acid, evaporation of the organic layer, redissolution into water and subsequent HPLC quantification of hippuric acid. The latter employed an ion-exchange column for the determination of hippuric acid in the filtrate after stopping the enzymatic reaction by boiling.

The procedure described here can be performed in a single tube; the analysis time of hippuric acid is 6 min per sample. No pretreatment other than deproteinization of the sample is required. Additionally, supernatant samples were found to be stable for at least five days at 4°C. These features are advantageous when handling a large number of samples as in the clinical screening of patients with elevated serum ACE [21], and for monitoring the effect of administrated ACE inhibitor [14]. In the latter case, there are many reports that action of a sulfhydryl group-containing ACE inhibitor, such as Captopril, changes depending on the time between blood sampling and assay of ACE activity [22—24]. This is a severe problem when an extremely low level of a highly potent inhibitor was present in the blood. There is no report using whole blood for the assay of blood ACE activity. Our observation that whole blood ACE activity directly reflects plasma ACE activity (Table II) is advantageous to evaluate in vivo action of ACE inhibitor since assay of ACE activity can start rapidly when using whole blood without having to separate serum or plasma. Furthermore, the present method can be applied to the assay of tissue ACE activity by combination with detergent. In quantitating tissue ACE, it is necessary to disrupt the cell membrane in order to solubilize ACE. Detergents such as Triton X-100 [25] and Nonidet-P40 [26] have been used widely to solubilize and isolate the membrane-bound ACE. However, these detergents have a high absorbance at 228 nm and cause uncertainty in the spectrophotometric assay of hippuric acid. The use of HPLC made it possible to determine hippuric acid even in the presence of detergent as shown in the present study.

Cushman and Cheung reported that ACE activity in 5000 *g* supernatant of tissues varied with species [27] and organs [2]. On the other hand, Soffer [1] pointed out a marked discrepancy among the reported values of the enzyme activity in tissues and the presence of methodological problems. We applied the present method to the assay of the enzyme in lung and kidney of some experimental animals, and also confirmed a high degree of species difference of the total activity in these tissues.

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CHROMBIO. 1405

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HYPOXANTHINE AND XANTHINE IN BIOLOGICAL FLUIDS

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SUMMARY

A rapid and selective reversed-phase high-performance liquid chromatographic method for the simultaneous determination of hypoxanthine and xanthine in biological fluids was developed. The identification of hypoxanthine and xanthine was confirmed by xanthine oxidase reaction. This method was applied to the investigation of purine metabolism in subjects with xanthine oxidase deficiency or gout. Hypoxanthine concentrations three to ten times higher than those determined in plasma were found in erythrocyte samples from normal subjects and from patients with xanthine oxidase deficiency or hyperuricemia under allopurinol therapy.

INTRODUCTION

Purine metabolism is complex. It is composed of five steps, one of which corresponds to purine nucleotide degradation and leads to hypoxanthine and xanthine formation. Hypoxanthine and xanthine are oxidized to uric acid by xanthine oxidase (EC 1.2.3.2.). Uric acid is the final product of purine metabolism in humans. A block of purine nucleotide degradation due to a deficiency of xanthine oxidase occurs in xanthinuria. This enzyme defect causes increased serum and urinary concentrations of hypoxanthine and

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xanthine and decreased serum and urinary uric acid concentrations. Hypoxanthine and xanthine are, therefore, the final products of purine degradation. This block also occurs during allopurinol therapy. Allopurinol, used in gouty subjects, is a potent inhibitor of xanthine oxidase.

Several methods for the determination of oxypurines (hypoxanthine and xanthine) in biological fluids have been published. Enzymatic spectrophotometric [1] and column chromatographic [2] methods have been used; but these methods presented a major disadvantage, namely they did not separate hypoxanthine and xanthine. Thin-layer chromatography [3] and gas—liquid chromatography coupled to mass spectrometry, requiring derivatization of compounds [4, 5] have also been used. Different methods have been reported for the separation of nucleosides or bases using high-performance liquid chromatography [6—15]. Ion-exchange chromatography using the possibility of ionisation of bases and nucleosides has been developed [9, 10]. Nowadays, reversed-phase systems have become the most popular method of liquid chromatography. The retention of charged molecules due to the presence of appropriate modifiers is a useful alternative to the ion-exchange method. Of the methods which have heretofore been considered, a great number did not resolve hypoxanthine from guanine [6, 7, 11—13]. Other works dis not consider the presence of guanine in the chromatographic systems [8, 14, 15]. It is important to separate hypoxanthine and guanine since, in purine metabolism, xanthine has two origins — from hypoxanthine after the action of xanthine oxidase and from guanine after the action of guanase. In xanthine oxidase deficiency, purine nucleotide degradation is disrupted. The possible presence of guanine at significant levels in biological fluids must not be ignored. Studies were principally carried out on serum or plasma [8, 12–14] but the specific analysis of hypoxanthine and xanthine in various biological fluids (especially urine and erythrocytes) has not been examined.

We therefore developed a reversed-phase method allowing a rapid and selective analysis of oxypurines in different biological fluids, without interference by purines and pyrimidines, especially guanine. Confirmation of peak identity was made by an enzymatic technique. This method was applied to the analysis of plasma, urine and erythrocytes in normal subjects, and in patients with xanthinuria and in hyperuricemic subjects undergoing allopurinol therapy.

EXPERIMENTAL

Reagents

Xanthine and hypoxanthine were purchased from Sigma (St. Louis, MO, U.S.A.), 9-methylxanthine was from Fluka (Buchs, Switzerland), and potassium dihydrogen phosphate, phosphoric acid and methanol (Uvasol) were from Merck (Darmstadt, G.F.R.).

Xanthine oxidase in suspension (activity 10 U/ml) was from PL Biochemicals (Milwaukee, WI, U.S.A.). Water throughout these experiments was deionized distilled water (Laboratoire Aguettant, Lyon, France).

Blank serum was a lyophilized control serum (serum Lyotrol N; Biomerieux, Charbonnières les Bains, France).

Apparatus and chromatographic conditions

The chromatograph was a Chromatem 800 (Touzart et Matignon, Vitry, France) equipped with a variable-wavelength absorbance detector LC—UV Pye Unicam (Philips, Bobigny, France).

The column (15 cm × 4.6 mm I.D.) was packed with Hypersil ODS 3 μm (Shandon, Cheshire, Great Britain) by the slurry packing technique as described by Coq et al. [16]. A precolumn (5 cm × 4.6 mm I.D.) used as a guard column was filled with Hypersil ODS 5 μm by the same technique. Separations were carried out using an isocratic method. The mobile phase consisted of a solution of 0.02 *M* KH_2PO_4, the pH of which was adjusted to 3.65 with phosphoric acid. The flow-rate was 1.5 ml min^{-1}; detection was performed at 254 nm.

Hypoxanthine and xanthine peaks were initially identified on the basis of their retention times. Peak identification was confirmed by the enzymatic peak-shift technique. Indeed, xanthine oxidase catalyses the following transformations

$$\text{Hypoxanthine} + O_2 + H_2O \rightarrow \text{xanthine} + H_2O_2$$

$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{uric acid} + H_2O_2$$

Plasma, urine and erythrocyte samples were incubated with xanthine oxidase at 25°C (pH 7.8) for 10 min (10 μl of xanthine oxidase + 50 μl of sample). Chromatographic profile observation before and after addition of xanthine oxidase makes it possible to confirm (or not) the presence of oxypurines in the samples.

Sample preparation

Standard solutions were prepared by dissolving hypoxanthine and 9-methylxanthine (internal standard) in water (1 mmol l^{-1}). Due to its weak solubility, xanthine was used at the concentration of 0.125 mmol l^{-1} in water. Standard solutions used to spike samples were first evaporated to dryness under nitrogen (to prevent sample dilution).

Blood (5 ml) was collected in heparinized tubes and immediately centrifuged. The plasma samples were then stored at −20°C. Erythrocytes were subjected to mild sonication and immediately analysed or stored at −20°C. A 1-ml aliquot of plasma spiked with the internal standard (25 μmol l^{-1} of 9-methylxanthine) was deproteinized by heating (2 min in a boiling water-bath). Deproteinized samples were centrifuged for 10 min at 23,500 *g* in an ultracentrifuge MSE Superspeed 65. Erythrocytes were treated in the same way.

Urine samples (24-h) were collected and stored at −20°C; 2 ml of spiked urine (25 μmol l^{-1} of internal standard) were analysed without further treatment.

RESULTS AND DISCUSSION

Chromatographic method

A preliminary study involving the separation of purine and pyrimidine bases and nucleosides led us to consider effect of the pH of the mobile phase on hypoxanthine/xanthine separation and possible interference with other com-

pounds, especially guanine. Fig. 1 shows that oxypurine separation is obtained without interference from other bases and nucleosides at pH 3.65. Separation of a standard mixture containing hypoxanthine, xanthine and 9-methylxanthine with the addition of allopurinol and guanine is shown in Fig. 2.

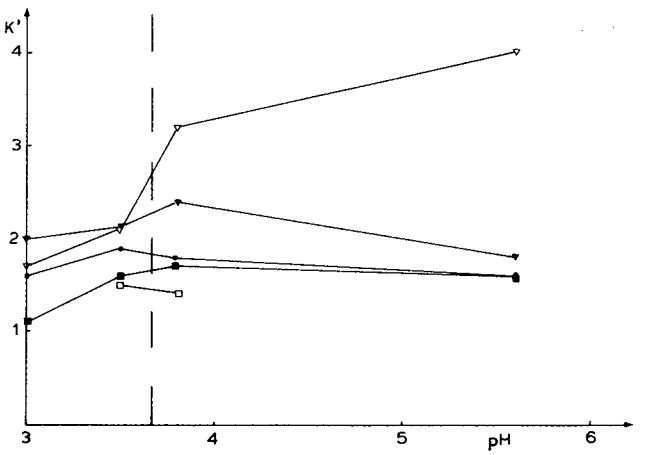

Fig. 1. Effect of eluent pH on retention of bases: (▽) adenine, (▼) xanthine, (●) hypoxanthine, (■) guanine, (□) uric acid.

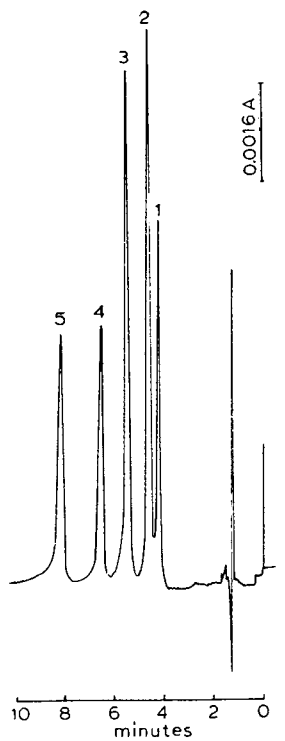

Fig. 2. Separation of guanine (1), hypoxanthine (2), xanthine (3), 9-methylxanthine (4), and allopurinol (5). Injection volume: 10 μl. Column: Hypersil ODS 3 μm. Precolumn: Hypersil ODS 5 μm. Mobile phase: 0.02 *M* KH_2PO_4 pH 3.65. Flow-rate: 1.5 ml min^{-1}. Detection: 254 nm.

Linearity (peak height versus concentration) was tested on standards in aqueous solution and in control serum spiked at various concentrations. The linearity is excellent up to 50 μmol l^{-1} for hypoxanthine, xanthine and 9-methylxanthine. Furthermore, slopes corresponding to aqueous solutions and serum are identical. This confirms that no compound of interest was lost during deproteinization.

Reproducibility and accuracy studies were performed at three different concentrations of hypoxanthine and xanthine in spiked control serum. The coefficient of variation was about 1.5%. The results are given in Table I.

The detection limit was 0.5 μmol l^{-1} for hypoxanthine and 1 μmol l^{-1} for xanthine (injection volume = 10 μl).

TABLE I

REPRODUCIBILITY AND ACCURACY OF HYPOXANTHINE AND XANTHINE ANALYSIS IN SPIKED CONTROL SERUM

	Amount added (μmol l^{-1})	Amount found (μmol l^{-1}; mean ± S.D., n = 10)	C.V. (%)
Hypoxanthine	2.5	2.51 ± 0.04	1.7
	5.0	5.01 ± 0.07	1.4
	15.0	14.95 ± 0.30	2.0
Xanthine	6.25	6.25 ± 0.07	1.2
	12.50	12.47 ± 0.05	0.4
	25.0	25.01 ± 0.25	1.0

The column used in this study has demonstrated a high degree of efficiency and a long lifetime (several hundred samples were injected on to the column for seven months). The above procedure was also applicable to the determination of hypoxanthine and xanthine in liver extracts. The use of an enzymatic technique to identify compounds provides a very specific technique. We observed in our study that hypoxanthine and guanine can be separated only at pH values below 4. This is why authors working at pH values ranging from 4.8 to 6.0 could not separate hypoxanthine from guanine.

Biological samples

This method was applied to biological samples from three normal subjects, two patients with xanthinuria and one with hyperuricemia undergoing allopurinol therapy. The results are listed in Table II. A chromatogram of control serum spiked with a standard mixture is shown in Fig. 3. Sample chromatograms of plasma, erythrocytes and urine from a normal subject are presented in Fig. 4. Sample chromatograms from a patient with xanthinuria are shown in Fig. 5.

In plasma and urine, as a consequence of the addition of xanthine oxidase, we observed a complete disappearance of hypoxanthine and xanthine peaks

TABLE II

HYPOXANTHINE AND XANTHINE LEVELS IN PLASMA, ERYTHROCYTE AND URINE SAMPLES FROM NORMAL SUBJECTS AND PATIENTS

	Normal subjects (mean conc. found, n = 3)		Patients with xanthine oxidase deficiency		Patient with hyperuricemia (allopurinol treatment)	
	Hypoxanthine	Xanthine	Hypoxanthine	Xanthine	Hypoxanthine	Xanthine
Plasma (μmol l^{-1})	3.2	2.0	24.7*	32.5*		
			43.5**	20*	6.6	26.5
Erythrocyte (μmol l^{-1})	31.8	<1	397*	<1*		
			106.2**	<1**	58	<1
Urine [μmol (24 h)$^{-1}$]	50.2	77	333*	2640*	—***	—
			399**	2620**		

*Patient S.A.
**Patient J.S.
***—, no sample.

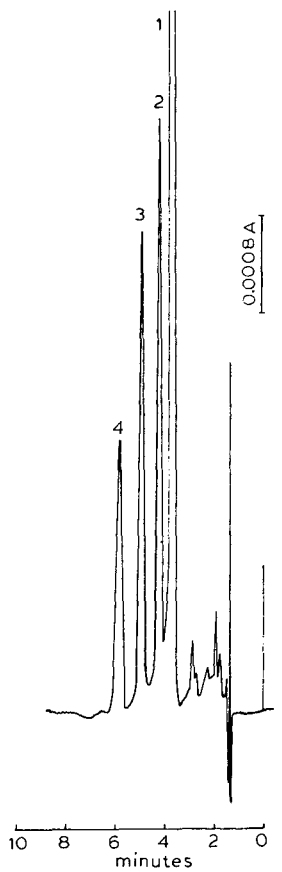

Fig. 3. Chromatogram of a control serum spiked with hypoxanthine (12.5 μmol l^{-1}), xanthine (30 μmol l^{-1}) and 9-methylxanthine (12.5 μmol l^{-1}). Peaks: 1 = uric acid, 2 = hypoxanthine, 3 = xanthine, 4 = 9-methylxanthine.

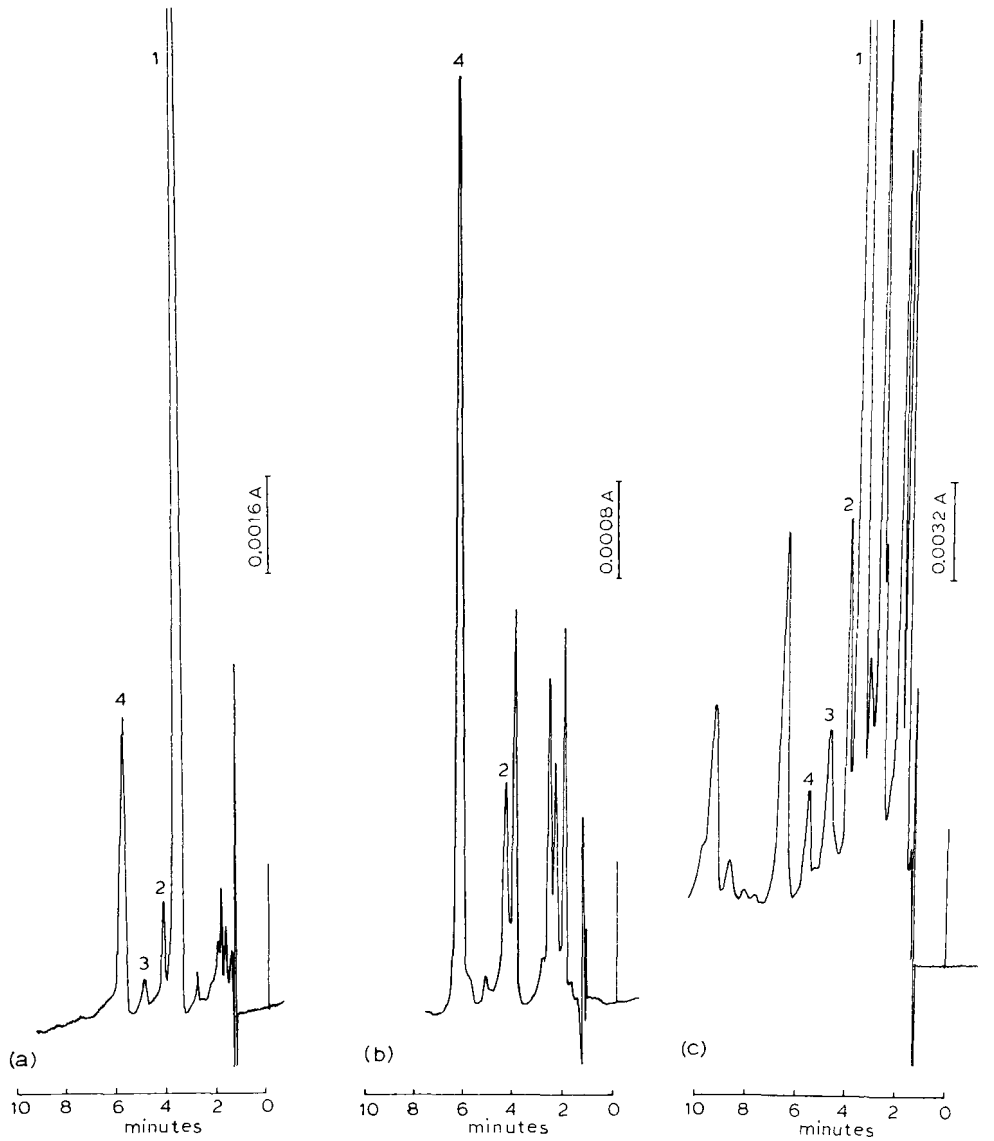

Fig. 4. Chromatograms of plasma (a), erythrocytes diluted five times (b), and urine (c) from a normal subject. Injection volume: 10 µl. Chromatographic conditions as in Fig. 2. Peaks: 1 = uric acid, 2 = hypoxanthine, 3 = xanthine, 4 = 9-methylxanthine.

and, simultaneously, an increase of the uric acid peak. For low xanthine oxidase activities, the oxidation of hypoxanthine into xanthine, and then of xanthine into uric acid, was observed successively and in that order. This result confirmed the presence of hypoxanthine and xanthine in the samples. In erythrocyte samples from patients, in spite of existence of two peaks which seemed to correspond to hypoxanthine and xanthine, enzymatic identification

showed that only hypoxanthine was present in erythrocytes, the other peak being due to the presence of an unidentified compound.

Results obtained from normal human samples agreed well with others in the literature; in serum samples, hypoxanthine concentrations ranged from 1.5 to 12.8 μmol l^{-1} and xanthine concentrations from 0.5 to 4.7 μmol l^{-1} [8]. The daily oxypurine excretion has been found by Desbois et al. [17] to range from 70 to 140 μmol $(24\ h)^{-1}$.

In patients with xanthinuria, only total oxypurine levels (hypoxanthine + xanthine) have usually been determined. They were found to range from 20 to 65 μmol l^{-1} in serum and from 700 to 4300 μmol $(24\ h)^{-1}$ in urine [17]. For the two patients studied, the sum of the values (hypoxanthine + xanthine) were in good agreement. Furthermore, our analysis being specific with regard to hypoxanthine and xanthine, makes it possible to specify that xanthine is predominant in urine whereas in plasma xanthine or hypoxanthine are found to be predominant depending on the samples. In the case of a high concentration of xanthine, therapy must be rapidly established to prevent xanthine stone formation due to the low solubility of xanthine.

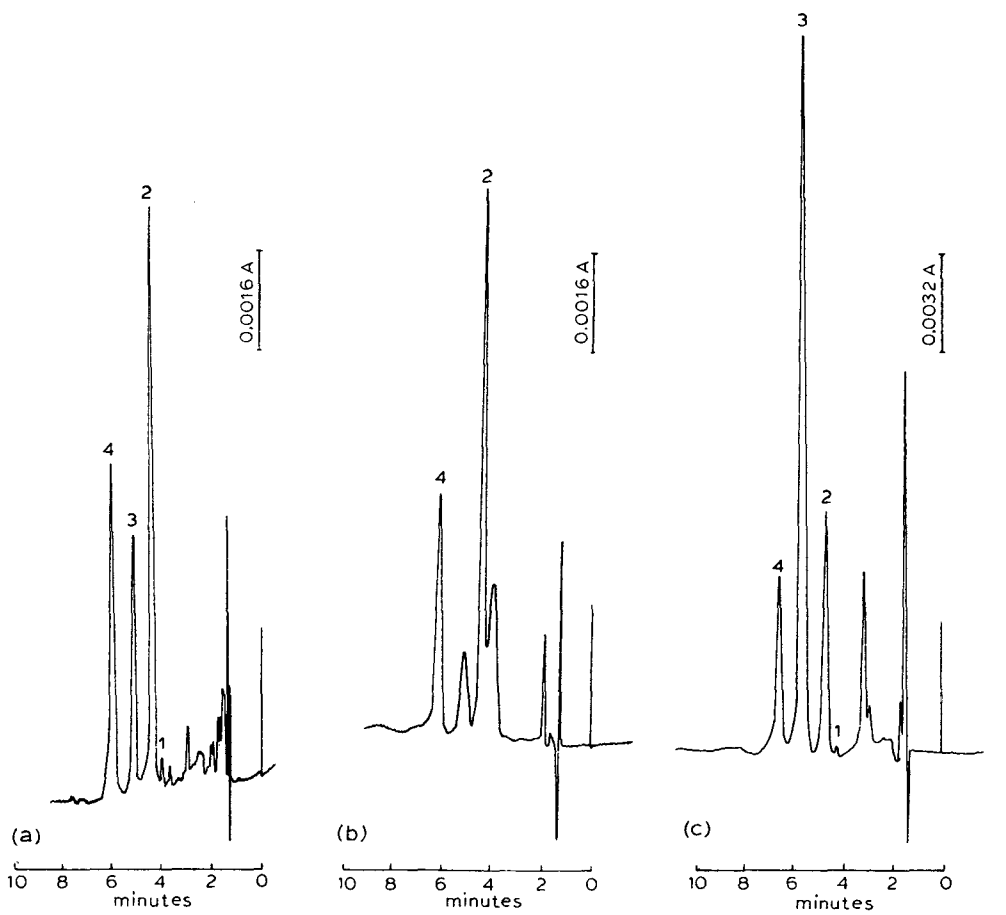

Fig. 5.

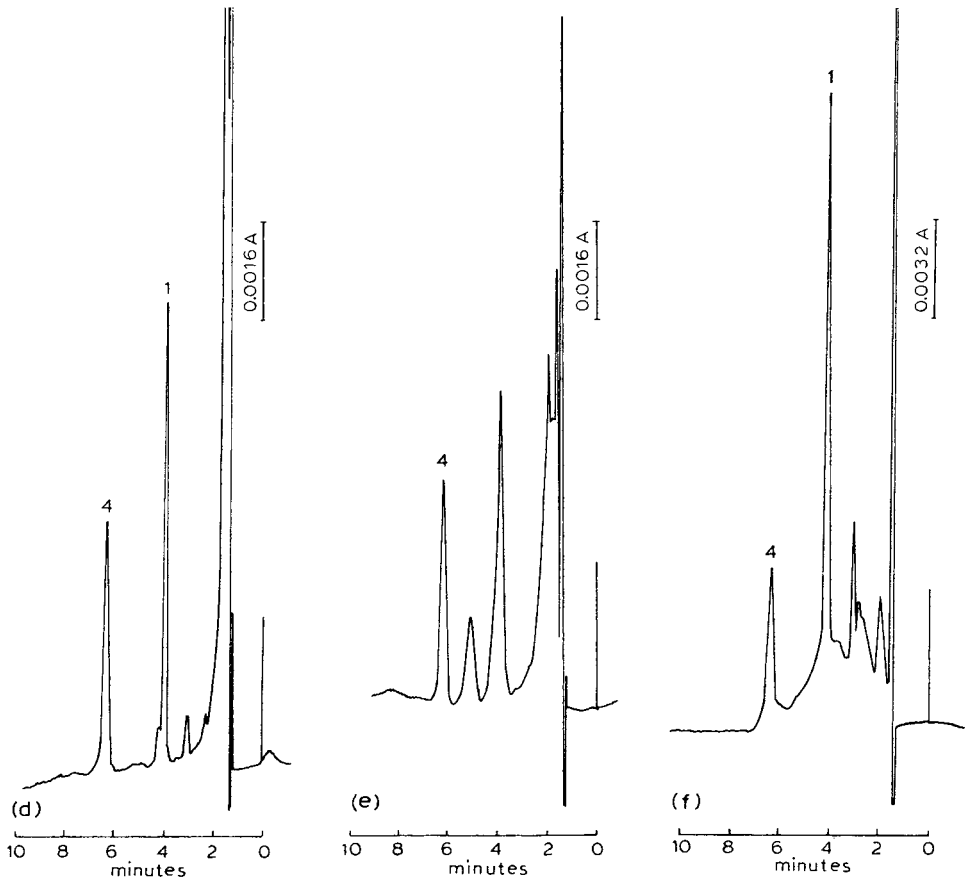

Fig. 5. (a—c) Chromatograms of samples from patient S.A. with xanthine oxidase deficiency. Plasma (a), erythrocyte diluted twenty times (b), urine diluted ten times (c). (d—f) Chromatograms of the same samples after incubation with xanthine oxidase: plasma (d); erythrocyte (e) and urine (f). Injection volume: 10 μl. Chromatographic conditions as in Fig. 2. Peaks: 1 = uric acid, 2 = hypoxanthine, 3 = xanthine, 4 = 9-methylxanthine.

For all the subjects studied, erythrocyte hypoxanthine concentrations were found to be three to ten times higher than those determined in plasma. This would explain the large differences in hypoxanthine concentrations noticed by Wung and Howell [13] in plasma, when the formed elements of blood were not immediately separated. Marz et al. [18] have found a rapid transport of hypoxanthine in cultured cells; they have shown that, at high or low concentrations of extracellular hypoxanthine, the steady-state concentrations of hypoxanthine in the cells approached those in the extracellular medium. Our results show that erythrocytes behave differently: they seem able to accumulate very large amounts of hypoxanthine. Furthermore, we have shown a specificity in the accumulation phenomenon of hypoxanthine: in spite of its non-negligible levels

in plasma there is no xanthine in erythrocytes. At the present time, this mechanism of accumulation is not known; it is under investigation in our laboratory.

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TWO SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR SIMULTANEOUS DETERMINATION OF 2'-DEOXYCYTIDINE 5'-TRIPHOSPHATE AND CYTOSINE ARABINOSIDE 5'-TRIPHOSPHATE CONCENTRATIONS IN BIOLOGICAL SAMPLES

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SUMMARY

Cytosine arabinoside (ara-C) has been used in the treatment of leukemia, but its exact mechanism of cytotoxicity is not yet known. One of the proposed mechanisms for the effectiveness of this drug in treating leukemias suggests that a metabolite of ara-C, i.e., 2'-deoxycytidine 5'-triphosphate (araCTP), competes with cytosine arabinoside 5'-triphosphate (dCTP) for binding to DNA polymerase. The ratio of the drug metabolite to the endogenous nucleotide (araCTP/dCTP) may, therefore, be important in determining the effectiveness of ara-C therapy. This ratio may also play a role in drug resistance. Previously published methods have focused on either araCTP or dCTP, along with metabolites and analogues of one of these compounds. The methods presented here provide two simple, sensitive ways to measure dCTP and araCTP in the same biological sample.

INTRODUCTION

It has been proposed that metabolites of cytosine arabinoside (ara-C), used in the treatment of leukemia, inhibit cell proliferation by several different mechanisms [1—3]. One of these mechanisms, i.e. competition by cytosine arabinoside 5'-triphosphate (araCTP) for the binding of 2'-deoxycytidine 5'-triphosphate (dCTP) to DNA polymerase, suggests that the araCTP/dCTP ratio may play an important part in determining the effectiveness of the drug. Until now, direct measurement of the levels of dCTP and araCTP in human white blood cells has been limited. Because the amount of sample obtained from patients is usually small, most studies have been done with cultured cell lines [4] and with cells from bone marrow aspirates, incubated with radioactive compounds [5]. With the advent of high dose ara-C therapy (HDARA-C) in leukemia [6], however, it has become advantageous to measure

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directly the levels of dCTP and araCTP in human white blood cells from bone marrow samples. Simultaneous measurement of these two compounds permits assessment of the importance of the ratio of araCTP/dCTP in the effectiveness of ara-C therapy, and minimizes the problem of small sample size.

Methods are available for determining concentrations of either araCTP or dCTP and related compounds [5, 7]; but a simple method for the simultaneous detection and quantitation of both compounds has not been reported. This paper reports two high-performance liquid chromatographic (HPLC) methods by which the concentrations of dCTP and araCTP can be measured in the same biological sample.

MATERIALS AND METHODS

HPLC grade $NH_4H_2PO_4$ was purchased from Fisher Scientific (St. Louis, MO, U.S.A.). Methanol was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Lymphoprep was purchased from Pharmacia (Piscataway, NJ, U.S.A.). All nucleotides and nucleosides used as chromatographic standards, and all other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Clinical samples

Refractory pediatric leukemic patients were treated with HDARA-C, i.e., 3.5 g/m^2/day by constant infusion over four consecutive days. Bone marrow aspirates or peripheral blood samples were taken prior to, 24 h after, and 72 h after the infusion of HDARA-C was begun. Heparin was used as anticoagulant in the collecting tubes. Mononuclear cells and blasts were then separated by the Ficoll—Hypaque method [8]. The number of cells in suspension was determined on a Model ZBI Coulter Counter. The cell samples were treated with perchloric acid (0.5 *N*), neutralized with potassium hydroxide, and then centrifuged to remove the potassium perchlorate precipitate [9]. The acid soluble supernatant fractions were stored at −70°C.

Preparation for chromatography

Immediately before analysis, the samples were treated by the periodate oxidation method of Garrett and Santi [10] to remove ribonucleotides which interfere with the resolution of dCTP and araCTP. This method quantitatively removed peaks of CTP and UTP in standard solutions from this area of the chromatogram.

HPLC

The high-pressure liquid chromatograph used was a Beckman (Altex) Model 332, with a Partisil SAX 10 anion-exchange column (25 cm × 4.6 mm).

Method I. The mobile phase was 500 m*M* $NH_4H_2PO_4$ (pH 3.5) with 2% methanol; the flow-rate was 0.5 ml/min. The methanol was added to the buffer at the beginning of each day and was used for one day only, because the concentration of methanol was critical. The solution was filtered and kept under vacuum for at least 30 min if the most sensitive scale, 0.005 absorbance units full scale (a.u.f.s.), was used, or baseline drift made interpreta-

tion of the graphs difficult. The difference in retention times of araCTP and dCTP was approximately 1.5 min.

Method II. The method used a gradient system and separated dCTP and araCTP peaks by greater than 2 min. The buffers used were: Buffer A, 150 m*M* $NH_4H_2PO_4$ (pH 3.5); Buffer B, 750 m*M* $NH_4H_2PO_4$ (pH 3.5) with 5% methanol. The flow-rate was 0.5 ml/min. The gradient was programmed into an Altex Microprocessor as follows:

Step	Time (min)	%B Buffer
1	0—10	0 → 50
2	10—20	50 → 10
3	20—50	10 → 100
4	50—60	100 → 0

If the descending gradient in Step 2 was omitted, dTTP coeluted with araCTP. After the program was completed, the baseline was allowed to stabilize with 100% Buffer A for 15 min before injection of the next sample.

The elution profiles obtained by both methods were monitored at 254 nm and 280 nm and the peak areas of the output of the 254-nm detector were quantitated by a Shimadzu Model E1B integrator. Because the detectors are connected in series, the peak corresponding to absorbance at 254 nm precedes the peak at 280 nm by several seconds. Quantitation of dCTP and araCTP by standard calibration curves based on measurements of peak heights and calculated peak areas agreed with the values obtained with the integrator at 0.01—0.16 a.u.f.s. sensitivity settings. Peak height measurement was a more reliable criterion for accurate measurements at the 0.005 a.u.f.s. sensitivity setting.

RESULTS

Figs. 1 and 2 illustrate the separations obtained by Method I with a standard solution of deoxyribonucleoside triphosphates (dNTP) and with a patient sample, equivalent to $4 \cdot 10^6$ cells, after treatment with ara-C. The sensitivity setting for each of these tracings was 0.01 a.u.f.s. As shown in Fig. 2, when the intracellular level of araCTP is extremely high as compared to dCTP, separation between these two compounds appears less complete than with standard solutions. This does not occur when concentrations of these compounds are more equal (as has been the case with most samples in our study); even when resolution appears less than optimal, quantitation by peak height measurement and computer calculation is reproducible and gives the same value (± 10%) as Method II.

The separations obtained with the gradient system of Method II are shown in Fig. 3 with an aliquot of the same standard solution as in Fig. 1, and in Fig. 4 with an aliquot of the same patient sample as in Fig. 2, equivalent to $5 \cdot 10^7$ cells. The sensitivity setting for each of these tracings was 0.08 a.u.f.s.

Retention times for dNTP standard solutions (Table I) were the same as retention times for these compounds when patient samples were injected and were reproducible ± 0.21 min for dCTP and araCTP for all patient samples.

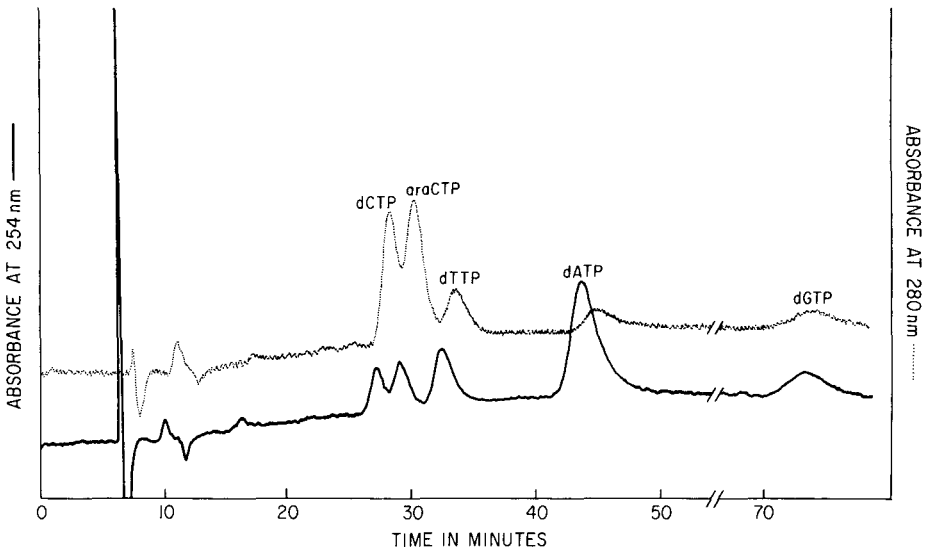

Fig. 1. Chromatogram (Method I) of a standard solution of deoxyribonucleoside triphosphates and araCTP. Peaks represent 300 pmole of each compound.

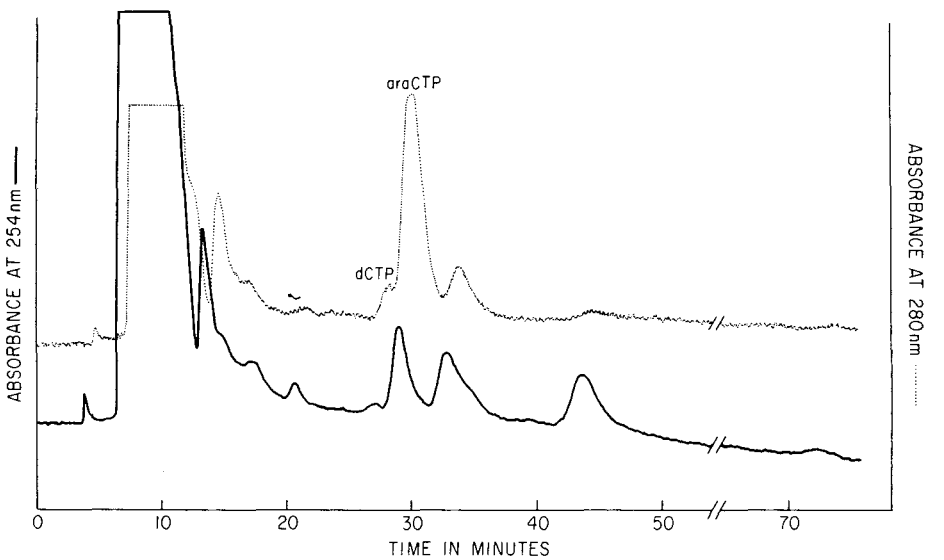

Fig. 2. Chromatogram (Method I) of acid-soluble fraction of the equivalent of $4 \cdot 10^6$ cells of a patient treated with HDARA-C therapy.

All other known metabolites of dCTP and araCTP elute within the first 20 min of the chromatogram by both methods [11—13]. While it is not impossible that unknown metabolites of these compounds are formed within the cell, it is unlikely that retention times and 254/280 absorbance ratios of both compounds in each system would remain constant and give equivalent results if significant contamination were present. These observations indicate that

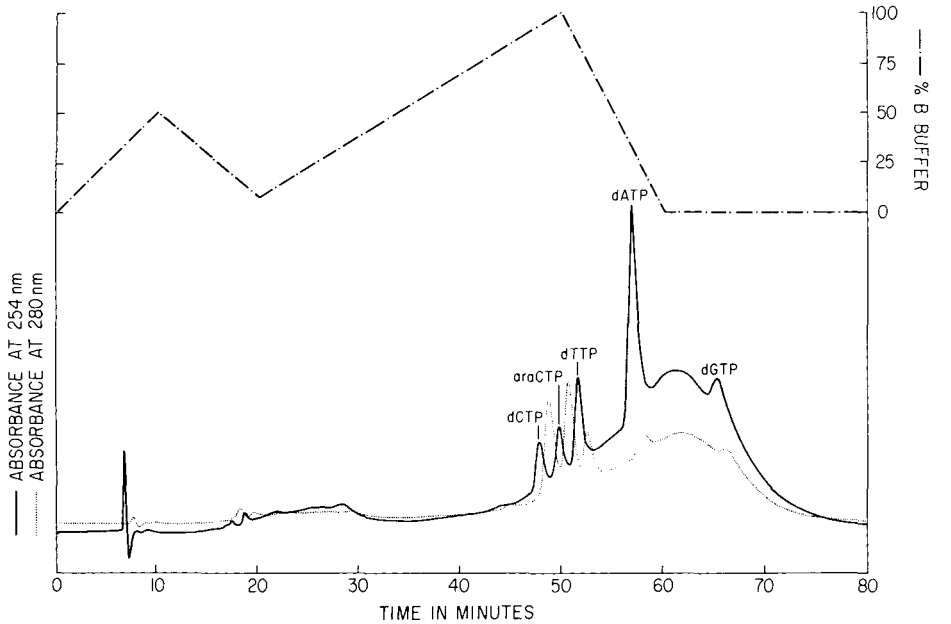

Fig. 3. Chromatogram (Method II) of a standard solution of deoxyribonucleoside triphosphates and araCTP. Peaks represent 2 nmole of each compound.

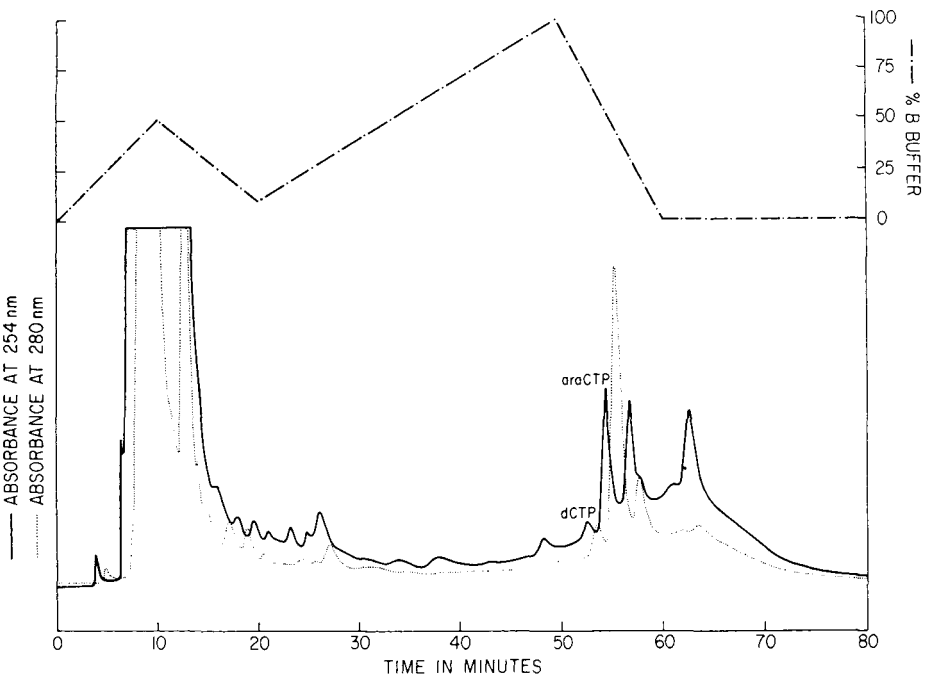

Fig. 4. Chromatogram (Method II) of acid-soluble fraction of the equivalent of $5 \cdot 10^7$ cells of a patient treated with HDARA-C therapy.

TABLE I

RETENTION TIMES OF dNTPs AND araCTP

	Compound	Retention time (min)
Method I	dCTP	26.81
	araCTP	28.63
	dTTP	32.25
	dATP	43.25
	dGTP	72.24
Method II	dCTP	47.82
	araCTP	51.96
	dTTP	53.82
	dATP	61.96
	dGTP	66.06

neither dCTP nor araCTP coeluted with other unidentified compounds. (The dTTP peak in patient samples appeared to coelute with another compound, possibly araUTP, which was not identified.) The dCTP concentrations were in the same range as those reported in cultured cells (ca. 25 pmole/10^6 cells) [4].

DISCUSSION

Both methods outlined in this paper gave quantitative, reproducible, simultaneous measurements of dCTP and araCTP in all samples studied. The number of cells required to give detectable levels of dCTP and araCTP by these methods will undoubtedly be related to dose and schedule of administration of ara-C as well as the time at which the tissue sample is taken. With the HDARA-C protocol given at this institution, dCTP and araCTP were both measureable in the 0.005 a.u.f.s. sensitivity range when the equivalent of $1.5—5.0 \cdot 10^6$ cells was injected onto the column.

Method I has the advantage of being an isocratic system, which allows instrument settings of maximum sensitivity with no baseline drift. This method has been useful in detecting the low levels of dCTP in the cells of patients prior to treatment with ara-C.

Method II has been most useful in evaluating samples of cells from patients who have been given HDARA-C. Using this method, the dCTP peak was separated from the araCTP peak by greater than 2 min, and so resolution of the two compounds was better than with Method I. Alterations of the methanol gradient, buffer concentrations, pH of one or both buffers, and flow-rate all gave poorer separations of dCTP and araCTP than the methods described. Baseline drift was evident at $NH_4H_2PO_4$ concentrations above 500 m*M* (Figs. 3 and 4). The integrator was programmed to correct for the change in baseline, and results based on integrated area units and manually measured peak heights and peak areas were equivalent. Quantitation of araCTP and dCTP concentrations was not impaired by the baseline drift. When standards were run in order to graph peak height or area vs. concentration of nucleotide,

the correlation coefficients of the lines generated were all between 0.989 and 0.999 for each compound for Method I and Method II. Further, if a known amount of standard was added to a patient sample as a tracer, and the sample and standard were injected onto the column together, the appropriate peak was increased by the predicted amount.

Each method outlined here gives adequate separation of dCTP and araCTP and has the added advantage of delaying elution of each of these peaks long enough to allow quantitation of these compounds in biological samples. When the equivalent of 10^5—10^7 cells is first oxidized and then injected onto an HPLC column, cell debris may obscure peaks which elute in the first 8—15 min of the run. (The exact time to reestablish the baseline after elution of debris will depend upon solvent, flow-rate, etc.) With each method reported here, elution is delayed long enough to circumvent this problem.

Additionally, Method II has the advantage of allowing quantitation of the deoxynucleoside diphosphates in the cell (data not shown). This is done simply by lengthening the 10-min gradient in Step 1 to a total of 30 min. In this way, deoxynucleoside diphosphates are separated from each other by a greater time interval than with an initial 10-min gradient, and separation and resolution of dCTP and araCTP are not adversely affected. Cell debris usually obscures the peaks of some of the deoxynucleoside monophosphates. While quantitation of deoxynucleoside diphosphates, as well as triphosphates, offered no advantage for our HDARA-C study, it may be useful in assessing other drug protocols.

Other possible methods for separating dCTP and araCTP include reversed-phase techniques with ion-pairing and anion-exchange techniques with either a $Na_2B_4O_7$—NH_4Cl gradient or a sodium citrate gradient [14—16]. Reversed-phase methods have usually been unsatisfactory because nucleotides elute very quickly and peaks of dCTP and araCTP are obscured by cell debris [17]. The method of Knox and Jurand [14] might possibly be adapted to measure araCTP and dCTP; but the approximate 30-min retention time (vs. a 26-min retention time for dCTP for Method I) of nucleotides offers no obvious advantage over the methods described here. Alternatively, other anion-exchange methods are available which could probably be adapted to separate dCTP and araCTP [15, 16]. If either of these methods could be adapted to give good resolution of araCTP and dCTP, it would have the benefit of simultaneous detection of bases, nucleosides, and nucleotides in the same sample. It would also have the disadvantage of taking over 2 h to run a single sample.

Obviously, each method has advantages and disadvantages which are related to time and equipment requirements, as well as compounds of interest other than dCTP and araCTP. This paper provides two simple, relatively short, reliable methods for quantitating dCTP and araCTP in biological samples.

ACKNOWLEDGEMENTS

The author wishes to thank Drs. Peter J. Houghton and Vernon L. Verhoef for their help, and Drs. Dewayne Roberts and Arnold D. Welch for their encouragement and helpful discussions. Dr. Judy Ochs and Susan Hilliard are also thanked for obtaining patient samples.

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CHROMBIO. 1435

DETERMINATION OF (−)-*threo*-CHLOROCITRIC ACID IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—POSITIVE CHEMICAL-IONIZATION MASS SPECTROMETRY

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SUMMARY

A method is described for measuring (−)-*threo*-chlorocitric acid in human plasma. Plasma is acidified to pH 1 to minimize lactonization and a ^{13}C analogue of (−)-*threo*-chlorocitric acid is added as internal standard. The acidified plasma is then extracted with ethyl acetate containing 10% methanol. The ethyl acetate—methanol extract is back-extracted with acetate buffer (pH 5). This extract, following adjustment to pH 1, is reextracted with ethyl acetate. The residue after removal of the ethyl acetate is treated with ethereal diazomethane. The wet residue is reconstituted in ethyl acetate and a portion of this solution is analyzed by gas chromatography—chemical ionization mass spectrometry. The mass spectrometer is set to monitor *m/z* 269 [MH^+ of trimethylated (−)-*threo*-chlorocitric acid] and *m/z* 270 [MH^+ of trimethylated (−)-*threo*-[^{13}C]chlorocitric acid] in the gas chromatographic effluent. The *m/z* 269 to *m/z* 270 ion ratio in a sample containing an unknown amount of (−)-*threo*-chlorocitric acid is converted to an amount of compound using a calibration curve. The calibration curve is generated by analyzing control plasma spiked with various known amounts of (−)-*threo*-chlorocitric acid and a fixed amount of (−)-*threo*-[^{13}C]chlorocitric acid. The limit of quantitation is 0.1—0.6 $\mu g\ ml^{-1}$, depending on the characteristics of the calibration curve generated with each set of samples. The precision (relative standard deviation) at a concentration of 2 $\mu g\ ml^{-1}$ is 3.3%.

INTRODUCTION

(−)-*threo*-Chlorocitric acid is currently undergoing testing as an anorectic agent [1—3]. This paper reports a specific and relatively simple gas chromatographic—mass spectrometric (GC—MS) procedure for (−)-*threo*-chlorocitric acid. The assay features the use of a ^{13}C-labeled analogue of (−)-*threo*-chlorocitric acid as internal standard and the GC- chemical ionization MS (CIMS)

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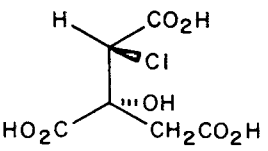
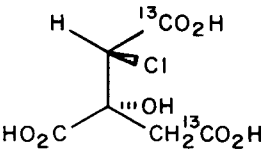
analysis of both (−)-*threo*-chlorocitric acid and its internal standard as their respective methyl tri-ester.



(−)-*threo*-chlorocitric acid



(−)-*threo*-[^{13}C]chlorocitric acid (50% labelled at each position)

EXPERIMENTAL

Equipment and operating conditions

Gas chromatography. A Finnigan Model 9500 gas chromatograph was equipped with a glass U-shaped column (152 cm × 2 mm I.D.) packed by Whatman (Clifton, NJ, U.S.A.) with OV-17 on μPartisorb®. The column was conditioned overnight at 295°C with a nitrogen flow-rate of 10 ml min^{-1}. The temperatures of the injector, column oven, interface oven and transfer line were 300, 170, 250 and 250°C, respectively. Methane (1.5 kg cm^{-2}) was used as carrier gas. The retention time of derivatized (−)-*threo*-chlorocitric acid under these conditions was approximately 95 sec.

Mass spectrometry. A Finnigan Model 3200 quadrupole mass spectrometer was tuned to give the maximum response consistent with reasonable ion peak shape and unit resolution. Methane (68 Pa) was used as CI reagent gas. The voltage across the continuous dynode electron multiplier was −2.0 kV and the voltage on the conversion dynode was +2.5 kV.

Peak monitor. A Finnigan Promim® was used to set the mass spectrometer to monitor *m/z* 269 and *m/z* 270. Each Promim channel was operated at 100 msec dwell-time, a 0.5-Hz frequency response, and a sensitivity of 10^{-8} A/V. Ion chromatograms were recorded on a four-channel Rikadenki pen recorder.

Glassware. Culture tubes (16 ml, Pyrex 9825), with Teflon®-lined screw caps, were used for plasma extraction and the ethyl acetate back-extraction. Conical centrifuge tubes (5 ml, Pyrex 8061) were used for the evaporation of the final solvent extract and subsequent reaction with diazomethane. Prior to use, all tubes were cleaned with detergent, rinsed with water, treated with Siliclad® (Clay Adams, Parsippany, NJ, U.S.A.), and finally rinsed with methanol and dichloromethane (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Solvent evaporator. Solvents were removed at 35°C using a nitrogen evaporator (N-Evap, Organomation Assoc.).

Shaker. Extractions were carried out by shaking (60 strokes min^{-1}) on a variable-speed reciprocating shaker (Eberbach).

Centrifuge. Centrifugations were done at 10°C using a Damon/IEC Model CRU-500 refrigerated centrifuge operated at 1600 *g*.

Chemicals

(−)-*threo*-Chlorocitric acid and (−)-*threo*-[^{13}C]chlorocitrate were obtained

from Dr. W. Scott and Dr. C.W. Perry, respectively, Hoffmann-La Roche, Nutley, NJ, U.S.A. Nanograde ethyl acetate and methanol were obtained from Burdick and Jackson Labs., Muskegon, MI, U.S.A. Glacial acetic acid, 12 *N* hydrochloric acid and sodium acetate were obtained from J.T. Baker, Phillipsburg, NJ, U.S.A. Diazald® and ethyl ether were obtained from Aldrich, Milwaukee, WI, U.S.A. and Mallinckrodt, St. Louis, MO, U.S.A., respectively.

Solutions

(−)-threo-Chlorocitric acid, stock solution. Dissolve 1.00 mg of titled compound in 1.00 ml of methanol.

(−)-threo-Chlorocitric acid, spiking solutions. Using the stock solution, prepare four solutions containing either 0.5, 2.5, 5 or 10 μg of the titled compound per 50 μl of methanol (solutions A, B, C, D, respectively).

(−)-threo-[^{13}C] Chlorocitric acid, stock solution. Dissolve 1.00 mg of the titled compound in 1.00 ml of methanol.

(−)-threo-[^{13}C] Chlorocitric acid, spiking solution. Using the stock solution, prepare one solution containing 5 μg of the titled compound per 50 μl of methanol (solution E).

10% methanol in ethyl acetate. 100 ml of methanol is diluted to 1 l with ethyl acetate.

0.2 M pH 5 acetate buffer. 16.41 g of sodium acetate and 5.2 ml of glacial acetic acid are dissolved in 1000 ml of distilled water.

6 N HCl. Dilute 1 volume of 12 *N* HCl with 1 volume of distilled water.

2 N HCl. Dilute 1 volume of 12 *N* HCl with 5 volumes of distilled water.

Ethereal diazomethane. Ethanol-free ethereal diazomethane is prepared from Diazald® using the manufacturer's suggested procedure.

Procedure

The calibration curve samples are prepared as follows. Transfer a 50-μl aliquot of methanol containing either 0, 0.5 μg (solution A), 2.5 μg (solution B), 5 μg (solution C) or 10 μg (solution D) of (−)-*threo*-chlorocitric acid to 16-ml culture tubes, followed by another 50-μl aliquot of methanol containing 5 μg of (−)-*threo*-[^{13}C] chlorocitric acid (solution E). Add 0.5 ml of 2 *N* hydrochloric acid and 1.0 ml of control human plasma and vortex each mixture thoroughly.

Spike the plasma samples (1 ml) containing unknown amounts of (−)-*threo*-chlorocitric acid with 5 μg of (−)-*threo*-[^{13}C] chlorocitric acid and then acidify and vortex them as described for the calibration curve samples.

Extract samples by shaking for 10 min with 5 ml of ethyl acetate containing 10% methanol. Centrifuge the tubes; transfer 4 ml of the top layer to another culture tube and back-extract by shaking the tubes for 5 min with 0.5 ml of 0.2 *M* acetate buffer pH 5. Centrifuge the tubes and discard as much of the organic layer as possible. Add 2 ml of ethyl acetate containing 10% methanol, vortex the tubes briefly, centrifuge the tubes and discard the organic solvent wash. Acidify the aqueous phase with 0.2 ml of 6 *N* hydrochloric acid and extract with 3 ml of ethyl acetate (without methanol). Shake the tubes for 5 min, centrifuge, and transfer a 2.5-ml aliquot of the ethyl acetate extract to a 5-ml conical centrifuge tube. Evaporate the ethyl acetate extract almost to dryness.

Add 0.6 ml of ethereal diazomethane solution and vortex the tubes. Allow the stoppered tubes to stand at room temperature for 10 min. To minimize losses of the derivative, evaporate the methylating solvent without heating by keeping the tubes positioned out of the water bath. Occasionally immerse the tubes in the water bath to remove condensation. Remove the tubes from the N-Evap just before the evaporation is completed. Add 100 μl of ethyl acetate to the wet residue and inject 2—5 μl of this solution into the GC—MS system. Turn off the GC valve 30 sec after injection. Turn on the ionizer 45 sec after injection.

Calculations. The peak heights are measured with a ruler and the *m/z* 269 to *m/z* 270 ion ratio is calculated. The data obtained from the calibration curve samples are fitted by a nonlinear least-squares program to the isotope dilution equation $R = (x + A)/(Bx + C)$ [4, 5]. In this equation x is the amount of (−)-*threo*-chlorocitric acid added to plasma, R is the ion ratio obtained from the spiked samples, and A, B, C are constants determined by the nonlinear least-squares fit. To find an unknown x given an experimentally determined R, the above equation is rearranged to solve for x: $x = (RC - A)/(1 - RB)$.

Clinical samples

A 90-kg healthy male volunteer did not eat anything for 7.5 h prior to receiving a 200-mg oral dose of (−)-*threo*-chlorocitric acid. Aliquots (10 ml) of whole blood were drawn at −0.25, 0.5, 0.67, 1, 1.25, 1.5, 2, 3 and 4 h post-dosing into heparinized Vacutainer® 6527 from Becton-Dickinson. Plasma is obtained following centrifugation of the blood at 2000 *g* at 5°C for 30 min. The plasma is then immediately acidified with 0.025 ml of 12 *N* hydrochloric acid per ml of plasma and stored at −10°C until analyzed.

RESULTS AND DISCUSSION

Complicating the analysis of (−)-*threo*-chlorocitric acid is the tendency of the drug, especially in an alkaline or neutral environment, to lactonize with generation of HCl. To minimize this possibility, plasma samples are acidified to pH 1 immediately after they are obtained from freshly drawn blood, and a mild derivatizing reagent, diazomethane, is used to convert the drug into a compound which can be gas chromatographed.

The methane positive CI mass spectra of derivatized (−)-*threo*-chlorocitric acid and derivatized (−)-*threo*-[^{13}C]chlorocitric acid are shown in Fig. 1. The mass spectra consist of MH^+ ions and base peak ions reflecting the loss of the elements of acetic acid from the MH^+ ions. The fact that the fragment ion in the mass spectrum of (−)-*threo*-[^{13}C]chlorocitric acid shows no loss of label suggests that the loss is of the carboxyl group attached to the carbon bearing the hydroxyl group. For the assay, the MH^+ ions at *m/z* 269 and *m/z* 270 are monitored in the GC effluent for (−)-*threo*-chlorocitric acid and its ^{13}C-labeled internal standard, respectively.

Typical selected ion current profiles from the analyses of 1 ml of either control plasma spiked with 0.5 μg of (−)-*threo*-chlorocitric acid (A), or plasma from a subject either 15 min before (B) or 2 h after (C) receiving a 200-mg dose of (−)-*threo*-chlorocitric acid are shown in Fig. 2. The small response in select-

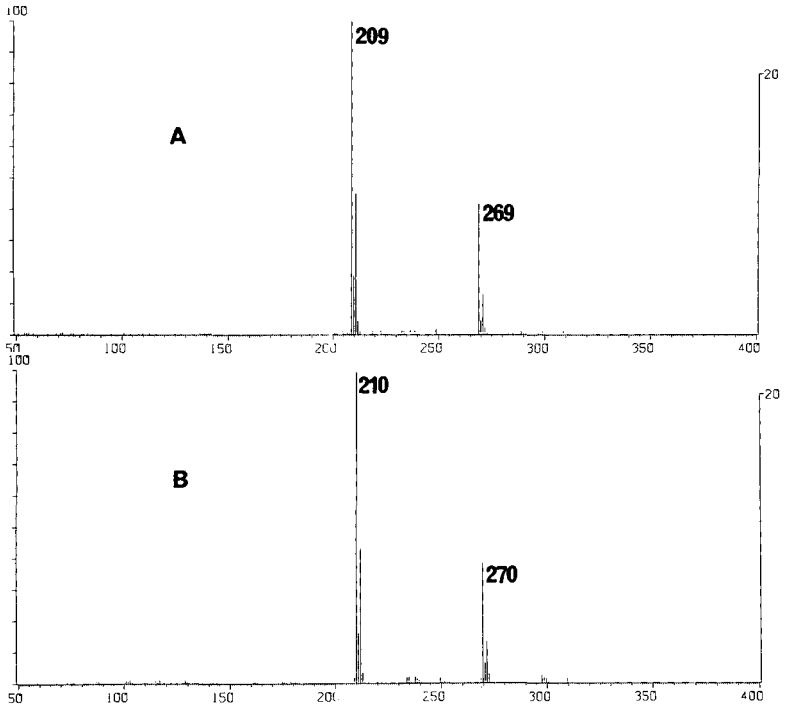


Fig. 1. Methane positive CI mass spectra of trimethylated derivatives of (−)-*threo*-chlorocitric acid (A) and (−)-*threo*-[^{13}C]chlorocitric acid (B).

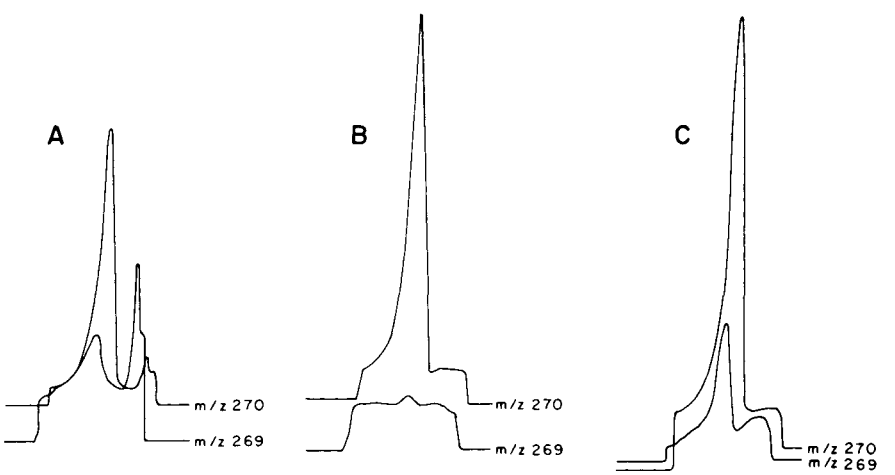


Fig. 2. Selected ion current profiles from the analyses of 1 ml of either control plasma spiked with 0.5 μg of (−)-*threo*-chlorocitric acid (A) or plasma from a subject either 15 min before (B) or 2 h after (C) receiving a 200-mg oral dose of (−)-*threo*-chlorocitric acid. All samples were spiked with 5 μg of (−)-*threo*-[^{13}C]chlorocitric acid. The measured concentration of (−)-*threo*-chlorocitric acid in the 2-h post-dose sample was 1.0 μg ml^{-1}.

ed ion current profile B in Fig. 2 at *m/z* 269 is from charge exchange ionization of (—)-*threo*-[^{13}C] chlorocitric acid and currently defines the limit of quantitation. When using 5 μg of the internal standard, this response typically represents between 0.05 and 0.2 μg of (—)-*threo*-chlorocitric acid. For any given set of samples, the limit of quantitation is considered to be three times the response at *m/z* 269 in the selected ion current profiles from the calibration curve plasma samples spiked only with (—)-*threo*-[^{13}C] chlorocitric acid. Typically this response is equivalent to a concentration of (—)-*threo*-chlorocitric acid of between 0.15 and 0.6 μg ml^{-1}.

Assay precision and recovery of (—)-*threo*-chlorocitric acid were determined by spiking six separate 1-ml plasma samples with 2.0 μg of authentic compound and analyzing the samples using the procedure described. The mean (± S.D.) concentration found was 2.10 ± 0.07 μg ml^{-1} and indicates a precision (relative standard deviation) of 3.3% at this concentration. The mean recovery (± S.D.) of the compound from these samples, based on a comparison of the responses of derivatized (—)-*threo*-chlorocitric acid from the calibration samples with the responses of external standard solutions containing known amounts of derivatized (—)-*threo*-chlorocitric acid, was 25 ± 6%.

The stability of (—)-*threo*-chlorocitric acid in acidified plasma upon prolonged storage was determined. To 20 ml of control plasma were added 100 μg of (—)-*threo*-chlorocitric acid. The plasma was acidified with 0.5 ml of 12 *N* hydrochloric acid. Duplicate 1-ml aliquots of the spiked plasma were analyzed six months later. The measured concentrations of (—)-*threo*-chlorocitric acid were within 6% of the initial plasma concentration measured on day 1.

The plasma concentration–time curve for (—)-*threo*-chlorocitric acid in a male volunteer following a 200-mg oral dose of the drug is shown in Fig. 3. The data can be fitted, using the program NONLIN [6], to a one-compartment open model [7] with complete absorption (correlation coefficient = 0.99). A peak

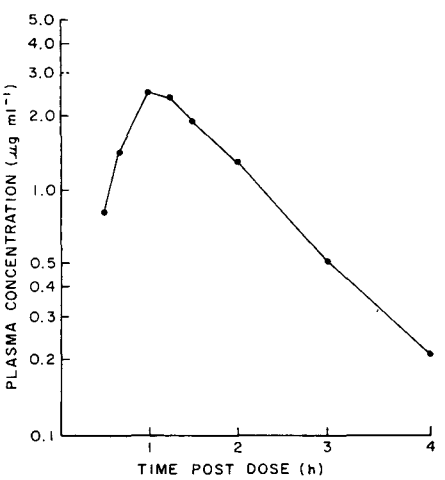


Fig. 3. Plasma concentration–time curve for a male volunteer who had received a 200-mg oral dose of (—)-*threo*-chlorocitric acid. The drug concentrations at —15, 10 and 20 min post-dose were all nonmeasurable.

plasma concentration of 2.5 μg/ml occurred at 1 h and the drug disappeared from the plasma with a half-life of 35 min. The volume of distribution, 39 liters, was 43% of body weight.

CONCLUSION

A specific and relatively simple GC—MS procedure has been described which can measure (−)-*threo*-chlorocitric acid in plasma for up to 4 h following a 200-mg dose of the drug.

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CHROMBIO. 1440

DETERMINATION OF MIDAZOLAM AND TWO METABOLITES OF MIDAZOLAM IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE CHEMICAL-IONIZATION MASS SPECTROMETRY

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(Received May 5th, 1982)

SUMMARY

A method is described for measuring midazolam, a new anesthesia induction agent and hypnotic, and its hydroxymethyl and desmethyl metabolites in human plasma. Deuterated analogues of each compound are added to plasma as internal standards. The compounds are extracted from plasma with benzene containing 20% 1,2-dichloroethane and after removal of the extracting solvent are dissolved in a solution of bis-(trimethylsilyl)acetamide and acetonitrile. An aliquot of this solution is analyzed by gas chromatography—mass spectrometry with the mass spectrometer set to monitor in the gas chromatographic effluent the M^- ions of drug, metabolites and internal standards generated by methane electron-capture negative chemical ionization. For all three compounds, the limit of quantitation is 1 ng ml^{-1}, and the precision (relative standard deviation) at a concentration of 5 ng ml^{-1} is less than 6%. Measurable amounts of the hydroxymethyl, but not the desmethyl, metabolite of midazolam could be found in the plasma of humans given either an intravenous or an oral dose of midazolam maleate.

INTRODUCTION

Midazolam (MDZ) maleate is a new water-soluble benzodiazepine salt which is currently undergoing testing as an intravenously administered anesthesia induction agent [1—3] and as an orally active hypnotic [4].

Electron-capture (EC) gas chromatographic (GC) [5, 6] and radioimmunoassay (RIA) [7] methods for MDZ have been published. This paper reports a sensitive, specific and relatively simple gas chromatographic—mass spectrometric (GC—MS) assay for MDZ, its principal metabolite in human urine [5], hydroxymethylmidazolam (HMMDZ) and a minor metabolite of midazolam in the dog [8], desmethylmidazolam (DMMDZ). If present in sufficient quantities, DMMDZ will interfere with the EC-GC and RIA assays for MDZ.

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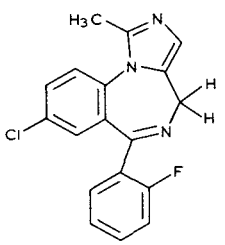
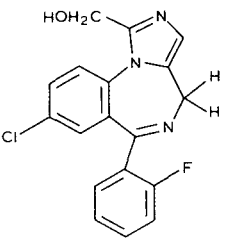

MDZ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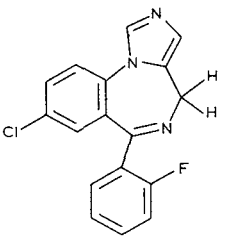
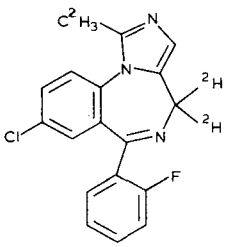
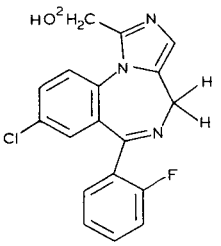
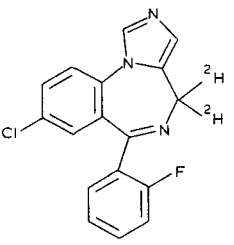

DMMDZ


MDZ–d_5


HMMDZ–d_2


DMMDZ–d_2

The reported assay features the use of MDZ-d_5, HMMDZ-d_2 and DMMDZ-d_2 as internal standards for MDZ, HMMDZ and DMMDZ, respectively, and the trimethylsilylation of the hydroxy group of HMMDZ to provide a compound with suitable GC properties.

EXPERIMENTAL

Equipment and operating conditions

Gas chromatograph. A Finnigan Model 9500 gas chromatograph was equipped with a 152 cm × 2 mm I.D. U-shaped borosilicate glass column packed with 3% Poly-S 176 on 80—100 mesh, high-performance Chromosorb W from Applied Science Labs., State College, PA, U.S.A. The column was conditioned at 320°C overnight with nitrogen as carrier gas. Methane (1.5 kg cm^{-2}) was used as GC carrier gas. The injector, column, interface oven and transfer line were operated at 310°C, 300°C, 250°C and 250°C, respectively. The retention times of MDZ, derivatized HMMDZ and DMMDZ under these conditions were 132, 72, and 156 sec, respectively. Prior to use, the GC column was conditioned daily with several injections of both Silyl-8® (Pierce Chemical Co., Rockford, IL, U.S.A.) and the reconstituted residue from an ethyl acetate extract of drug-free plasma.

Mass spectrometer. The ion source parameters of a Finnigan Model 3200 quadrupole mass spectrometer were set to give the maximum response consistent with reasonable ion peak shape and unit resolution. The modifications to the instrument to permit the detection of negative ions have been described [9]. The continuous dynode electron multiplier was set at −1.7 kV and the conversion dynode was set at +2.5 kV. Methane (Liquid Carbonic, 99%) at an ion source pressure of 68 Pa was used as negative chemical-ionization

reagent gas. To avoid "ghosting", the MS tuning and GC column conditioning were optimized using the response from the injection of microgram amounts of Ro 21-3547, the desfluoro analogue of MDZ.

Peak monitor. A Finnigan Promim® with a Rikadenki four-channel recorder was used to set the mass spectrometer to monitor *m/z* 325 (MDZ), *m/z* 330 (MDZ-d_5), *m/z* 311 (DMMDZ), *m/z* 313 (DMMDZ-d_2), *m/z* 413 (trimethylsilylated HMMDZ) and *m/z* 415 (trimethylsilylated HMMDZ-d_2). All channels were operated at a gain of 10^{-8} A/V, 100 msec dwell time and a filter setting of 0.5 Hz.

Glassware. Culture tubes (16 ml, Pyrex 9825) provided with Teflon®-lined screw caps were used for plasma extraction. Conical centrifuge tubes (5 ml, Pyrex 8061) were used for the evaporation of the benzene extract. All the tubes were purchased from SGA Scientific, Bloomfield, NJ, U.S.A. Prior to use, the glassware was treated with Siliclad® (Clay Adams, Parsippany, NJ, U.S.A.) and rinsed with methanol and dichloromethane.

Solvent evaporator. Solvents were removed at 60°C using a nitrogen evaporator (N-Evap®, Organomation Assoc.).

Shaker. Extractions were done by shaking (60 strokes min^{-1}) on a variable-speed reciprocating shaker (Eberbach Inc.).

Centrifuge. Centrifugation was carried out on a Damon/IEC Model CRU-500 refrigerated centrifuge operated at 1600 *g* and 10°C.

Chemicals

MDZ (maleate salt), HMMDZ, DMMDZ and Ro 21-3547 were prepared by Dr. A. Walser, and the deuterated compounds, MDZ-d_5, HMMDZ-d_2 and DMMDZ-d_2, were prepared by Dr. Yu-Ying Liu. Both chemists work for the Chemical Research Department, Hoffmann-La Roche Inc., Nutley, NJ, U.S.A. Nanograde methanol, benzene, 1,2-dichloroethane and acetonitrile were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Bis-(trimethylsilyl)acetamide (BSA), boric acid (analytical reagent), potassium chloride and anhydrous sodium carbonate were purchased from Pierce Chemical Co., Mallinckrodt Chemical Co. (St. Louis, MO, U.S.A.), Fisher Scientific Co. (Pittsburgh, PA, U.S.A.) and J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.), respectively.

Solutions

Stock solutions. MDZ: 13.6 mg of MDZ maleate are dissolved in 10.0 ml of methanol to give solution A (1 mg ml^{-1} of MDZ free base). HMMDZ: 10.0 mg are dissolved in 10.0 ml of methanol to give solution B (1 mg ml^{-1}). DMMDZ: 10.0 mg are dissolved in 10.0 ml of methanol to give solution C (1 mg ml^{-1}). MDZ-d_5: 20.0 mg are dissolved in 10.0 ml of methanol to give solution D (2 mg ml^{-1}). HMMDZ-d_2: 2.0 mg are dissolved in 2.0 ml of methanol to give solution E (1 mg ml^{-1}). DMMDZ-d_2: 2.0 mg are dissolved in 2.0 ml of methanol to give solution F (1 mg ml^{-1}).

Tuning solution. 1.0 mg of Ro 21-3547 is dissolved in 10.0 ml of methanol (100 $\mu g\ ml^{-1}$).

Spiking solutions for MDZ, HMMDZ and DMMDZ. Aliquots (0.2 ml) of solutions A, B, and C are transferred into one 100-ml volumetric flask which

is brought to volume with methanol (2 μg ml^{-1}). Individual aliquots of this solution (either 3, 2, 1, 0.5 or 0.2 ml) are transferred to 10-ml volumetric flasks and brought to volume with methanol to give solutions containing 30, 20, 10, 5 or 2 ng of MDZ, HMMDZ and DMMDZ per 0.05 ml of solvent.

Spiking solutions for internal standards. A 0.1-ml volume of solution D and 0.2 ml of solutions E and F are transferred into one 100-ml volumetric flask which is brought to volume with methanol (2 μg ml^{-1}). Then 2 ml of this solution are transferred to a 10-ml volumetric flask and brought to volume with methanol to give a solution containing 20 ng of MDZ-d_5, HMMDZ-d_2 and DMMDZ-d_2 per 0.05 ml of solvent.

1 M pH 10 borate buffer. Boric acid (61.8 g) and potassium chloride (74.7 g) are dissolved in 1000 ml of distilled water. This solution is then titrated to pH 10 with a solution containing 106 g of sodium carbonate per 1000 ml of distilled water.

20% 1,2-dichloroethane in benzene. 200 ml of dichloroethane are added to 800 ml of benzene.

BSA. The contents of one BSA ampule (1 ml) are added to 4 ml of acetonitrile. This solution is prepared just prior to its use.

Procedure

Calibration curve samples (prepared in duplicate). Transfer 1 ml of drug-free control human plasma to each of twelve culture tubes containing either 30 ng, 20 ng, 10 ng, 5 ng, 2 ng or 0 ng of MDZ, together with equal amounts of HMMDZ and DMMDZ and 20 ng of each of the deuterated analogues.

Experimental ("unknown") samples. Transfer 1-ml aliquots of experimental plasma to culture tubes containing 20 ng of each of the deuterated analogues.

Extraction. To each plasma sample add 2 ml of 1 *M* pH 10 borate buffer followed by 6 ml of benzene—1,2-dichloroethane (4:1). Shake the tubes for 20 min, centrifuge the tubes and transfer 5 ml of each benzene extract to a centrifuge tube. Evaporate the solvent. Place tubes in a desiccator and further dry the residues under vacuum for 30 min. Dissolve the residues in 50 μl of BSA—acetonitrile (1:4) and allow the tubes to stand at room temperature for 20 min.

GC—MS analyses. The tuning solution containing Ro 21-3547 is injected and the mass offset value of this compound's $M^{\cdot-}$ ion is determined. The same mass offset value is used to set the Promim to monitor the $M^{\cdot-}$ ions of MDZ, DMMDZ and their respective deuterated analogues.

Aliquots (2—5 μl) from each sample extract are injected into the GC—MS system. Approximately 30 sec after an injection, the GC divert valve is closed and 15 sec later the ion source is turned on. After all the extracts have been analyzed, the mass spectrometer is tuned to monitor the $M^{\cdot-}$ ions of silylated HMMDZ and silylated HMMDZ-d_2. Aliquots of the extracts are then injected using the same protocol just described above for the analysis of MDZ and DMMDZ.

Calculations. The peak heights in the ion chromatograms are measured and the ion ratios for *m/z* 325 vs. *m/z* 330, *m/z* 311 vs. *m/z* 313, and *m/z* 413 vs. *m/z* 415 are calculated. The calibration curve for MDZ is analyzed by linear regression. Calibration curves for HMMDZ and DMMDZ, however, are non-

linear and are analyzed using the computer program NONLIN [10]. Concentrations of MDZ in the experimental plasma samples are calculated using the equation x (ng) = $(R - b)/m$, where R is the experimental m/z ion ratio and b (intercept) and m (slope) are constants generated by the linear-regression analysis of the calibration curve data. Concentrations of DMMDZ and HMMDZ are calculated by using the rearranged isotope dilution equation x (ng) = $(RC - A)/(1 - RB)$, where R is the experimental ion ratio and A, B, and C are parameters generated by the NONLIN program.

Clinical samples

Two healthy male volunteers did not eat anything for 8 h before dosing. One volunteer received a 10-mg dose of MDZ maleate orally and the other volunteer received a 20-mg dose of MDZ maleate intravenously. Blood samples (10 ml) were obtained using heparinized Vacutainer® 6527 from Becton-Dickinson. Samples from the volunteer given the intravenous dose were obtained at −0.25, 0.025, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 17 and 24 h post dosing. Samples from the volunteer given the oral dose were obtained at −0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, and 12 h post dosing. The blood was centrifuged for 0.5 h and the resulting plasma was isolated and stored at −20°C until analyzed.

RESULTS AND DISCUSSION

The negative chemical-ionization mass spectra of MDZ and DMMDZ consist only of M^- ions, while the spectrum of trimethylsilylated HMMDZ shows an $[M - (CH_3)_3SiOH]^-$ fragment ion at m/z 323 in addition to an M^- ion (Fig. 1). The negative CI mass spectra of the deuterated analogues used in the assay are identical to those of the protio compounds, except the masses are shifted by the appropriate number of daltons. As would be expected from GC—MS assays of benzodiazepines previously developed in our laboratory [11, 12], the ionization efficiencies of negative CI for these compounds are quite good. Only 25—50 pg of either MDZ, HMMDZ or DMMDZ are needed to generate a selected ion current profile for each M^- ion which has a signal-to-noise ratio of better than 5:1.

Fig. 2 shows typical selected ion current profiles at m/z 325 and 330 from the analyses of 1 ml of either control or experimental plasma spiked with 3.7 ng of MDZ (A) or plasma from a subject either 0.25 h before (B) or 12 h after (C) receiving a 10-mg oral dose of MDZ. Selected ion current profiles at m/z 413 and 415 from the analyses of 1 ml of either control plasma spiked with 5 ng of HMMDZ (A) or plasma from a subject either 0.25 h before (B) or 8 h after (C) receiving a 10-mg oral dose of MDZ are shown in Fig. 3. The limit of quantitation of this assay is 1 ng ml^{-1} for MDZ, HMMDZ and DMMDZ.

Assay precision and recovery were determined by spiking six separate 1-ml plasma samples with 5 ng ml^{-1} each of MDZ, HMMDZ and DMMDZ and analyzing the samples by the procedure described. The mean (± S.D.) concentrations found, 5.10 ± 0.10 ng ml^{-1} for MDZ, 5.00 ± 0.20 ng ml^{-1} for HMMDZ and 5.30 ± 0.30 ng ml^{-1} for DMMDZ, indicate a precision (relative standard deviation) of less than 6% at this concentration for all three compounds. The mean recoveries ± S.D. from these samples, based on a com-

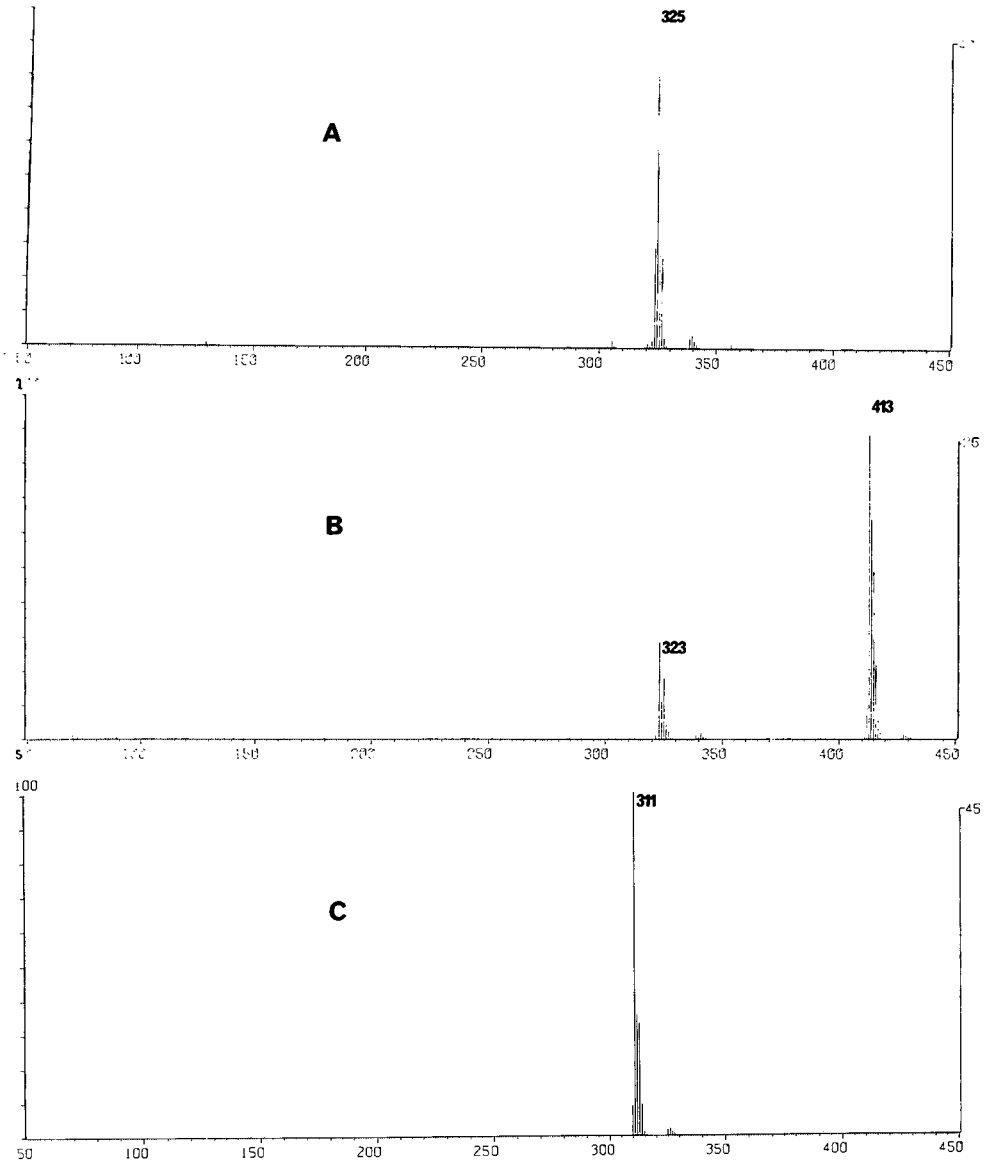


Fig. 1. Methane negative chemical-ionization mass spectra of MDZ (A), trimethylsilylated HMMDZ (B) and DMMDZ (C).

parison of the ion responses from the processed samples to the responses from the injection of known amounts of MDZ, trimethylsilylated HMMDZ and DMMDZ, are 105 ± 6% for MDZ and DMMDZ and 110 ± 3% for HMMDZ.

The plasma concentration—time curves for MDZ and HMMDZ in male volunteers following either an intravenous or an oral dose of MDZ are shown in Figs. 4 and 5, respectively. Assuming that distribution is complete after 2 h, the elimination half-lives of MDZ in the subjects given the intravenous

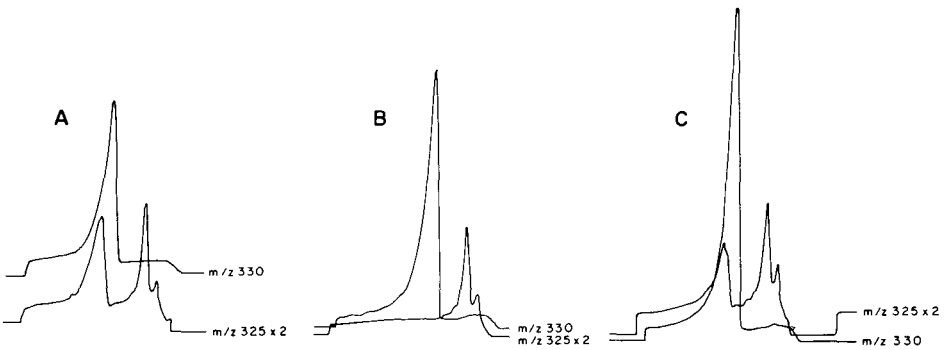


Fig. 2. Selected ion current profiles from the analysis of 1 ml of either control plasma spiked with 3.7 ng of MDZ (A) or plasma from a subject either 0.25 h before (B) or 12 h after (C) receiving a 10-mg oral dose of MDZ maleate. All plasma samples were spiked with 20 ng of MDZ-d_5. The measured concentration of MDZ in the 12-h post-dose sample was 1.3 ng ml^{-1}.

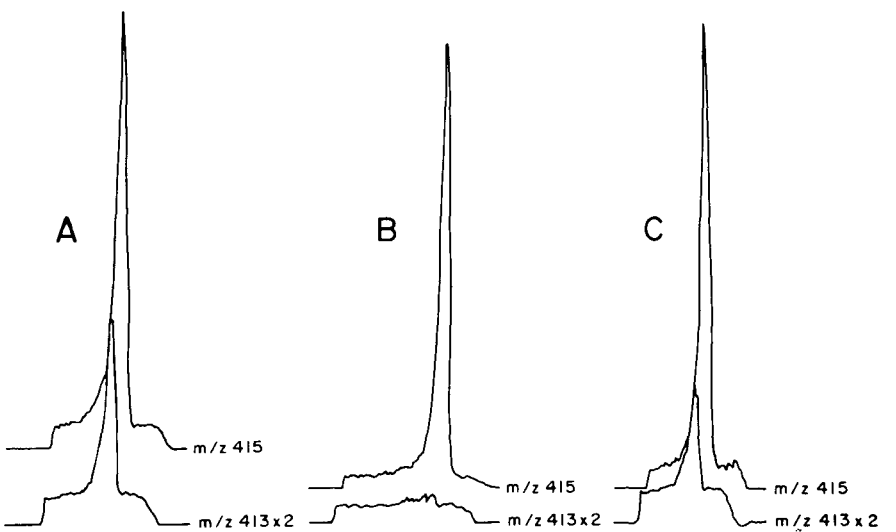


Fig. 3. Selected ion current profiles from the analysis of 1 ml of either control plasma spiked with 5 ng of HMMDZ (A) or plasma from a subject either 0.25 h before (B) or 8 h after (C) receiving a 10-mg oral dose of MDZ maleate. All plasma samples were spiked with 20 ng of HMMDZ-d_2. The measured concentration of HMMDZ in the 8-h post-dose sample was 2.5 ng ml^{-1}.

and oral doses of MDZ are 4 and 3 h, respectively. Following an intravenous dose of MDZ the area under the plasma concentration—time curve for HMMDZ is less than 10% of the area under the plasma concentration—time curve for MDZ. Following oral dosing, however, these areas are approximately equal. The half-times for HMMDZ following both routes of administration are similar, approximately 3 h, which suggests that HMMDZ will not accumulate following daily administration of MDZ.

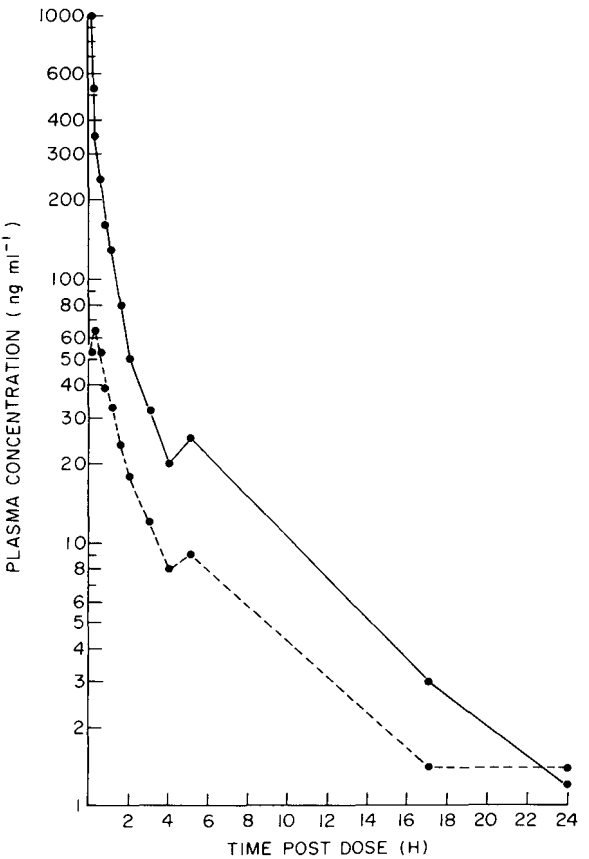


Fig. 4. Plasma concentration—time curve for a subject who had received a 20-mg intravenous dose of MDZ maleate. (——) MDZ; (— — —) HMMDZ.

In all of the plasma samples whose analyses are given in Figs. 4 and 5 and in all other plasma samples analyzed using this GC—MS method, no measurable levels of DMMDZ were found, although in a few of the samples a small, but non quantifiable, response for DMMDZ was observed. Eberts [13] has suggested that the desmethyl metabolite of triazolam, a benzodiazepine structurally similar to MDZ, results from the spontaneous loss of CO_2 from a carboxylic acid metabolite of triazolam which is generated from further oxidation of triazolam's hydroxymethyl metabolite. Experiments in our laboratory suggest that the corresponding carboxylic acid metabolite of MDZ will decompose to DMMDZ on standing at room temperature in plasma for many days. Nevertheless, our results suggest that DMMDZ is not present in sufficient amounts in normally preserved plasma from subjects given MDZ to interfere with the published EC-GC or RIA assays for MDZ at their quoted limits of quantitation.

CONCLUSIONS

A sensitive and specific GC—MS procedure has been described which can measure MDZ and HMMDZ in plasma following a therapeutic dose of the

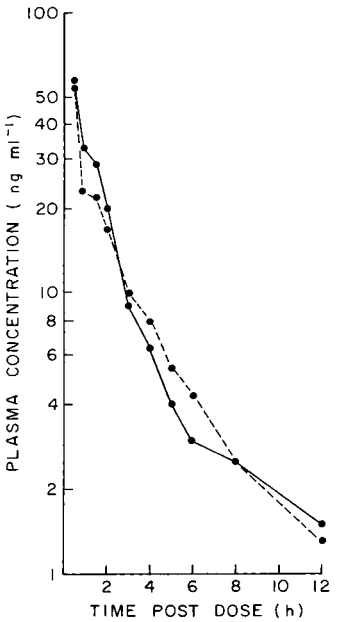


Fig. 5. Plasma concentration–time curve for a subject who had received a 10-mg oral dose of MDZ maleate. (——) MDZ; (— — —) HMMDZ.

drug. Another metabolite of MDZ, DMMDZ, could not be measured in plasma following a therapeutic dose of the drug.

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DETERMINATION OF IMIDAZOBENZODIAZEPINE-3-CARBOXAMIDE, A NEW ANXIOLYTIC AGENT, IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE CHEMICAL-IONIZATION MASS SPECTROMETRY

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SUMMARY

A method is described for measuring imidazobenzodiazepine-3-carboxamide, a new anxiolytic agent, in human plasma. A tetradeuterated analogue of the analyte is used as the internal standard. The drug and its internal standard are (1) extracted from plasma at pH 9 with benzene containing 20% 1,2-dichloroethane, (2) derivatized with pentafluoropropionic anhydride in the presence of triethylamine and (3) the nitrile derivative of the analyte and internal standard are analyzed by gas chromatography (GC)—negative chemical-ionization mass spectrometry (CIMS) using methane as both GC carrier gas and CI reagent gas. The mass spectrometer is set to monitor the intense $(M—HCl)^-$ ions of imidazobenzodiazepine-3-nitrile and its tetradeuterated analogue at *m/z* 316 and *m/z* 320, respectively. Quantitation of an experimental plasma sample is based on the comparison of the *m/z* 316 to *m/z* 320 ion ratio in each sample to that obtained from the analyses of control plasma spiked with various amounts of the drug and a fixed amount of internal standard. The limit of quantitation of the method is approximately 100 pg ml^{-1} of plasma and the precision (relative standard deviation) at a plasma concentration of 1 ng ml^{-1} is 4%.

INTRODUCTION

Imidazobenzodiazepine-3-carboxamide, 8-chloro-6-(2′-chlorophenyl)-4H-imidazo[1,4]benzodiazepine-3-carboxamide [1], is currently undergoing testing as an anxiolytic agent. The compound is significantly more potent than diazepam in animal tests used to detect anxiolytic properties [2].

A published high-performance liquid chromatographic procedure [3] for imidazobenzodiazepine-3-carboxamide with a limit of quantitation of 50 ng/ml is too insensitive to measure the less than 2 ng/ml concentrations of imidazo-

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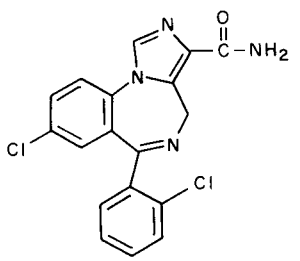
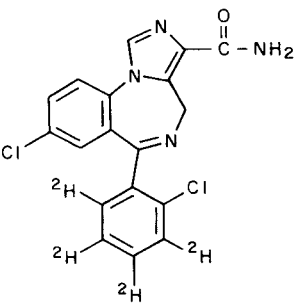


Imidazobenzodiazepine-3-carboxamide



Tetradeuterated imidazobenzodiazepine-3-carboxamide

benzodiazepine-3-carboxamide found in human plasma following a therapeutic 0.2-mg oral dose of the drug.

This paper reports a sensitive, specific and relatively simple gas chromatographic (GC)—negative chemical-ionization mass spectrometric (CIMS) procedure for imidazobenzodiazepine-3-carboxamide. The method is based on the conversion of imidazobenzodiazepine-3-carboxamide to a more easily chromatographed nitrile derivative. Chromatographic peak shape is improved by using a WSCOT capillary GC column. The assay features the use of a tetradeuterated analogue of imidazobenzodiazepine-3-carboxamide as internal standard.

EXPERIMENTAL

Equipment and operating conditions

Gas chromatograph. A Finnigan Model 9500 gas chromatograph was equipped with a WSCOT CP^{TM} SIL 8 capillary column (24.3 m × 0.48 mm I.D.) obtained from Chrompack, Santa Fe Springs, CA, U.S.A. Methane (0.2 kg m^{-2}) was used as carrier gas. The injector, column, interface oven and transfer line were operated at 325, 320, 250 and 250°C, respectively. The retention time of derivatized imidazobenzodiazepine-3-carboxamide was 115 sec. Prior to use, the column was conditioned overnight at 300°C with a 2 ml min^{-1} flow of nitrogen and then by several daily injections of the reconstituted residue from the ethyl acetate extract of drug free plasma.

Mass spectrometer. A Finnigan Model 3200 quadrupole mass spectrometer was adjusted to give the maximum response consistent with reasonable ion peak shape and unit resolution. The modifications to the instrument to permit the detection of negative ions have been described [4]. Methane, at an ion source pressure of 67 Pa, was used as reagent gas. To avoid "ghosting", the MS tuning and GC conditions were optimized using the response from the injection of μg amounts of 8-deschloro-imidazobenzodiazepine-3-carboxamide into the GC—MS system. The voltage across the continuous dynode electron multiplier was −2.0 kV and the conversion dynode was operated at +2.5 kV.

Peak monitor. A Finnigan Promim® with a Rikadenki recorder was used to set the mass spectrometer to monitor *m/z* 316 and *m/z* 320 in the GC effluent. Each Promim channel was operated at 100-msec dwell time, a 0.5-Hz frequency response and a gain of 10^{-8} A/V.

Glassware. Culture tubes (16 ml, Pyrex 9825), provided with Teflon®-lined screw caps, were used for plasma extractions. Conical centrifuge tubes (5 ml, Pyrex 8061) were used for the derivatization procedure and for the evaporation of the final extract. All tubes were washed with detergent and water, were treated with Siliclad® (Clay Adam, Parsippany, NJ, U.S.A.) and, prior to use, were rinsed with methanol and dichloromethane (Fisher Scientific).

Solvent evaporator. Solvents were removed at 60°C with a nitrogen evaporator (N-Evap, Organomation Associates).

Shaker. Extractions were performed by shaking (60 strokes min^{-1}) on a variable speed reciprocating shaker (Eberbach) for 20 min.

Centrifuge. A Damon/IEC Model CRU-500 refrigerated centrifuge was operated at 1600 *g* and 10°C.

Chemicals

Imidazobenzodiazepine-3-carboxamide and 8-deschloro-imidazobenzodiazepine-3-carboxamide were obtained from Dr. W. Scott, Hoffmann-La Roche, Nutley, NJ, U.S.A. Tetradeuterated imidazobenzodiazepine-3-carboxamide was prepared by Dr. Yu-Ying Liu, Hoffmann-La Roche. Nanograde benzene, methanol, ethyl acetate, N,N-dimethylformamide, chloroform, hexane and 1,2-dichloroethane were purchased from Burdick and Jackson Labs.

Solutions

Imidazobenzodiazepine-3-carboxamide. A 1 mg ml^{-1} stock solution was prepared in N,N-dimethylformamide. This solution was diluted with methanol to give solutions A, B, C and D containing 0.1, 0.5, 1 and 5 ng of imidazobenzodiazepine-3-carboxamide per 50 μl of solvent, respectively.

Tetradeuterated imidazobenzodiazepine-3-carboxamide. A 1 mg ml^{-1} stock solution was prepared in N,N-dimethylformamide. This solution was diluted with methanol to give solution E containing 10 ng of tetradeuterated imidazobenzodiazepine-3-carboxamide per 50 μl of solvent.

20% 1,2-dichloroethane in benzene. 200 ml of 1,2-dichloroethane were diluted to 1000 ml with benzene.

2% triethylamine in chloroform. Triethylamine (0.1 ml) was diluted with 4.9 ml of chloroform. This solution was prepared immediately prior to use.

Molar borate buffer (pH 9). Boric acid (61.8 g) and potassium chloride (74.7 g) were dissolved in 1000 ml of distilled water. This solution was then used to titrate a solution of 106 g of sodium carbonate in 1000 ml of distilled water to pH 9.

0.25 M borate buffer (pH 9). One volume of 1 *M* borate buffer pH 9 was diluted with 3 volumes of distilled water.

Procedure

Calibration curve samples are prepared in duplicate by spiking 1 ml of drug-free control plasma with 0 or 50 μl of either solution A, B, C or D (0, 0.1, 0.5, 1 or 5 ng of imidazobenzodiazepine-3-carboxamide, respectively). A volume of 50 μl of solution E (10 ng of tetradeuterated imidazobenzodiazepine-3-carboxamide) are added to both calibration curve and experimental samples and each resulting mixture is vortexed briefly. A volume of 1 ml of 1 *M*

pH 9 borate buffer is added and the samples are extracted with 5 ml of 20% 1,2-dichloroethane in benzene. The samples are centrifuged for 10 min, 4 ml of the benzene extract are transferred to a 5-ml centrifuge tube and the benzene is evaporated. The residue is dissolved in 50 μl of the 2% triethylamine in chloroform solution, and 20 μl of pentafluoropropionic anhydride are added. The solution is vortexed and allowed to stand at room temperature for 15 min. The solution is evaporated to dryness, the residue is dissolved in 0.5 ml of hexane and 0.2 ml of 0.25 *M* pH 9 borate buffer is added. The mixture is vortexed briefly and then centrifuged. As much hexane as possible is transferred to another 5-ml centrifuge tube, the solvent is evaporated, and the residue is reconstituted in 40 μl of ethyl acetate. Aliquots (3—5 μl) of this solution are injected into the gas chromatograph—mass spectrometer with the mass spectrometer set to monitor *m/z* 316 and *m/z* 320 in the effluent of the gas chromatograph. Thirty sec after injection the divert valve of the gas chromatograph is turned off and 15 sec later the ionizer is turned on.

The ion ratio of *m/z* 316 to *m/z* 320 from an unknown sample is converted to a known amount of imidazobenzodiazepine-3-carboxamide using a calibration curve. The calibration curve is constructed by fitting the *m/z* 316 to *m/z* 320 ion ratios from the calibration curve samples to their respective concentration values using a linear least squares computer program. The resulting slope

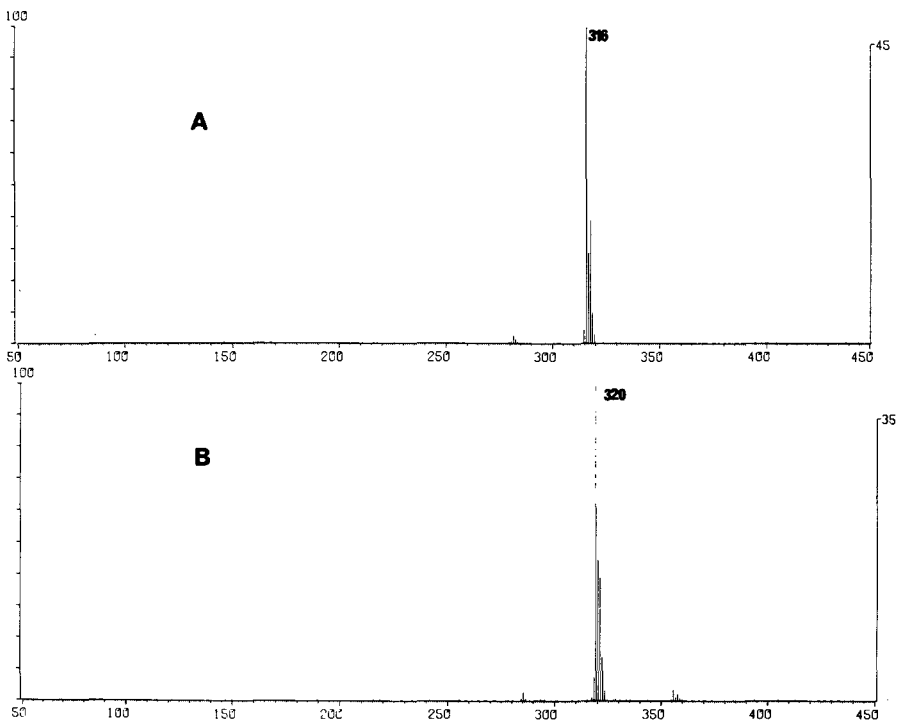


Fig. 1. Methane negative chemical-ionization mass spectra of the nitrile derivatives of imidazobenzodiazepine-3-carboxamide (A) and tetradeuterated imidazobenzodiazepine-3-carboxamide (B).

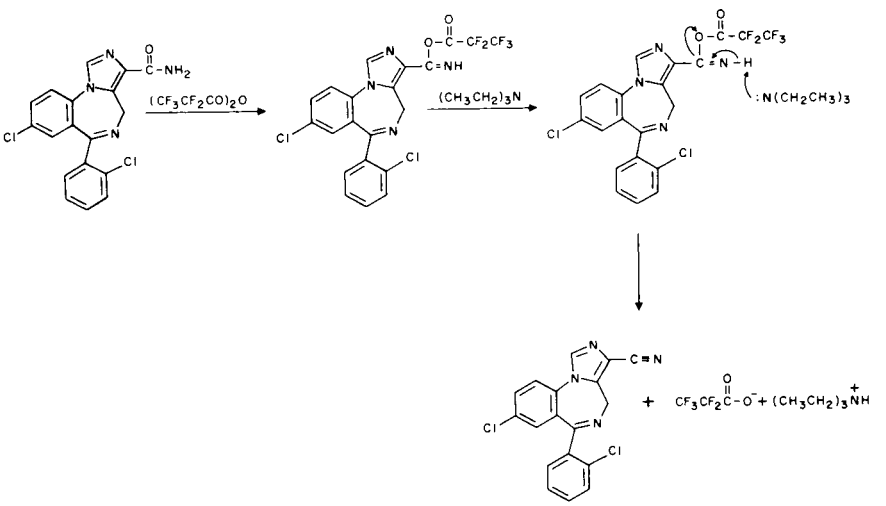
(m) and intercept (b) values are then used to calculate the amount (x) of imidazobenzodiazepine-3-carboxamide corresponding to each ion ratio (R) in experimental samples by the equation: $x = (R-b)/m$.

Clinical samples

A healthy, male volunteer did not eat anything for 7.5 h prior to receiving a 0.2-mg oral dose of imidazobenzodiazepine-3-carboxamide. Aliquots (10 ml) of whole blood were drawn at −0.25, 0.5, 1, 2, 3, 4, 6, 9, 12, 18, 24, 36, 48, 60 and 72 h post dosing into a Vacutainer® 6527 containing heparin (Becton-Dickinson). The blood was centrifuged for 30 min and the resulting plasma was isolated and stored at −10°C until analyzed.

RESULTS AND DISCUSSION

Several years ago while searching for a volatile derivative of imidazobenzodiazepine-3-carboxamide we discovered that treatment of the drug with pentafluoropropionic anhydride and triethylamine converted the primary amide function of imidazobenzodiazepine-3-carboxamide into a nitrile function. The conversion is quantitative using even picogram amounts of imidazobenzodiazepine-3-carboxamide. A possible mechanism for this reaction is shown below.



Gal et al. [5] and Stogniew and Callery [6] have recently used a similar reaction to convert the amide functions of disopyramide and gabamide into their respective nitrile derivatives.

Analysis by thin-layer chromatography and direct insertion probe-MS of reacted imidazobenzodiazepine-3-carboxamide suggest that nitrile formation occurs in the reaction tube and not in the injection port of the gas chromatograph.

The methane negative CI mass spectra of the nitrile derivative of imidazobenzodiazepine-3-carboxamide and tetradeuterated imidazobenzodiazepine-3-carboxamide are shown in Fig. 1. Neither mass spectra shows an intense molecular

anion at either *m/z* 352 or *m/z* 356, respectively. However, intense $(M-HCl)^-$ fragment ions occur at *m/z* 316 for imidazobenzodiazepine-3-carboxamide and at *m/z* 320 for tetradeuterated imidazobenzodiazepine-3-carboxamide. Negative CI was used, in spite of the disadvantage of requiring the monitoring of a fragment ion, because an assay with a low limit of quantitation was required. Previous work in this laboratory with other 1,4-benzodiazepines have shown that negative CI provides extremely efficient ionization of this class of compounds [4, 7, 8]. Also, the loss of HCl is not expected to occur on metabolism of imidazobenzodiazepine-3-carboxamide and specificity is accordingly maintained.

Typical selected ion current profiles from the analysis of 1 ml of either control plasma spiked with 0.1 ng of imidazobenzodiazepine-3-carboxamide (A) or plasma from a subject either 15 min before (B) or 72 h (C) after receiving a 0.2-mg oral dose of imidazobenzodiazepine-3-carboxamide are shown in Fig. 2. The small response at *m/z* 316 in selected ion current profile B is from undeuterated imidazobenzodiazepine-3-carboxamide in the tratradeuterated imidazobenzodiazepine-3-carboxamide and "ghosting" from previous injections. Using 5 ng of tetradeuterated imidazobenzodiazepine-3-carboxamide as internal standard, this response typically represents 0.03 ng ml^{-1} of imidazodiazepine-3-carboxamide. The limit of quantitation for this assay is considered to be three times the response at *m/z* 316 in the selcted ion current profiles from the calibration curve plasma samples spiked only with tetradeuterated imidazobenzodiazepine-3-carboxamide, and is typically 0.1 ng ml^{-1}.

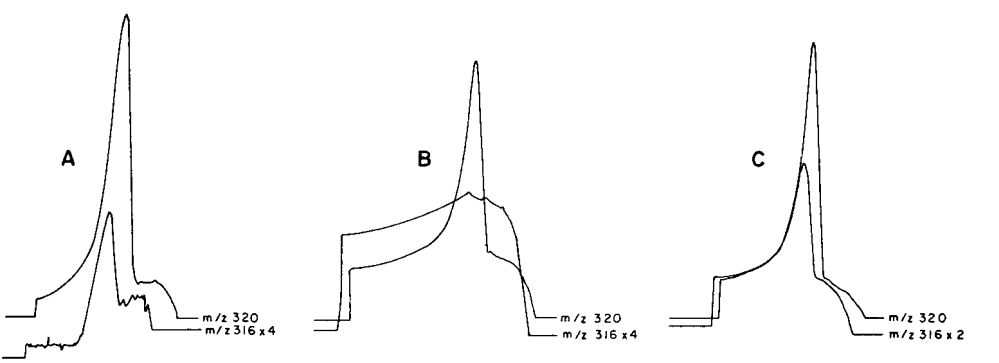


Fig. 2. Selected ion current profiles from the analysis of 1 ml of either control plasma spiked with 0.1 ng of imidazobenzodiazepine-3-carboxamide (A) or plasma from a subject either 15 min before (B) or 72 h after (C) receiving a 0.2-mg oral dose of imidazobenzodiazepine-3-carboxamide. All samples were spiked with 2.5 ng of tetradeuterated imidazobenzodiazepine-3-carboxamide. The measured concentration of imidazobenzodiazepine-3-carboxamide in the 72-h post dose sample was 0.64 ng/ml.

Assay precision and recovery of imidazobenzodiazepine-3-carboxamide were determined by spiking 1-ml plasma samples with either 0.1, 0.5, 1.0 or 5.0 ng of authentic compound and analyzing the samples using the procedure described. The relative standard deviations (n = 4) of the determinations were 17%, 9%, 3% and 0.5%, respectively. The mean recovery (± S.D.) of imidazobenzodiazepine-3-carboxamide from these samples, based on the responses to

injection of external standard solutions containing known amounts of derivatized imidazobenzodiazepine-3-carboxamide, was 42 ± 6%.

The plasma concentration—time curve for imidazobenzodiazepine-3-carboxamide in a male volunteer following a 0.2-mg dose of the drug is shown in Fig. 3. Assuming that distribution is complete after 6 h, the elimination half-life of the drug in this volunteer is 39 h.

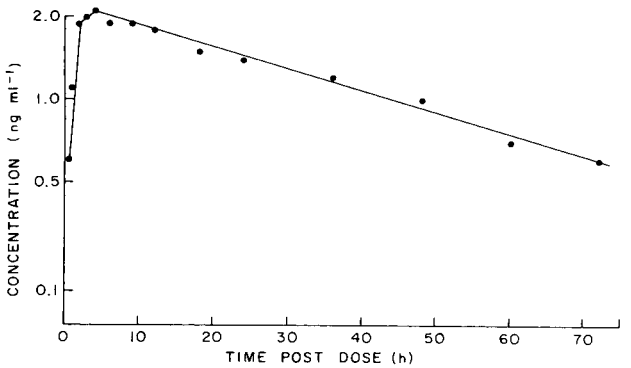


Fig. 3. Plasma concentration—time curve for a male volunteer who had received a 0.2-mg oral dose of imidazobenzodiazepine-3-carboxamide.

CONCLUSION

A sensitive and specific GC—MS procedure has been described which can measure imidazobenzodiazepine-3-carboxamide in human plasma for up to 72 h following a 0.2-mg dose of the drug.

ACKNOWLEDGEMENT

The authors wish to thank Dr. P. Blumenthal of Roche's Clinical Pharmacology Department for providing the clinical samples.

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CHROMBIO. 1455

DETERMINATION OF CLOFILIUM, A NEW ANTIFIBRILLATORY AGENT, IN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A sensitive and selective method for the assay of the new quaternary amine antifibrillatory agent clofilium is described. Plasma samples were extracted with dichloromethane (98.5 ± 0.2% recovery) and analyzed by gas chromatography—mass spectrometry operating in the electron-impact mode. The method involves a Hofmann elimination of an N-alkyl radical from clofilium and the internal standard in the presence of a strong nucleophile in the injector of the gas chromatograph. The resulting tertiary amines are chromatographed and detected by selective ion monitoring. The ratio of the clofilium base peak (*m/z* 224) to the internal standard peak (*m/z* 210) was linear relative to the plasma clofilium concentration over the range of 25—1000 ng/ml plasma.

INTRODUCTION

Clofilium (4-chloro-N,N-diethyl-N-heptylbenzene butanaminium phosphate) is a new quaternary amine antifibrillatory agent which has been shown to elevate the ventricular fibrillation threshold, reduce defibrillation threshold and allow electrically induced ventricular fibrillation to convert to normal sinus rhythm in pentobarbital anesthetized dogs [1, 2]. [^{14}C] Clofilium has been used to study the plasma and tissue kinetics of clofilium since a suitable non-radioactive assay for clofilium had not been available. However, myocardial levels of radioactivity after intravenous (i.v.) administration of [^{14}C] clofilium have been shown to closely parallel the kinetics of the biological response in dogs [3].

Various investigators have shown that pyrolytic degradation of quaternary amines may reproducibly lead to products which can be resolved by gas chromatography. The quaternary compound may be subjected to chemical degradation prior to injection into the gas chromatograph [4, 5] or they may be thermally decomposed in a gas chromatograph equipped with a pyrolyzer fitted to

the injection port [6]. In this paper, we report on the development of a gas chromatography—mass spectrometry (GC—MS) assay for clofilium which utilizes an on-column Hofmann elimination reaction and selective ion monitoring.

METHODS

Plasma extraction

To 200-μl samples of plasma were added 100 ng of internal standard (4-chloro-N,N-dimethyl-N-heptylbenzene butanaminium bromide) in aqueous solution, 1.5 ml of 0.1 *M* sodium bromide and 5 ml of dichloromethane. After centrifugation, the organic phase was transferred to a glass stoppered centrifuge tube previously treated with 1% dichlorodimethylsilane. The aqueous phase was re-extracted with 5 ml of dichloromethane and the organic phase was combined with the first organic extract. The extract was evaporated to dryness at room temperature under vacuum. The walls of the centrifuge tube were washed with 150 μl of methanol and again evaporated to dryness. The residue was then taken up in 10 μl of methanol containing 0.01 *M* potassium hydroxide and 3 μl were injected into the gas chromatograph—mass spectrometer. Extraction efficiency studies were performed in triplicate using dog plasma to which [^{14}C]clofilium (3.7 μCi/mg) was added to a final concentration of 2 μg/200 μl plasma. Samples were extracted as outlined above and radioactivity in the organic fraction was quantitated. The efficiency of extraction was 98.5 ± 0.2.

Gas chromatography—mass spectrometry

An LKB-9000 gas chromatograph—mass spectrometer (electron-impact mode) was used throughout. Samples were injected into a 0.61-m silanized glass column containing 1% (w/w) SP-2100 on Supelcoport (100—120 mesh) maintained at 195°C. The injector and separator were maintained at 295 and 230°C, respectively. The helium carrier gas flow-rate was 30 ml/min. The retention times of the internal standard and clofilium were 2.0 and 2.4 min, respectively. The mass spectrometer was operated in the selective ion monitoring mode at mass settings of *m/e* 210 (I.S.) and *m/e* 224 (clofilium). Ionizing voltage was 22 eV.

Alternatively, a Hewlett Packard 5840A gas chromatograph equipped with a flame ionization detector (FID) was used to investigate the parameters for the dequaternization of clofilium. Samples of clofilium or tertiary amine standard (4-chloro-N-ethyl-N-heptylbenzene butanamine) were chromatographed on a 0.61-m silanized glass column containing 3% (w/w) SP-2100 on Supelcoport (100—120 mesh) maintained at 200°C. The detector temperature was held at 300°C and the injector temperature was varied between 250 and 375°C. Helium carrier gas flow-rate was 38 ml/min. The area under the peak after injection of clofilium was compared to the area under the peak after injection of tertiary amine standard and expressed as percent N-deethylation of clofilium.

Standard curve

Standard solutions of 10 μg/ml clofilium and internal standard were prepared in water. Appropriate volumes of clofilium standard were added to

plasma samples to give final concentrations of 25—1000 ng/ml plasma. Internal standard was added to a final concentration of 500 ng/ml plasma. Plasma samples were then extracted and assayed for clofilium as outlined above.

Dosing

Clofilium was administered i.v. to 3 female mongrel dogs weighing approximately 12 kg at a dose of 5 mg/kg in saline. Blood samples were collected at various times in heparinized Vacutainer tubes. Samples were centrifuged and plasma was separated and stored at −20°C until assayed.

RESULTS AND DISCUSSION

The goal of the present investigation was to develop a selective and sensitive assay for clofilium in plasma. Although clofilium can be chromatographed using ion-exchange high-performance liquid chromatography (HPLC), the ultraviolet absorbance is too small to detect the low plasma concentrations of drug. Although flame ionization or electron-capture detection might be sensitive enough, ordinary GC was not successful because of the quaternary amine structure of the drug. However, the possibility of performing a Hofmann elimination in the injector of the gas chromatograph prompted the following investigation. When clofilium was injected into the gas chromatograph—mass spectrometer with injector temperatures between 290 and 375°C, a peak was detected which had a mass spectrum indicating the formation of 4-chloro-N-ethyl-N-heptylbenzene butanamine (Fig. 1). This deethylation is analogous to the pyrolytic dealkylation of choline esters observed by Szilagyi et al. [7]. The effect of injector temperature on the formation of the tertiary amine is shown in Fig. 2. Comparison of the peak area after injection of clofilium to the peak area after injection of an equimolar quantity of standard 4-chloro-N-ethyl-N-heptylbenzene butanamine showed that only 30% of the clofilium was deethylated at

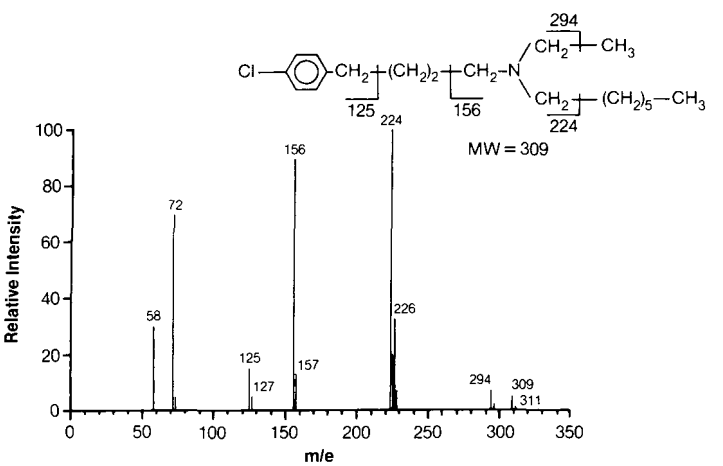


Fig. 1. Mass spectrum obtained after GC of clofilium with an injector temperature of 300°C. No molecular ion was observed at *m/e* 338 corresponding to the molecular weight of clofilium. A parent peak at *m/e* 309 was observed corresponding to N-deethylated clofilium.

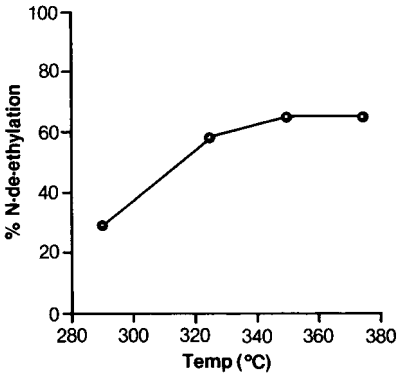
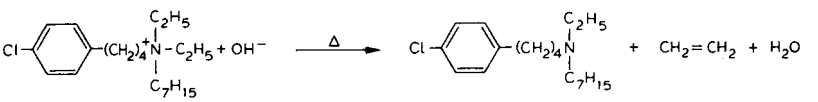


Fig. 2. The effect of injector temperature upon the N-deethylation of clofilium.

290°C which increased to a maximum of 65% at 350°C. Hofmann elimination reactions involve nucleophilic attack on quaternary amine alkyl groups eliminating ethylene which carries the smallest number of alkyl substituents. Therefore, the addition of a nucleophilic hydroxide ion in methanol to the extract before injection onto the gas chromatograph should improve the efficiency of the elimination reaction consistent with the following scheme:



At a constant injector temperature, an increase in hydroxide ion concentration lead to increased deethylation of clofilium as shown in Fig. 3. Using a hydroxide ion concentration of 0.01 *M*, the deethylation reaction was quantitative and independent of injector temperature between 250 and 325°C (data not shown). Thirty repetitive injections of 650 ng of clofilium in 3 μl of 0.01 *M* potassium

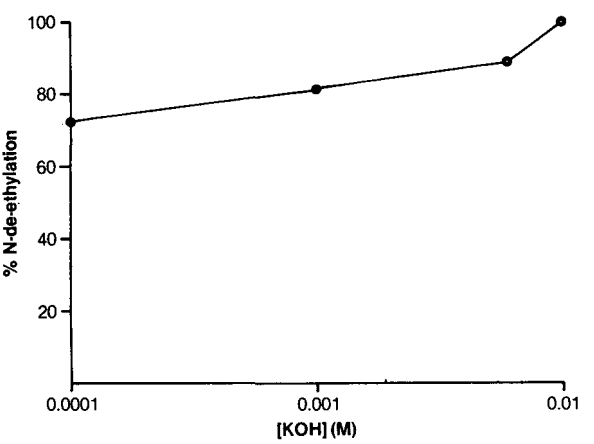


Fig. 3. The effect of hydroxide ion concentration upon the N-deethylation of clofilium at a constant injector temperature of 325°C.

hydroxide—methanol did not result in any loss of column or detector efficiency (standard error of the mean peak areas is 0.6%).

Although the GC of clofilium was possible, the sensitivity of flame ionization or electron-capture detection was not sufficient for the low plasma levels of clofilium anticipated. Therefore, the base peak (*m/e* 224) in the mass spectrum of clofilium originating from the Hofmann elimination reaction was used for a sensitive selective ion monitoring method. The clofilium analogue, 4-chloro-N,N-dimethyl-N-heptylbenzene butanaminium bromide, also undergoes N-dealkylation in the gas chromatograph to yield the 4-chloro-N-methyl-N-heptyl tertiary amine (Fig. 4) and has chromatographic properties similar to

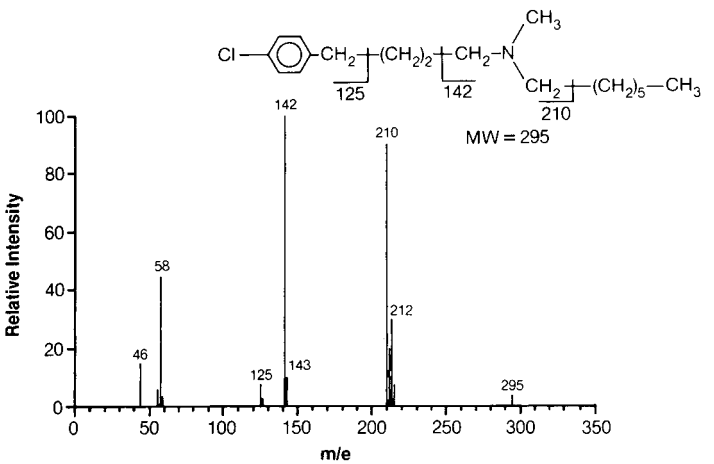


Fig. 4. Mass spectrum obtained after GC of the internal standard with an injector temperature of 300°C. No molecular ion was observed at *m/e* 310 corresponding to the molecular weight of the internal standard. A parent peak at *m/e* 295 was observed corresponding to N-demethylated internal standard.

clofilium. In addition, the compound has a prominent ion at *m/e* 210 in the mass spectrum. These properties allowed its use as an internal standard. A standard curve was then constructed with control dog plasma to which was added clofilium and internal standard. By monitoring the ratio of the peak height of the *m/e* 224 ion (clofilium) to the peak height of the *m/e* 210 ion (internal standard), a linear relationship was observed relative to clofilium plasma concentration. Linearity was observed between 25 and 1000 ng clofilium per ml plasma. The accuracy and precision of the assay are shown in Table I. The limit of detection was 10 ng clofilium per ml plasma.

TABLE I

ACCURACY AND PRECISION OF THE GC—MS ASSAY OF CLOFILIUM

Clofilium added to plasma (ng/ml plasma)	Clofilium assayed in plasma (ng/ml plasma)	
	Mean ± S.D. (n=4)	C.V. (%)
100	99.5 ± 11.0	11.0
500	478.0 ± 20.0	4.2

Dogs were administered 5 mg/kg of clofilium by i.v. injection and plasma samples were assayed for clofilium at various times. The results are shown in Fig. 5. Clofilium plasma levels rapidly decreased within the first hour and continued to decline throughout the 24-h observation period. Within 24 h, plasma levels had decreased to 36 ng/ml plasma. The selectivity and sensitivity of this assay will allow its use in further characterizing the plasma kinetics of clofilium in the dog and other laboratory animals.

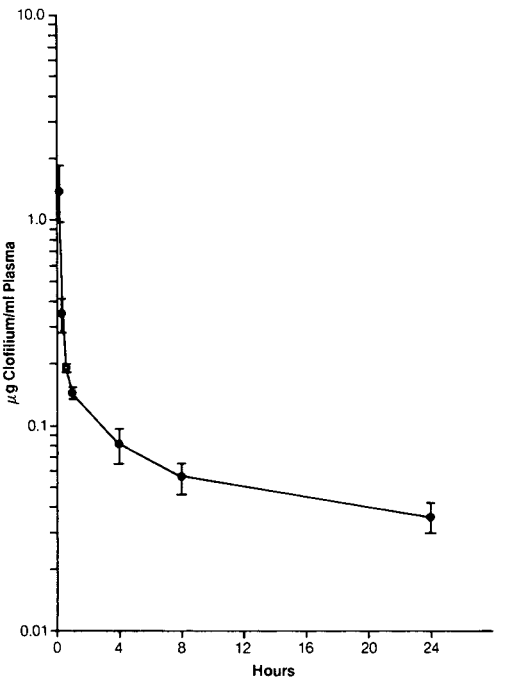


Fig. 5. Plasma levels of clofilium in dogs following a single intravenous injection of 5 mg/kg of clofilium. The values are the mean ± standard error for 3 dogs.

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CHROMBIO. 1412

DETERMINATION OF 3-HYDROXY-GUANFACINE IN BIOLOGICAL FLUIDS BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

An electron-capture gas—liquid chromatographic method was developed for measuring 3-hydroxy-guanfacine, the main metabolite of guanfacine in human plasma and urine. After extraction, the metabolite was derivatized by condensing the amidino group with hexafluoroacetylacetone and by methylating the NH and OH groups with methyl iodide. The obtained derivative possessed good bioanalytical gas chromatographic properties, using a capillary column. The O-glucuronide was measured after enzymatic hydrolysis. Unchanged guanfacine could be determined in urine together with its 3-hydroxy metabolite by this method.

INTRODUCTION

Guanfacine (I) is a new derivative of guanidine which possesses antihypertensive activity and a central site of action [1]. Biotransformation has been studied in animals and man [2]. Metabolism occurs on the aromatic moiety of the molecule; the main route of biotransformation yields 3-hydroxy-guanfacine (II) which is then conjugated to the O-glucuronide (III) or O-sulphate (IV) (Fig. 1).

The present study describes an analytical procedure for the determination of 3-hydroxy-guanfacine in biological fluids (plasma and urine). After extraction, the metabolite is derivatized by condensing the amidino group with hexafluoroacetylacetone and by methylating the NH and OH groups with methyl iodide. The obtained derivative possesses good bioanalytical properties, allowing the quantitation of the OH metabolite in plasma and urine; the O-glucuronide can be determined after enzymatic hydrolysis.

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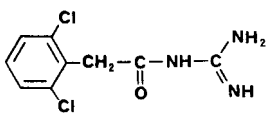
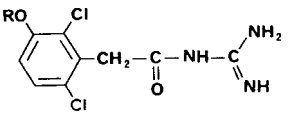


Guanfacine
I



II R = H
III R = Gluc
IV R = SO_3H

Fig. 1. Chemical formulae of guanfacine and its main metabolites.

EXPERIMENTAL

Reagents

Dichloromethane and methyl isobutyl ketone (MIBK) were obtained from Baker (Deventer, The Netherlands) and glass distilled under nitrogen prior to use. Toluene and hexane RS for pesticide analysis were obtained from Carlo-Erba (Milan, Italy) and were used without prior distillation. Tetrabutylammonium hydroxide was purchased from O.S.I. (Paris, France) and the final concentration used was 0.15 *M* in water. The 10% dilution in methanol of hexafluoroacetylacetone (HFAA), obtained from Fluka (Buchs, Switzerland), was prepared just before use.

The solution of 0.5 *M* methyl iodide was obtained by dissolution of 3.12 ml of CH_3I in 100 ml of toluene just before use.

Extraction solvent was prepared by mixing 50 volumes of MIBK with 50 volumes of dichloromethane.

β-Glucuronidase was obtained from Sigma (St. Louis, MO, U.S.A.). It was diluted to 40,000 UF/ml in pH 4.6 acetate buffer. Ethyl- and butylanthraquinone were purchased from Aldrich (Milwaukee, WI, U.S.A.).

Gas chromatography

A Hewlett-Packard 5710 A gas chromatograph equipped with a 15 mCi ^{63}Ni electron-capture detector was used for analysis.

Packed column. The glass column (2 m × 2 mm I.D.) was packed with 3% OV-225 on 100—120 mesh Chromosorb W (Erba-Sciences, Paris, France). The injector port, detector and oven were maintained at 250°C, 300°C and 210°C, respectively. Flow-rate was 30 ml/min for the argon—methane (90:10) carrier gas.

Ethylanthraquinone was used as internal standard. The retention times were 4 min and 5.5 min for the guanfacine metabolite and the internal standard, respectively.

Capillary column. Separation was performed on a glass capillary column (25 m × 0.25 mm I.D.) coated with CP Sil 5 from Chrompack (Middelburg, The Netherlands). The chromatograph was equipped with a glass solid injector (moving needle). The column was operated at 150°C for 2 min, then the oven temperature was increased at 8°C/min to 210°C. Flow-rates were 3 ml/min for the hydrogen carrier gas and 30 ml/min for the argon—methane (90:10) auxiliary gas.

The internal standard was butylanthraquinone. Retention times were 8 min

30 sec and 9 min 45 sec for the guanfacine metabolite and the internal standard, respectively.

Gas chromatography—mass spectrometry

Analyses by gas chromatography—mass spectrometry (GC—MS) were carried out on a Nermag R 10-10 Sidar 11A. A Girdel gas chromatograph was connected to the mass spectrometer and the system was completed by a PDP/8 computer system (Nermag, Rueil-Malmaison, France).

The chromatograph was equipped with a glass solid injector (moving needle) and a glass capillary column (30 m × 0.3 mm I.D.) coated with SE-52 from Chrompack. Helium at a pressure of 1.9 bars was the carrier gas. The oven temperature was programmed from 150°C to 210°C at 8°C/min. Injector and interface temperatures were 300°C. The source temperature was 200°C, the ionisation voltage was 70 eV for the electron-impact mode and 100 eV for the chemical-ionisation mode; the emission current was 250 μA. The chemical-ionisation (CI) spectra were obtained with methane as reactant gas.

Glassware

All glassware was washed with a 1% diluted cleaning solution of Liquinox from Alconox (New York, NY, U.S.A.) and rinsed thoroughly with water, distilled water and methanol. Then it was silanized with 10% hexamethyldisilazane in hexane.

Standard stock solutions

Guanfacine and its 3-hydroxy derivative were synthesized in the Sandoz Laboratories in Basle (Switzerland).

The standard stock solutions of pure butylanthraquinone, ethylanthraquinone and 3-hydroxy-guanfacine were prepared by dissolving 5 mg in 50 ml of methanol. They were stable for one month when stored at 4°C. The dilutions of internal standard, butylanthraquinone (capillary column) or ethylanthraquinone (packed column), were prepared every day. They were first diluted 1 to 20 with methanol and diluted again 1 to 10 with hexane to give a solution containing 1 ng per 2 μl.

Analytical procedure

To 2 ml of plasma or urine were added 1.4 g of sodium chloride, and 5 ml of solvent for extraction. The tube was sealed with a glass cap and shaken for 15 min. After centrifugation (5 min, 2400 *g*), the upper organic phase was transferred to a reaction tube and then evaporated to dryness under a nitrogen stream at 35°C. The dry extract was redissolved in 200 μl of 10% HFAA in methanol. The tube, stoppered with Polytef capsules, was heated in an aluminium heating block at 100°C for 1 h and then evaporated to dryness under a nitrogen stream at 50°C. The resulting dry residue was then taken up in 2.5 ml of methyl iodide solution and 200 μl of tetrabutylammonium hydroxide to which 2 ml of 1 *N* sodium hydroxide were added. The tube was shaken for 20 min. After a brief centrifugation, the organic phase was transferred to a small glass-stoppered tube and evaporated to dryness under a stream of nitrogen at 40°C. The dry residue was dissolved in 400 μl of internal stan-

dard solution, then 1 ml of 1 *N* sodium hydroxide was added to eliminate the excess reagent. The sample was vortexed, centrifuged and 2 μl of the hexane phase were injected into the chromatograph.

In the first collections of urine, the concentration was high, so the samples were first diluted 1 to 4 with distilled water.

Enzymatic hydrolysis

Urine or plasma (2 ml) was buffered at pH 4.6 with acetate buffer and incubated with 0.5 ml of β-glucuronidase solution at 37°C. After 48 h, 1.4 g of sodium chloride were added and the extraction was carried out as for the unchanged drug.

RESULTS AND DISCUSSION

In order to determine the optimal conditions of derivatization and extraction of II, some preliminary experiments were performed.

(1) The derivative obtained by condensing the amidino group of the metabolite with HFAA, as reported previously for guanfacine [3], possesses unsatisfactory GC properties, probably due to the high polarity of the hydroxyl group. After methylation of this group, the response of the resulting O-CH_3 derivative analyzed by GC with an electron-capture detector was more satisfactory.

(2) The extraction conditions used for I do not allow the extraction of the metabolite and therefore different solvents and pH conditions have been tested.

(3) III was essentially recovered in human urine as the O-glucuronide. So, the enzymatic hydrolysis was worked out.

(4) Finally, in order to increase the sensitivity amd specificity, the method has been adapted to a capillary column.

Derivative identification

The structure of the ultimate derivative obtained after the two chemical reactions on I and its metabolite II was determined by GC—MS.

(A) Methylation of the pyrimidino derivative of guanfacine. After condensation of I with HFAA (derivative A), methylation with methyl iodide led to V (Fig. 2), the structure of which was elucidated by GC—MS. A comparison of the spectra of V and of the pyrimidino guanfacine derivative A is shown in Fig. 3.

The following observations are of relevance. (1) For the two compounds, in the electron-impact mode, the molecular ions are very small, If the spectrum is taken in the chemical-ionisation mode (Fig. 4), the quasi-molecular ion MH^+ confirms the structure for V. (2) The characteristic ions of the aromatic moiety were seen in both spectra of V and A. (3) All the ions containing the nitrogen atoms had a mass increase of 14 units for derivative V. The methylation of derivative A occurs on the nitrogen atom in the α-position to the carbonyl group.

The fragmentation pattern of V is displayed in Fig. 5.

(B) Methylation of the pyrimidino derivative of 3-hydroxy-guanfacine. The

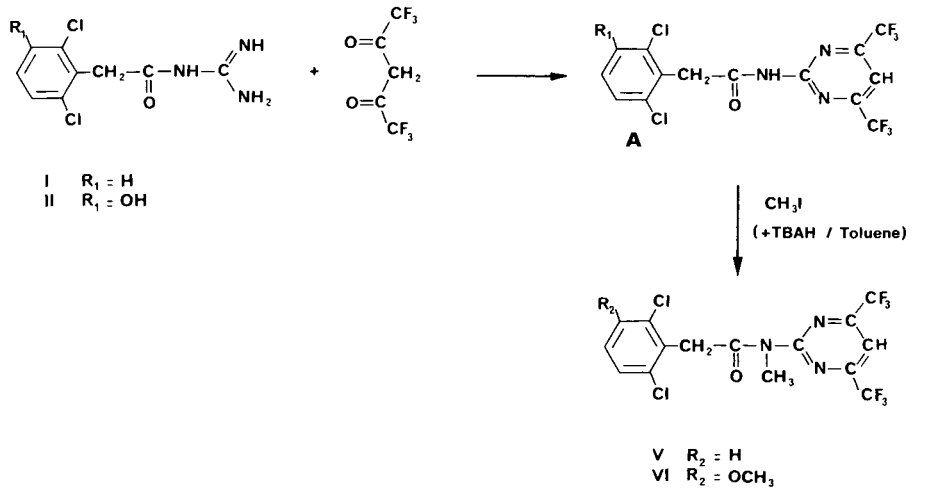
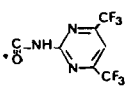
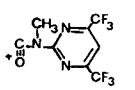
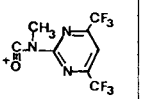
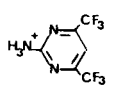
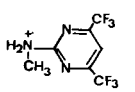
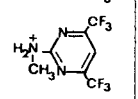
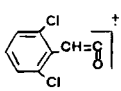
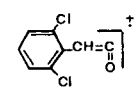
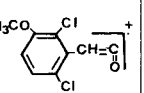
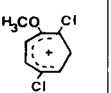


Fig. 2. Formation of guanfacine derivatives by condensation with HFAA and extractive alkylation.

Derivative A			Derivative V			Derivative VI		
m/e	Characteristic ions	A*%	m/e	Characteristic ions	A*%	m/e	Characteristic ions	A*%
417-419	M^+	10	431-433	M^+	3	461-463	M^+	1
382-384	$M^+\_35 = M^+\_Cl$	30	396-398	$M^+\_35 = M^+\_Cl$	18	426-428	$M^+\_35 = M^+\_Cl$	75
258		10	272		25	272		20
232		20	245		20	246		15
186-188		98	186-188		45	216-218		60
159-161		75	159-161		45	189-191		50

*: A % = ABUNDANCE PEAK in % of BASIC PEAK

Fig. 3. Main fragment ions obtained in electron-impact mass spectra of guanfacine derivatives.

structural similarity of I and II allows us to presuppose that methylation on the nitrogen atom in the α-position of the carbonyl group also occurred for the metabolite. However, for the hydroxyl group on the benzene ring, methylation is also likely in addition. This was confirmed by GC—MS analysis.

In the chemical-ionisation mode (Fig. 6) the quasi-molecular ion MH^+ obtained is 30 units higher than that of the guanfacine derivative V, showing that one hydrogen was substituted by the O-CH_3 group. The assumed struc-

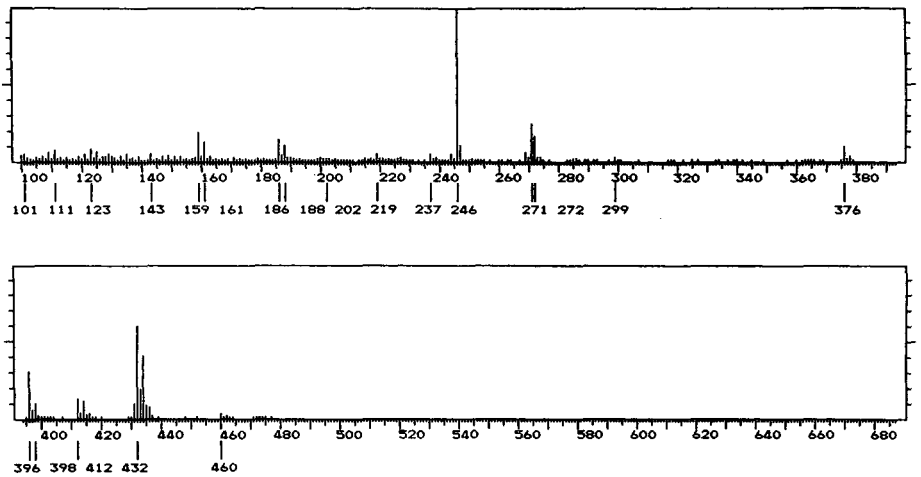


Fig. 4. Chemical-ionisation mass spectrum of guanfacine derivative V.

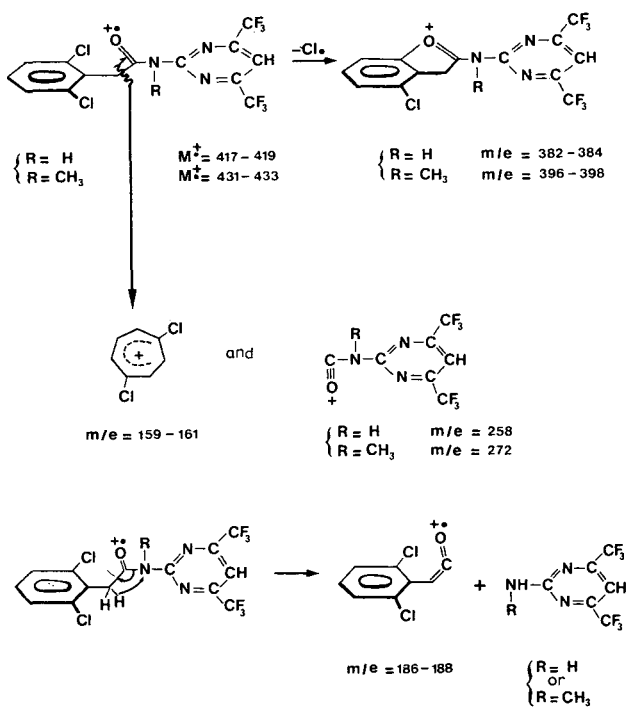


Fig. 5. Fragmentation pattern of guanfacine derivatives.

ture for this compound (VI, Fig. 2) is corroborated by examination of the main fragment ion, displayed in Fig. 3.

It is possible to conclude that the hydroxyl group of the metabolite is a position of methylation. This is confirmed by the presence of two fragments at *m/e* 216 and *m/e* 189, respectively, homologous to ions *m/e* 186 and *m/e*

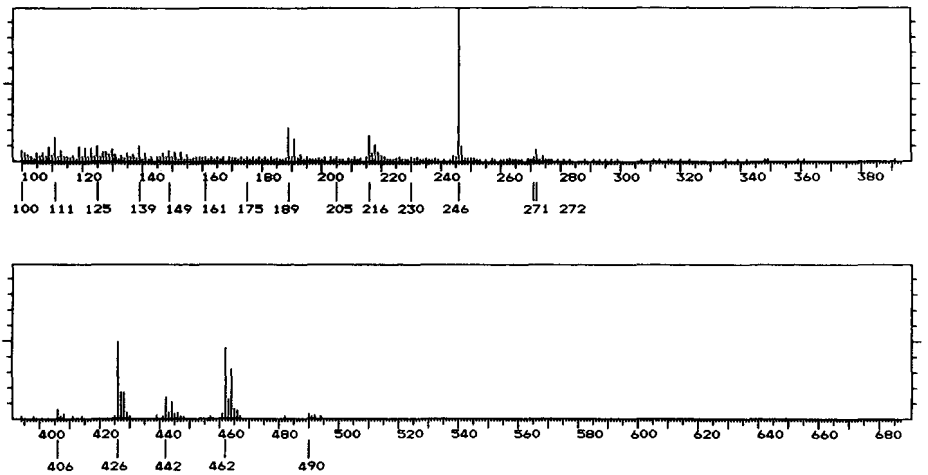


Fig. 6. Chemical-ionisation mass spectrum of 3-hydroxy-guanfacine derivative VI.

159 seen in the spectra of the two guanfacine derivatives A and V in the electron-impact mode. As for guanfacine, the second position of methylation is the nitrogen atom in the α-position to the carbonyl group. This is confirmed by the presence of fragments at *m/e* 272 and *m/e* 246, which are also seen in the spectrum of V. Furthermore, the pattern of fragmentation for the derivative of II is the same as that of the guanfacine derivatives A and V, as shown in Fig. 5.

Derivative synthesis

The formation of the pyrimidino derivative by condensation of the amidino group of II with HFAA was carried out as reported previously for I [3]. It has been verified by thin-layer chromatography that the reaction was complete. The methylation was performed as for the extractive alkylation [4] described previously for a diuretic, Brinaldix [5].

Solvents of the methylation reaction. Among the different solvents used for the extractive methylation, chloroform, dichloromethane, benzene and toluene gave good yields, but toluene was chosen on account of some advantages: indeed, the organic phase is the top layer in contrast to chloroform and dichloromethane, and the toxicity of toluene is lower than of benzene. Extraction by chloroform or dichloromethane leads to a larger, unfavorable residue. Extraction and methylation are as complete with 2.5 ml as with 5 ml of solvent. So, 2.5 ml are used to accelerate the evaporation step.

Temperature and heating time. Assays with different temperatures (ambient, 45°C and 60°C) and heating times (15 to 45 min) were performed. At 60°C the reaction was nearly complete after 20 min and the reproducibility was better than at 45°C..

Under these conditions, I derivatized by condensation with HFAA is completely methylated without degradation.

Sample extraction

Various parameters of the extraction procedure were analyzed. Different solvents (chloroform, dichloromethane, benzene, diethyl ether or MIBK) resulted in less than 10% yield at different pH values (1.4, 5.4, 7, 9, 13).

Since metabolite II is water-soluble, saturation with sodium chloride was necessary. Instead of MIBK which gave the best yield but took a long time to be evaporated, a mixture of MIBK and dichloromethane was preferred.

Recovery

The recovery of II was determined by adding various known amounts of II to human urine (Table I) and analyzing each sample in triplicate according to the described procedure. Compared to a similar series of unextracted reference standard of II, the recoveries varied from 81.5 to 101.5% with an average of 87.7 ± 3.0%, and were independent of the concentration within the tested range. In plasma the recovery of II was 80.1 ± 5.2%.

TABLE I

RECOVERY OF 3-HYDROXY-GUANFACINE ADDED TO HUMAN URINE SAMPLES

Assays were performed on 2 ml of urine.

3-Hydroxy-guanfacine added (ng/ml)	Recovery (mean* ± S.E.M.)
25	101.5 ± 3.6
50	83.4 ± 2.5
100	93.4 ± 0.9
200	83.0 ± 0.1
400	81.5 ± 2.3
600	83.5 ± 2.0

*Average of three determinations.

Selectivity

Evidence of selectivity of the method was furnished by characteristic GC retention times of the reference compounds and the lack of interfering peaks in plasma or urine extracts from subjects who had not received guanfacine. Figs. 7 and 8 show chromatograms obtained after determination on a packed column or on a capillary column. Some drugs, usually prescribed with guanfacine, which might interfere in the assay were tested, e.g. diuretics (furosemide or clopamid) and β-blocking agents. No interfering peak was observed under the described conditions.

Reproducibility and accuracy

Reproducibility and accuracy of the determination were estimated by preparing urine standards of several concentrations for triplicate analysis. The results presented in Table II for the packed column and the capillary column show that the overall accuracy of the procedure was good.

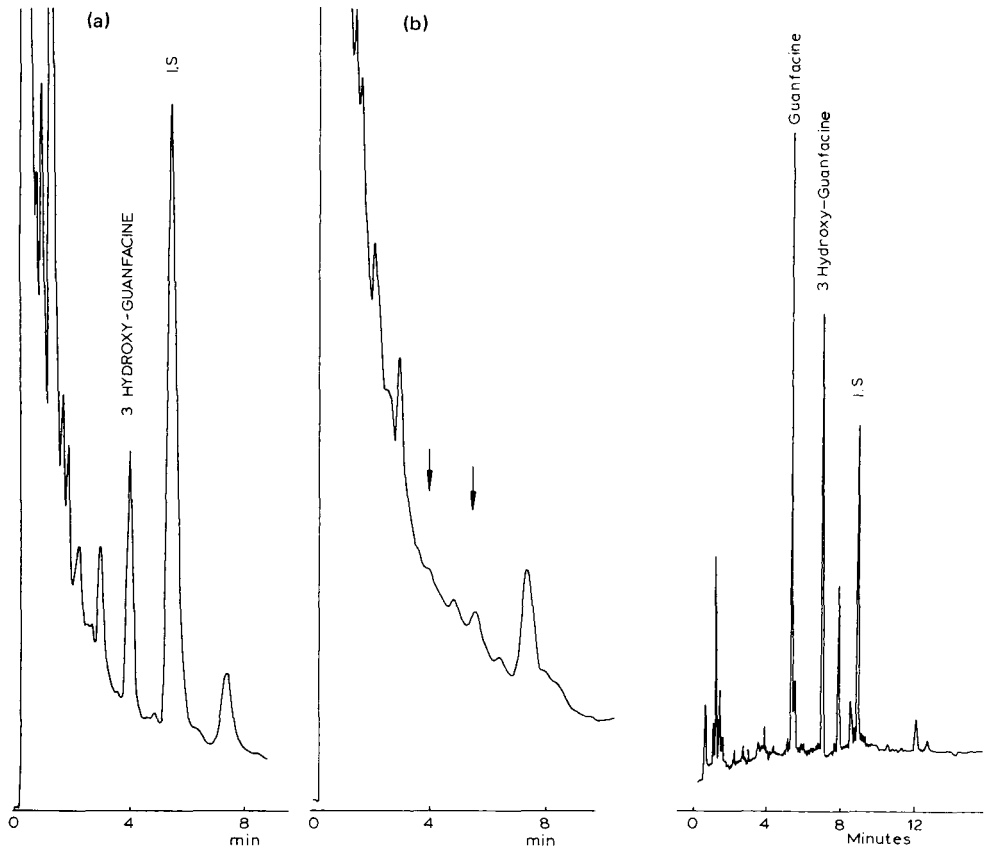


Fig. 7. Gas chromatogram of a 0—24-h urine extract from a subject given 2 mg of guanfacine orally, on a packed column (a). Blank urine (b).

Fig. 8. Gas chromatogram of a 0—24-h urine extract from a subject given 2 mg of guanfacine orally on a capillary column.

The relative variations of the determination are better using capillary columns (range 0.2—6.3) than packed columns (range 5.1—9.6).

The accuracy of the method expressed by the mean percentage deviation of all concentrations from the theoretical value, ranged from 3.6 to 10.1% (mean 6.8%) for the packed column and from 1.6 to 9.9% (mean 4.8%) for the capillary column.

Linearity and sensitivity

The linearity of the method was demonstrated by calculation of the linear correlation coefficients of peak area ratio versus metabolite II concentration.

On the packed column the coefficients were $Y = 0.0080X - 0.0063$ ($r = 0.9951$).

On the capillary column the obtained curve was split into two straight lines. For concentrations below 25 ng/ml the coefficients were $Y = 0.0091X$

TABLE II

REPRODUCIBILITY AND ACCURACY OF DETERMINATION OF 3-HYDROXY-GUANFACINE ADDED TO HUMAN URINE

Urine samples were 2 ml.

Amount added (ng/ml)	Amount recovered (ng/ml) (mean ± S.E.M.)	Coefficient of variation (%)	Accuracy (mean ± S.E.M.)
Packed column			
10	10.62 ± 0.51	8.2	6.21 ± 1.80
20	20.17 ± 0.70	6.0	4.87 ± 0.80
50	44.93 ± 2.50	9.6	10.13 ± 6.01
100	81.70 ± 5.25	9.1	6.57 ± 2.13
120	123.02 ± 6.32	8.9	9.25 ± 1.53
200	201.27 ± 7.25	5.1	3.63 ± 0.64
Capillary column			
5	5.22 ± 0.16	5.31	9.01 ± 2.36
25	26.81 ± 0.98	6.34	7.84 ± 2.23
50	44.80 ± 1.50	4.73	6.42 ± 2.80
100	108.07 ± 1.15	1.51	8.07 ± 1.15
200	196.82 ± 0.23	0.17	1.59 ± 0.12
400	392.54 ± 10.47	4.62	4.14 ± 0.86
600	607.17 ± 14.98	3.49	2.50 ± 1.19

(r = 0.9986). For concentrations between 25 and 600 ng/ml the coefficients were $Y = 0.00434X + 0.11021$ (r = 0.9994).

The sensitivity, defined as a signal-to-noise ratio greater than 3, was 10 ng/ml from 2 ml of urine with packed columns and 2 ng/ml from 1 ml of urine with capillary columns. In the latter case, the reproducibility was about 10%. In plasma, from a 1-ml sample, the sensitivity is about 5 ng/ml though lower levels can be detected with a less good precision.

In biological fluids, the 3-hydroxy metabolite (II) is also found as an O-glucuronide conjugate and its determination can be performed after enzymatic hydrolysis. The optimal conditions were determined on a 0—24-h urine sample from a subject who received 2 mg guanfacine orally. The optimal pH was determined previously and was found to be 4.6 (acetate buffer).

Incubations with β-glucuronidase using various reaction times showed that after two days the hydrolysis appears to be complete. The same assays with 30,000 UF instead of 20,000 UF of β-glucuronidase enzyme led to comparable results.

Application

The described procedure can be used for the determination of II in biological fluids. The use of a wall-coated capillary column with a high efficiency allows both high specificity and very good sensitivity. In addition, guanfacine itself, after the derivatization steps of this procedure, elutes on packed column at the beginning of the chromatogram together with interfering sub-

stances from the reaction mixture. In contrast, on the capillary column, the peak of guanfacine occurs on the chromatogram at a retention time of about 6 min (Fig. 4), and is well separated from peaks of interfering substances.

Although the sensitivity for I is not as good as that of the direct method described previously [3], the determination of I is possible together with its metabolite II in urine by using capillary columns since the levels of guanfacine in urine are sufficiently high. This procedure has the advantage of reducing the analysis time. In Table III are displayed the precision and the accuracy for the assay of the parent drug in urine.

TABLE III

REPRODUCIBILITY AND ACCURACY OF DETERMINATION OF GUANFACINE ADDED TO HUMAN URINE ASSAYED AS DESCRIBED FOR 3-HYDROXY-GUANFACINE (GC WITH CAPILLARY COLUMN)

Amount added (ng/ml)	Amount recovered (ng/ml, mean ± S.E.M.)	Coefficient of variation (%)	Accuracy (mean ± S.E.M.)
25	26.24 ± 1.65	10.91	9.91 ± 7.89
50	48.03 ± 0.71	2.55	3.93 ± 1.41
100	99.03 ± 3.74	6.55	5.04 ± 1.34
200	201.18 ± 7.67	6.60	6.76 ± 1.45
400	400.15 ± 15.86	6.87	5.19 ± 1.50
600	602.50 ± 9.96	2.87	2.12 ± 0.72

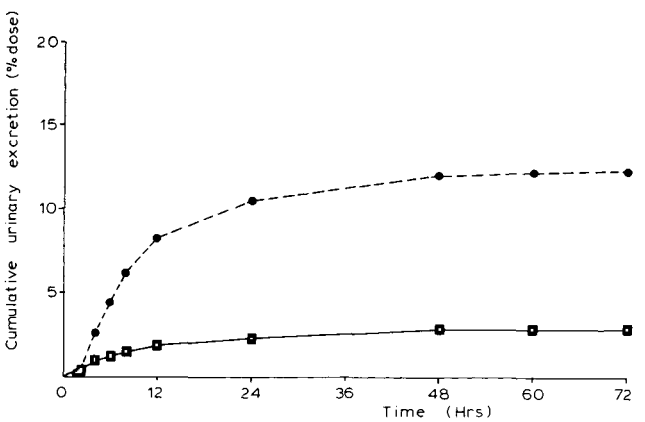


Fig. 9. Mean urinary cumulative excretion curve (n = 6) as a function of time for 3-hydroxy-guanfacine (——) and its glucuronide (·····).

Nevertheless, for the determination of I in plasma, the previous method [3] should preferably be used since the therapeutic levels are lower.

The described method was used for the determination of both II and III in the urine of six subjects who received 2 mg of guanfacine orally. The mean urinary cumulative excretion (percentage of administered dose) and the mean urinary excretion rate (expressed in μg/h) are shown in Figs. 9 and 10, re-

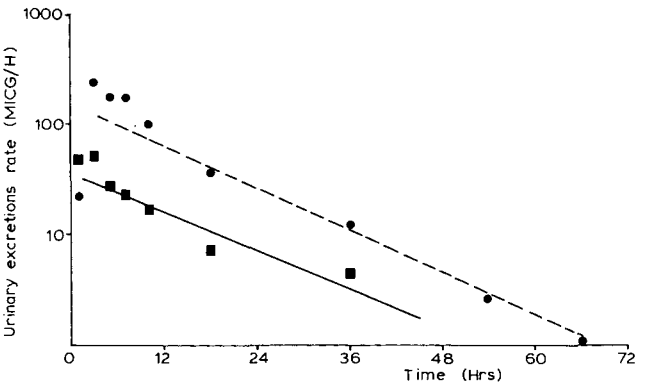


Fig. 10. Mean urinary excretion rate curve (n = 6) as a function of time for 3-hydroxyguanfacine (——) and its glucuronide (·····).

spectively. Fig. 9 shows that the hydroxy metabolite is excreted in urine essentially as the O-glucuronide. The apparent half-life of III (about 13 h) is not significantly different from that of metabolite II.

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RAPID EXTRACTION OF LEUKOTRIENES FROM BIOLOGIC FLUIDS AND QUANTITATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Previous methods for the recovery and quantitation of leukotrienes have involved tedious extraction procedures, and high-performance liquid chromatographic (HPLC) techniques with significant limitations. We have designed a method to extract leukotrienes from biologic fluids using commercially available silica mini-columns requiring minimal preparation. Sample clarification is followed by a sensitive and reproducible HPLC technique which separates and quantifies the leukotrienes LTC_4, LTD_4, LTB_4 (and at least three of their isomers). The entire procedure requires less than one hour per sample.

INTRODUCTION

Leukotrienes are a family of peptido-lipids derived from the metabolism of arachidonic acid and related long-chain polyunsaturated fatty acids via the lipoxygenase pathway. Since these compounds have potent effects upon white cell function, bronchial tone, and several other biological target systems, there has been considerable recent interest in quantifying these compounds in biological samples. Early assays employed one or more biologic endpoints for quantitation (for example, the contraction of the guinea pig ileum) [1]. Although such assays are sensitive to a few nanograms of leukotrienes, they are cumbersome and do not readily distinguish and quantify each of the various leukotrienes, such as LTB_4 and the components of slow-reacting substance of anaphylaxis, or "SRS-A" (leukotrienes C_4, D_4, and E_4). More recent techniques have employed high-performance liquid chromatography (HPLC). Early HPLC methods, using primarily preparative chromatography, also did not separate well the several components of SRS-A and/or did not simultaneously measure LTB_4 [2—6].

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Furthermore, most extraction methods employed the laborious combination of sequential XAD resin columns and silicic acid open-column chromatography [2, 3, 5—7]. A more recent method reported by us [8], using self-packed HPLC columns, was adequately sensitive and selective; however, use of commercially available HPLC columns was not assessed and the extraction procedure (XAD-7 resin) required extensive (1–2 days) preparation prior to use. We report herein a rapid and reliable extraction procedure for leukotrienes that employs commercially available extraction columns which retain leukotrienes (and several of their isomers), which can then be readily eluted and assayed. We also report several modifications of our original HPLC technique which permit improved reproducibility and sensitivity in the quantitation of leukotrienes when using commercial (rather than self-packed) HPLC columns.

MATERIALS AND METHODS

Silica and C_{18} (ODS) extraction columns (Sep-Paks) were purchased from Waters Assoc. (Milford, MA, U.S.A.). Chromatography was carried out using a 5-μm ODS column (25 cm length) purchased from Altex Scientific (Berkeley, CA, U.S.A.). (Similar results to those reported were also obtained in preliminary studies using a 10-μm ODS column from Waters Assoc.) All solvents including water were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) or Waters Assoc. Samples were injected in 50—500 μl of water—methanol (30:70) through a Waters U6K injector. A Waters Assoc. guard column packed with Corasil (ODS) pellicular packing, a Waters 6000A pump, and a Model 441 UV detector with a 280-nm filter (generally set at 0.01 a.u.f.s.) were used for chromatography. Murine mastocytoma cells were stimulated to produce leukotrienes, as previously described [9]; the leukotrienes were then extracted and purified by HPLC [8]. Free fatty acid-free bovine serum albumin was purchased from Sigma (St. Louis, MO, U.S.A.) and A23187 (divalent ionophore) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Reagents for cell culture medium were purchased from GIBCO (Grand Island, NY, U.S.A.).

RESULTS

Chromatography technique

As we have previously observed, the chromatography of leukotrienes is exquisitely sensitive to changes in mobile phase pH (which has major effects on selectivity, α) and in methanol content (which has major effects on capacity factor, k', and lesser effects on α). Despite rigorous control of these variables, the HPLC system previously described by us proved not to be entirely adequate when commercially available columns (Waters C_{18}, 10 μm; Altex C_{18}, 5 μm) were used. Most dramatically, the recoveries (especially of leukotriene C_4) were variable and very poor; in addition, both α and k' varied unacceptably. After extensive empirical changes in mobile phase, optimum solvent characteristics were ascertained: 67% methanol, 33% water, 0.08% acetic acid, 0.04% ammonium hydroxide, brought to a final pH of 6.2. The addition of an excess of acetic acid and ammonium hydroxide (beyond that required to achieve an optimal pH) was empirically discovered to maximize peak sharpness and to

achieve reproducible and acceptable α and k' values. Although not pursued further, this effect was presumed to be due to the increase of ionic strength (ammonium acetate) of the mobile phase. Using this mobile phase at a flow-rate of 1 ml/min, a chromatogram could be obtained in which three leukotrienes (LTB_4, LTC_4, LTD_4), as well as the 11-*trans* isomer of LTC_4 and two non-enzymatically formed 5,12-dihydroxy isomers of LTB_4 could be separated in less than 30 min (Fig. 1). k' values using this mobile phase were: prostaglandin (PG) B_2, 3.8; LTC_4, 4.4; 11-*trans* LTC_4, 4.8; LTD_4, 7.6; LTB_4, 8.4. Although retention times proved reproducible from day to day, a standard curve using PGB_2 (as internal standard) and each of the leukotrienes was derived each day prior to injecting unknown samples.

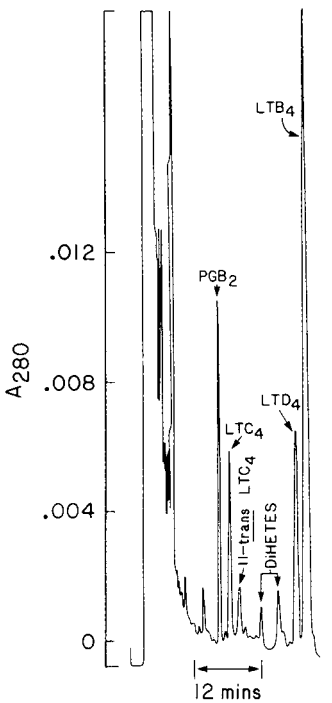

Fig. 1. Elution pattern of varying amounts of standards: PGB_2, LTC_4, 11-*trans* LTC_4, LTD_4, two non-enzymatically formed Δ^6-*trans*-LTB_4 [dihydroxyeicosatetraenoic acid (DiHETE)] isomers of LTB_4, and LTB_4. Column, 5 μm ODS (Altex); flow-rate 1 ml/min; mobile phase, methanol—water—acetic acid— ammonium hydroxide (67:33:0.08:0.04), pH 6.2.

Although it solved the problem of achieving a reproducible elution pattern, the above technique did not correct the variable and inadequate recovery of leukotrienes (especially LTC_4 and LTD_4) from the Altex column when nanogram to microgram quantities of standard (of known spectrophotometric absorbance) were injected onto the column. This problem was less severe for LTB_4 and minimal for the internal standard PGB_2, and therefore was presumptively ascribed to interactions between the peptide sequences of LTC_4 and LTD_4 and the column, leading to excessive retention. This problem was obviated by flushing the column overnight with several hundred ml of 3% diso-

dium EDTA (Fig. 2a) and maintaining the improvement by injecting 2–3 ml of EDTA through the U6K injector, guard column and column every morning prior to chromatography (Fig. 2b). This technique markedly increased recovery of LTC_4 (and the consequent sensitivity of the assay), such that reproducible and linear standard curves were achieved. Minimum detectable quantities were approximately 1 ng for LTB_4 and 2–3 ng for LTC_4 and LTD_4.

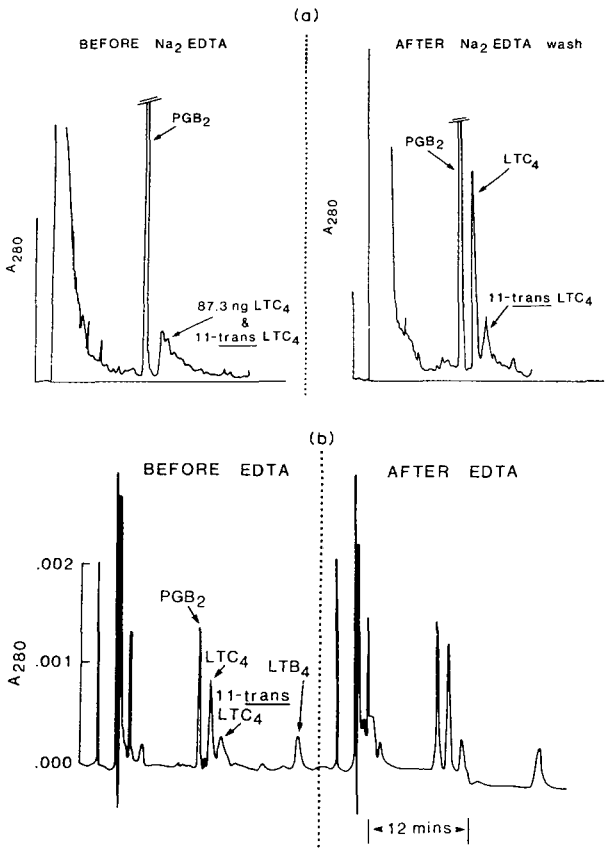

Fig. 2. (a) Effect on recovery of LTC_4 and 11-*trans* LTC_4 of an initial overnight rinse of the chromatographic column (Altex ODS) with disodium EDTA. (b) Effect of a brief (10 ml) morning rinse of EDTA to regenerate the HPLC column for the recoveries of leukotrienes after several hours' usage the preceding day. Note that the recoveries of LTC_4, 11-*trans* LTC_4 and LTB_4 are increased 44%, 25% and 22%, respectively, by EDTA, whereas that of PGB_2 is increased insignificantly (5%).

Extraction procedure

Initial attempts were made to develop a simple extraction technique using an ODS extraction column, following the work of Powell [10], who had previously observed greater than 90% recovery into methyl formate of prostaglandin standards (and about 50% recovery of 15-hydroxyeicosatetraenoic acid) added to biologic fluids, using this Sep-Pak. However, our initial studies demonstrated that recoveries of leukotrienes into methyl formate were poor. Although these

TABLE I

RECOVERY OF STANDARDS ADDED TO MASTOCYTOMA BUFFER (MCT, ref. 9) OR CULTURE MEDIUM (NCTC 135/MEDIUM 199) IN THE ABSENCE OR PRESENCE OF 1% FATTY ACID-FREE BOVINE SERUM ALBUMIN

Recoveries (mean ± S.D.) were assessed by measurement of peak heights of the samples and comparing them to values for a "standard curve" derived from external standard injections on the same day.

	PGB_2	LTB_4	LTC_4	LTD_4
Silica Sep-Pak				
MCT, no BSA*	82 ± 7 (n = 8)	76 ± 8 (n = 7)	93 ± 12 (n = 7)	86 ± 8 (n = 2)
MCT, with BSA	85 ± 8 (n = 5)	75 ± 6 (n = 5)	65 ± 10 (n = 5)	—
Medium, no BSA	91 ± 10 (n = 12)	76 ± 11 (n = 9)	81 ± 20 (n = 10)	—
Medium, with BSA	87 ± 10 (n = 3)	77 ± 6 (n = 3)	75 ± 5 (n = 3)	—
ODS (C_{18}) Sep-Pak				
Medium, no BSA	87 ± 15 (n = 7)	57 ± 20 (n = 6)	39 ± 18 (n = 6)	44 ± 8 (n = 2)

*BSA = bovine serum albumin.

recoveries were somewhat improved using methanol as the eluent (Table I), recoveries (particularly of LTC_4) were still unacceptable. Initial attempts to extract samples on silica columns instead of ODS were hindered by several artifactual peaks co-eluting near or with the leukotriene standards. These artifacts were traced to an unidentified contaminant washed off the silica in the Sep-Pak (and/or its container) after exposure to aqueous samples or water. It was empirically discovered that samples could be clarified of these peaks by a thorough pre-washing of the Sep-Pak using 120 ml of water followed by 20 ml of methanol and 20 ml of hexane. This procedure not only cleaned, but also pre-wetted, the Sep-Pak; contrary to the manufacturer's instructions, the latter was necessary for retention of compounds such as leukotrienes. (It was also noted that certain brands of plastic syringes used in the extraction procedure led to similar artifacts; therefore, only glass syringes were used thereafter.)

Following these washes, samples were placed on the extraction column and successively eluted with 10 ml of hexane, 5 ml of methylene chloride and 20 ml of pure methanol. The sample was then dried down using a rotary evaporator, brought up in methanol—water (30 : 70) and an aliquot injected on the column. For samples to which bovine serum albumin (1 g per 100 ml) had been added, the protein was first precipitated using 3 volumes of cold acetonitrile and 1 volume of cold methanol, followed by vigorous vortexing, 30 min of refrigeration and centrifugation. Following this, the supernatants were decanted, evaporated and the residue brought up in 8 ml of water and placed on the Sep-Pak as described above.

Using these techniques, recoveries of leukotrienes and internal standard from

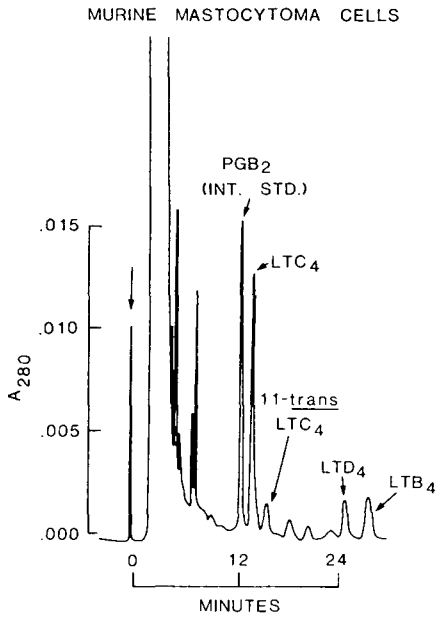

Fig. 3. Chromatogram of leukotrienes (and internal standard PGB_2) synthesized by murine mastocytoma cells, after protein precipitation, centrifugation, and sample extraction on silica Sep-Pak. Not labelled are several smaller peaks migrating between 11-*trans* LTC_4 and LTD_4 representing compounds which co-migrate with the non-enzymatically formed 5,12-dihydroxy isomers of LTB_4. Medium, minimum essential medium; extraction, silica (methanol); column, ODS (5 μm); mobile phase, methanol—water—acetic acid—ammonium hydroxide, pH 6.2; flow-rate 1 ml/min.

either buffer or culture medium were acceptably high and reproducible (in the presence or absence of exogenous bovine serum albumin) (Table I). However, it should be noted that in the case of LTC_4, the recovery appeared to be somewhat decreased by the presence of protein (Table I). Since the internal standard PGB_2 was not always recovered in an exact and equal proportion to each leukotriene under all circumstances, we generally correct the measured value of leukotrienes in biologic samples first for the recovery of the internal standard (PGB_2) and then for the ratio of the recovery of each leukotriene relative to PGB_2 for the sample type studied (i.e. medium or buffer) (Table I).

Using this system we measured the quantities of leukotrienes in one sample generated by murine mastocytoma cells [9] after extracting identical aliquots using three techniques: XAD-7 alone, XAD-7 followed by open silicic acid columns, and the simplified Sep-Pak extraction system described above (Fig. 3; Table II). Although recoveries after XAD-7 column alone were somewhat greater than with the silica Sep-Pak, the latter was comparable to the combination of XAD-7 and silicic acid open columns. Thus LTC_4 had a slightly greater recovery, LTB_4 isomers an identical recovery, and LTB_4 a slightly reduced recovery after Sep-Pak extraction, compared to XAD-7 and silicic acid columns (Table II).

TABLE II

COMPARISON OF THE RECOVERIES OF LEUKOTRIENES AFTER DIFFERENT EXTRACTION/PURIFICATION PROCEDURES

Aliquots of medium from one sample of murine mastocytoma cells, treated as previously described [9], were extracted on silica Sep-Pak columns (as described in text), or on XAD-7 columns (with or without open silicic acid columns) as previously described [8, 9]. Amounts of leukotrienes recovered after each method of extraction were quantified by HPLC on the same days using absolute absorbance units for quantitation. Values are expressed as percentage recovered (mean of two determinations each) relative to recovery from XAD-7 column alone (taken as 100%).

	Sep-Pak	XAD-7 and open silicic acid columns
LTC_4	83	69
11-*trans* LTC_4	63	63
Δ^6-*trans*-LTB_4 (isomer I)	66	68
Δ^6-*trans*-LTB_4 (isomer II)	63	63
LTB_4 (isomer III)	59	74

DISCUSSION

Most previous methods for the HPLC quantitation of leukotrienes either lack the required sensitivity, specificity, and selectivity for use in analytical separation of leukotrienes, or else they require tedious and time-consuming extraction processes (see, for example, refs. 2–7). For example, a new lot of XAD-7 can require more than a day in preparation alone before it can be used, and variable recoveries can result from impurities in some batches. We developed earlier an HPLC method which partly solved the first series of problems [8]. However, when adapted for use on commercially available columns, the reproducibility of the method and the recovery of leukotrienes proved to be inadequate. We discovered that a combination of increasing the ionic strength of the mobile phase and washing the column with 3% disodium EDTA, as well as exact preparation of solvent batches, resolved these problems. Although the mechanisms of the first two effects were not pursued further, they might be explicable in the first case, in part, by the increased buffering capacity of the system (thereby preventing even minimal changes in pH during the course of the HPLC runs), or, more likely, by a form of ion pairing with ammonium and acetate ions acting essentially to suppress both free carboxyl (COO^-) and amino (NH_3^+) functions on leukotrienes C_4 and D_4 which could not be simultaneously ion-suppressed by either acetic acid or ammonium hydroxide alone. It may be fruitful in the future to attempt to apply more conventional ion-pairing techniques (e.g. tetrabutylammonium ion in phosphate buffer) to the HPLC assay of leukotrienes. The EDTA effect suggests that negatively charged moieties on the leukotrienes were interacting with some cations retained on the column, leading to a destruction of the triene chromophore or to marked retention of all molecules except for a small proportion which were ion-suppressed at the pH used.

Although our initial attempts to use an ODS extraction column were not

encouraging, we found that we could reproducibly extract samples using a normal-phase silica extraction column providing that it was extensively washed beforehand to remove several spurious peaks co-eluting with leukotrienes and absorbing at 280 nm. Since extraction was carried out on a silica column using organic solvents, many unwanted contaminants with a lesser polarity than prostaglandins and leukotrienes (such as some neutral lipids) might be expected to be removed by the hexane or methylene chloride washes. This can be contrasted to XAD which primarily clarifies the sample of salts and polar compounds. Other investigators may wish to modify the choice of solvents used for particular purposes (e.g. substitute chloroform or ether for hexane or methylene chloride during the extraction), but ethyl acetate should be avoided since it will remove the internal standard PGB_2 and 5,12-dihydroxyeicosatetraenoic acids from retained LTC_4 and LTD_4 [9]. Some more polar compounds elute from the silica Sep-Pak with the leukotrienes but they migrate from the HPLC column in, or close to, the solvent front, with little or no baseline disturbance at 280 nm remaining by the elution time of the compounds of interest. However, proteins should first be removed by a denaturation/centrifugation step and cells, cell fragments and other particulates should likewise be removed by filtration or centrifugation. Thus, the current technique combines an extraction technique based on the retention of hydrophilic (polar) compounds on silica with an HPLC column which retains progressively more hydrophobic (non-polar) compounds with increasing avidity. The leukotrienes (being long-chain fatty acids with polar constituents) are selectively recovered after the combination of these two steps.

A sample can be extracted and quantitated on HPLC using the system described above in less than 1 h compared to 1—1.5 days required for the initial clean-up of the XAD, the extraction, then open silicic acid column chromatography, and finally HPLC. Furthermore, data obtained measuring aliquots of a sample of leukotrienes generated by mastocytoma cells suggest that the current technique yields results similar to those derived using the more time-consuming extraction techniques. Although recoveries are lower than those after XAD alone, the loss is modest and more than compensated for by the ease and speed of processing samples. Furthermore, this system can be used analytically or semi-preparatively (i.e., microgram quantities of leukotrienes). Thus this system should facilitate the measurement of leukotrienes in large numbers of biologic samples.

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QUANTITATION OF HARRINGTONINE AND HOMOHARRINGTONINE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Harringtonine and homoharringtonine are naturally occurring alkaloids with demonstrated antineoplastic activity against certain types of leukemias in cell cultures, experimental animals, and initial clinical trials. Sample preparation consists of addition of the internal standard (one compound used as the internal standard for the other), solvent extraction with methylene chloride, washing with ammonium formate, and evaporation to dryness. The residue is dissolved in the mobile phase (40% methanol—60% 0.1 *M* ammonium formate) and an aliquot is chromatographed on a μC_{18} reversed-phase column (flow-rate 1.5 ml/min). Peaks are detected with a spectrophotofluorimeter by monitoring the emission at 320 nm with excitation wavelength of 280 nm. Limit of detection is 10 ng/ml (20 n*M*) for both compounds; reproducible quantitation can be made to 30 ng/ml (60 n*M*).

INTRODUCTION

Extracts from a yew-like coniferous tree (*Cephalotaxus hainanensis* Li) have been used for the treatment of tumors in the Fujian province in China for a long time. Paudler et al. [1] isolated several active principles from a related plant. Among the esters of cephalotaxine several exhibited activity against L-1210 lymphoid leukemia and P-388 lymphocytic leukemia [2]. The alkaloids that can be isolated from the genus *Cephalotaxus* have been reviewed [3].

Harringtonine and homoharringtonine (Fig. 1) have been characterized [4] and partial synthesis has been attempted [5, 6]. They have been tested in a variety of experimental tumor systems; a clinical brochure on homoharringtonine summarizes available toxicological as well as antitumor activity informa-

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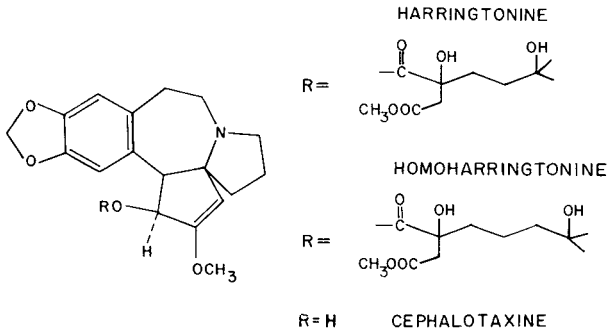

Fig. 1. Chemical structures of harringtonine and homoharringtonine, derivatives of cephalotaxine.

tion [7]. After encouraging initial clinical trials in China [8], both drugs are now being used in several cancer treatment centers in China [9]. Current research with these drugs, including the proposed mechanism of action, has been reviewed [10]. Homoharringtonine is now available in the U.S.A. for Phase I clinical trials, and harringtonine is also expected to be studied in humans.

Analytical methodologies are obviously required for the study of the pharmacokinetics and metabolism of these drugs. Some of the active principles in crude alkaloid extracts were characterized by electron ionization mass spectrometry after gas chromatographic separation [11]. A mass spectrometric method, utilizing chemical ionization, has been developed for the analysis of both drugs [12]. The authors are not aware of any other published method for the quantitation of these drugs. The present method utilizes high-performance liquid chromatography (HPLC) in a simple technique requiring equipment frequently available in laboratories supporting Phase I clinical trials.

EXPERIMENTAL

Drugs and reagents

Pure harringtonine and homoharringtonine, and also radiolabeled harringtonine, were supplied by the Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, People's Republic of China). The composition of the pure drugs was confirmed by precise mass measurements: molecular weights agreed within 3—4 millimass units with calculated values. The purity of the compounds was 97—99% as determined by HPLC and thin-layer chromatography. The tritium-labeled harringtonine (universal labeling) had a specific activity of 454 mCi/mg and a radiochemical purity of 99% (purified by HPLC).

All solvents were of HPLC grade: methanol was from Fisher Scientific (Pittsburgh, PA, U.S.A.), water and other solvents were of "distilled in glass" quality from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ammonium formate was purchased from Sigma (St. Louis, MO, U.S.A.); the Aquafluor scintillation cocktail was from New England Nuclear (Boston, MA, U.S.A.).

Stock solutions of harringtonine and homoharringtonine were prepared by dissolving 1.0 mg of each in 0.2 ml of 0.1 *N* hydrochloric acid at room temperature. The solutions were diluted with HPLC grade water and neutralized with

0.1 *M* sodium hydroxide. These stock solutions could be kept in a refrigerator for two weeks. Aliquots of these stock solutions were diluted with HPLC grade water as needed for spiking serum samples or for addition as internal standards. For the animal experimentation, the drugs were weighed as dry powder (provided without excipients) and prepared as described above for the stock solutions, except that sterile saline solution (0.85%) was used instead of water; these solutions were prepared immediately prior to injection into the animals.

Instrumentation

HPLC was carried out using a system consisting of a single pump (Model 110A, Altex, Berkeley, CA, U.S.A.), a manual injector (Model U6K, Waters Assoc., Milford, MA, U.S.A.), a spectrophotofluorimeter (Model FS-970, Schoeffel/Kratos Co., Westwood, NJ, U.S.A.), a 10-mV potentiometer recorder (Omniscribe, Houston Instruments, Houston, TX, U.S.A.), and an electronic filter amplifier (Model 1021A, Spectrum, Newark, DE, U.S.A.). The filter was placed in series with the spectrophotofluorimeter and was used to improve the quality and intensity of the signals.

Samples were evaporated to dryness with a nitrogen evaporator (N-Evap, Organomation Assoc., Shrewsbury, MA, U.S.A.). The spectrophotofluorimeter used in the initial experiments to study the pure compounds was a Model MPF-3 instrument from Perkin-Elmer Co. (Stamford, CT, U.S.A.). Radioactivity counting was made with a conventional scintillation counter (Model 32-55 Tricarb, Packard, New York, NY, U.S.A.). High-resolution mass spectra were obtained with a Model ZAB-1F mass spectrometer system (VG Analytical, Altrincham, Great Britain).

High-performance liquid chromatography

A stainless-steel column (30 cm × 3.9 mm I.D.) filled with a reversed-phase C_{18}-type material of 10-μm particle size (μBondapak C_{18}, Waters Assoc.) was the analytical column. A guard column, filled with Bondapak C_{18}/Corasil (Waters Assoc.), was placed between the injector and the analytical column. The guard column was changed after 75—100 runs or as needed.

The mobile phase was 40% methanol—60% ammonium formate buffer (0.1 *M*, pH 6.8) which was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and thoroughly vacuum-degassed prior to use. The flow-rate was 1.5 ml/min, resulting in an average pressure of 14 MPa in the chromatographic column.

The spectrophotofluorimeter was operated at an excitation wavelength of 280 nm, and emission was monitored at 320 nm. Detector sensitivity was usually 0.05 μA (time constant: 9). The particular settings of filtering and gain on the electronic filter-amplifier were adjusted as needed by the quantity of the drug present; typical settings were 0.02 Hz for cut-off and 5 for the gain.

Sample preparation

Serum samples were obtained from whole blood after clotting for 10—15 min at room temperature and centrifuging at 1000 *g* for 15 min. Plasma samples were obtained with heparin with no preservatives added. Either serum or plasma may be used in the technique developed (Fig. 2).

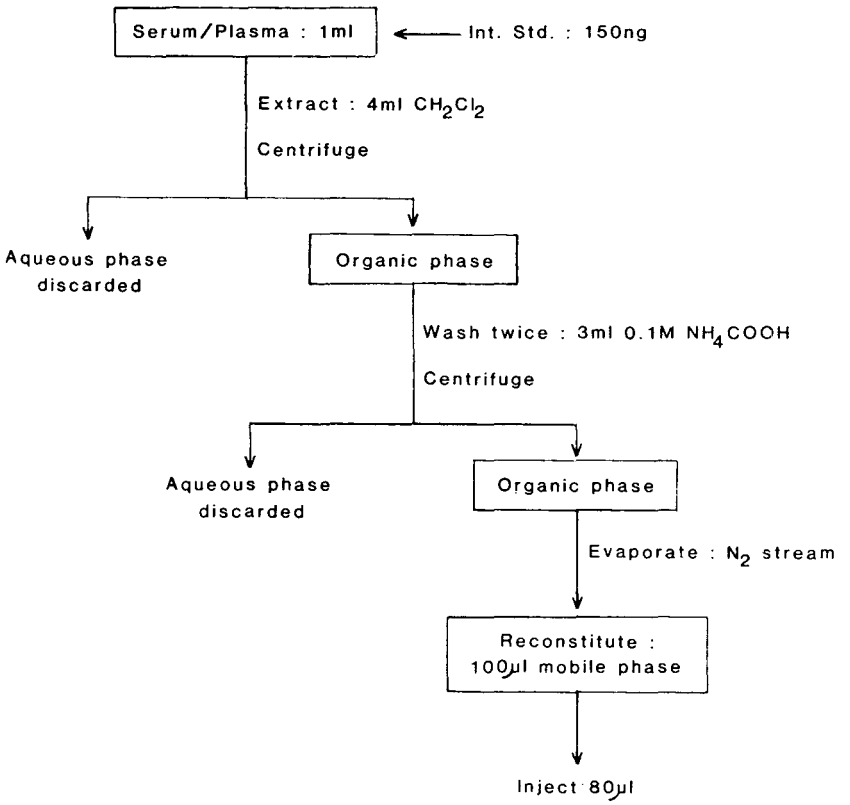

Fig. 2. Scheme of the procedure of extraction of harringtonine and homoharringtonine from serum or plasma.

Starting with a sample volume of 1 ml, the first step was the addition of the internal standard: harringtonine and homoharringtonine served as internal standard for each other. In most experiments the level of the internal standard was 150 ng/ml. The samples were next extracted with 4 ml of methylene chloride. After vortexing for 1 min, the samples were placed in a centrifuge at 50,000 *g* for 15 min at 4°C. After discarding the aqueous layer (top layer), the methylene chloride extract was washed twice with 0.1 *M* ammonium formate buffer. Next, the organic layer was evaporated with a gentle flow of nitrogen in a water bath maintained at 40°C. The dry residue (white or pale yellow color) was dissolved in 100 µl of mobile phase and an aliquot of 80 µl was injected into the liquid chromatograph.

Recovery experiments

Harringtonine was universally labeled with tritium by gas exposure. It was purified by HPLC using the same type of column and mobile phase utilized for analytical work. For the recovery experiments, 6 µg of unlabeled harringtonine was spiked by adding labeled harringtonine corresponding to 3605–17,094 counts. Seven different concentrations were used within this range and all samples were analyzed in duplicate. The spiked samples were extracted as described

for sample preparation. The dry residues were dissolved in 100 μl of mobile phase; 8 ml of scintillation cocktail were added and the mixtures were counted.

Calibration samples

Pure samples of harringtonine and homoharringtonine, dissolved in the mobile phase, were used to establish retention times, to determine sensitivities and detection limits for the pure compounds, and also for routine daily checking of instrument performance.

An appropriate amount of internal standard (usually 150 ng/ml) was added to every calibration sample prior to sample preparation. The same amount of internal standard was added to all samples within a set. Calibration curves were established by spiking normal serum samples with increasing quantities of the drug. A blank sample, i.e. no drug added, was always included in the calibration sets. To compensate for possible experimental errors, a full set of calibration samples (spiked) were analyzed with every set of samples from the experimental animals and from patients undergoing experimental chemotherapy. The concentration range of the drugs in the calibration set covered the expected range in the samples (30–150 ng/ml).

RESULTS AND DISCUSSION

Spectrophotofluorimetry

When harringtonine and homoharringtonine were dissolved in 0.1 *M* hydrochloric acid or methanol and their fluorescence properties were investigated using a conventional spectrophotofluorimeter, maximum emission sensitivity was observed at 320 nm for excitation wavelengths of 290 and 292 nm. It was also observed, in agreement with expectations based upon structure, that these compounds do not have particularly favorable fluorescence properties. However, both selectivity and sensitivity, and also overall performance, were still found to be superior with spectrophotofluorimetry than techniques based on UV absorption. As discussed later, sensitivity that is adequate for initial clinical trials could be obtained when the detector signal was improved electronically. It was also observed that the optimum excitation wavelength (in terms of highest attainable sensitivity) should best be determined individually for the particular spectrophotofluorimetric detector (and overall HPLC system) employed; any value in the 280—292-nm range may be found optimal.

Recovery experiments

A scheme of the sample preparation technique is shown in Fig. 2. Radioactively labeled harringtonine was used to establish the efficiency of the method developed. Unlabeled harringtonine was spiked as described, and seven different concentrations were analyzed in duplicate. Recovery was determined by taking the ratios of the initial counts added with respect to counts obtained after the entire sample preparation procedure was carried out. There was no need for corrections because the quenching of the mobile phase was below 6%. The overall recovery was determined as 71.2% ± 2.04% with a range of 68.2–74.1%. This was considered to be adequate for Phase I clinical trials.

Detection limits, quantitation

The limit of detection of pure harringtonine and homoharringtonine is approximately 0.8 ng of injected material. The limit of detection (detectability) in serum is defined as the amount of substance needed to produce a peak height of the particular drug analyzed twice that of the noise level in the blank sample. Fig. 3A shows that there is no interference in the blank at the retention times of harringtonine (6.0 min) and homoharringtonine (8.5 min). Fig. 3B shows the detection of 10 ng/ml (20 n*M*) for both drugs in human plasma. It is noted that there is a negative peak of rather high intensity just before the harringtonine peak. The intensity of the negative peak appeared to vary from sample to sample. The one shown in Fig. 3 represents a case of a "large" negative peak; usually they are much smaller. At any rate, the appearance of this peak does not affect the quantitation of harringtonine. It is also noted that the negative peak does not occur in mice or rat plasma (see Fig. 5). Finally, it is noted that the limit of detection can only be reached with the full utilization of the filter-amplifier accessory. At these very low levels it is often necessary to make several runs at various cut-off frequencies and gain settings to provide the best signal-to-noise ratio.

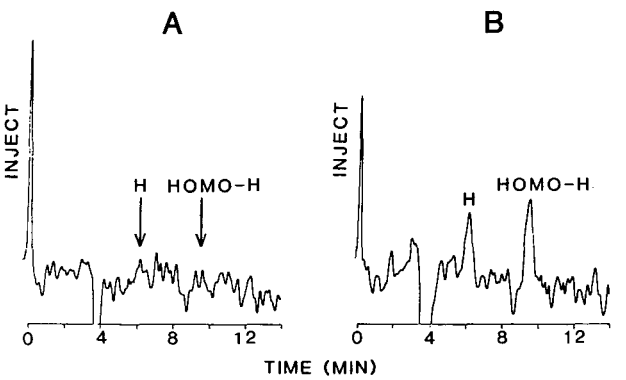

Fig. 3. Detection limit of harringtonine (H) and homoharringtonine (HOMO-H). A = blank serum with no drugs added; B = detection of 10 ng/ml (20 n*M*) of each drug added.

The limit of quantitation was established as three times that of the limit of detection, i.e. 30 ng/ml (60 n*M*), for both harringtonine and homoharringtonine. An illustration of this is shown in Fig. 4. Here different settings were used on the filter-amplifier to demonstrate the variations obtainable in noise levels. It was concluded that the sensitivity of the technique was adequate for the expected use in Phase I trials in all except the lowest initial dose levels. It may be possible to extend detectability by producing a derivative of the compounds with enhanced fluorescence. This, however, would involve more complex sample preparation and will probably not be needed because blood levels greater than 60 n*M* are expected with therapeutic doses.

Quantitation of either drug was accomplished with the aid of calibration curves consisting of a plot of observed peak height ratios of the drug analyzed/internal standard against known amounts of the drug used in spiking the cali-

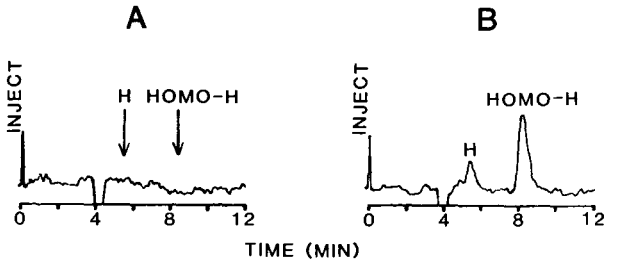

Fig. 4. Quantitation limit of harringtonine (H) and homoharringtonine (HOMO-H). A = blank serum with no drugs added; B = quantitation of 30 ng/ml (60 n*M*) of H in serum with 150 ng/ml HOMO-H added as internal standard.

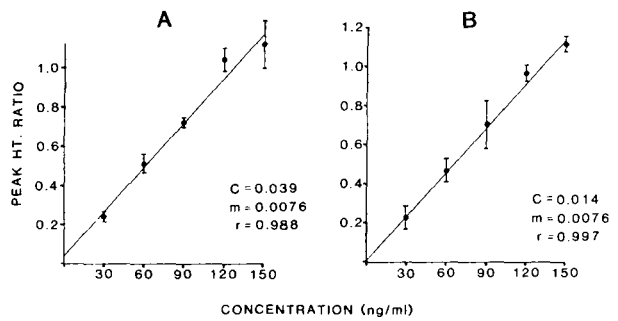

Fig. 5. Calibration curves for the quantitation of harringtonine (H) and homoharringtonine (HOMO-H) in human serum. The drugs served as internal standards for each other. Ordinates: peak height ratios of drugs measured/internal standard. Reproducibility of individual points is indicated for $n = 5$. The values c, m, and r were obtained by conventional regression analysis.

bration samples. The calibration curves (Fig. 5) were straight lines passing through or very near the origin with correlation coefficients (r) routinely in the 0.988–0.997 range. The reproducibility of the technique for both drugs in human plasma is illustrated in Fig. 5 ($n = 4$).

Applications

The technique is now being used for quantitation of both harringtonine and homoharringtonine in experimental studies (both mice and rats) and will be applied for patient monitoring when Phase I clinical trials commence. Fig. 6 illustrates a typical analysis of harringtonine in BDF1 mice with a dose of 3 mg/kg drug injected intraperitoneally. The amount of homoharringtonine added as internal standard was 150 ng/ml. Using a calibration curve, the amount of harringtonine in this sample was determined to be 149 ng/ml. (It is noted that the blank here did not exhibit the negative peak which usually appears in human samples.) Fig. 6 shows the analysis of a sample collected 20 min after dosing. Maximum blood level was attained in 5 min, and by 120 min the level was below detectability. It was estimated that the half-life in this case was approximately 15 min, in agreement with determinations made by radioactivity

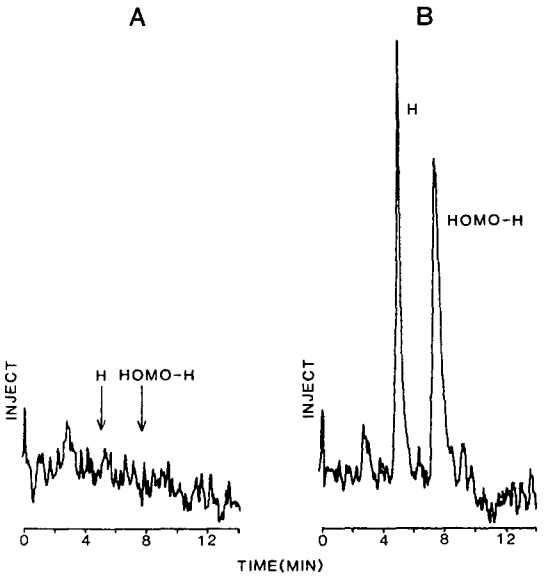

Fig. 6. Quantitation of harringtonine (H) in mouse serum. A = blank serum, no H or internal standard (homoharringtonine, HOMO-H) added. B = analysis of sample 20 min after the intraperitoneal injection of 3 mg/kg H. The amount of H present = 149 ng/ml.

measurements (Han, unpublished data). Pharmacokinetic data on experimental animals will be published elsewhere.

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DETERMINATION OF HEROIN AND ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the simultaneous determination of heroin (3,6-diacetylmorphine, DAM) and its two active metabolites 6-acetylmorphine and morphine in blood by high-performance liquid chromatography using a normal-phase column and a UV detector at 218 nm. The compounds are stabilized in blood by rapid freezing and recovered by a multistep liquid—liquid extraction. The mobile phase is acetonitrile—methanol (75:25, v/v) buffered to apparent pH 7 with ammonium hydroxide and acetic acid. Using *l*-α-acetylmethadol as an internal standard, UV detection and a 1-ml biofluid sample, the lower limit of sensitivity is 12.5 ng/ml. Commonly used narcotic analgesics including codeine, propoxyphene, meperidine, methadone and levorphanol do not interfere with the analysis. The method has been applied to blood samples from humans and rats. Extracts of blood from a patient who had received an intravenous dose of 14 mg of DAM contained DAM and both of its active metabolites.

INTRODUCTION

Heroin (3,6-diacetylmorphine, DAM) is a commonly abused narcotic agonist which has been studied as an analgesic for the management of pain due to advanced cancer [1]. DAM is enzymatically deacylated to 6-acetylmorphine (AM) and subsequently to morphine (M) (Fig. 1) by a variety of tissues from man, rat, rabbit, mouse, dog [3, 4] and other species.

The clinical evaluation of DAM [4] has stimulated pharmacokinetic studies in man [5] and laboratory animals. DAM disposition has previously been studied using a number of analytical techniques including counter current distribution followed by spectrophotometric quantitation of methyl orange complexes or folin determination of free phenol content [2, 6], paper chromatography [6, 7], thin-layer chromatography [8—10], combined gas chro-

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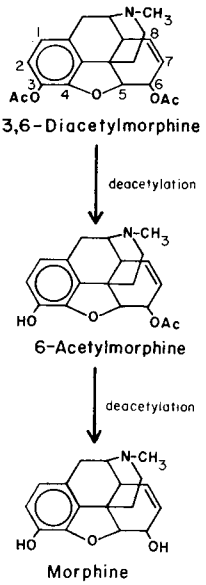


Fig. 1. Biotransformation pathway for heroin (diacetylmorphine, DAM), and structural formulae for DAM, acetylmorphine (AM) and morphine (M).

matography—mass spectrometry [11] and differential UV spectrophotometry [12]. High-performance liquid chromatography (HPLC) has been used in the analysis of DAM from pharmaceutical, illicit and other non-biological sample preparations [10, 13—15]. The lability of DAM's 3-ester at alkaline pH and to hydrolases in serum and red cells, coupled with the wide range in lipophilicity of the three compounds of interest have, however, hindered the development of a generally applicable analytical method with the requisite sensitivity for the routine simultaneous determination of DAM, AM and M in biofluid samples. Furthermore, Garrett and Gurkan's [10] method for stabilizing DAM in dog blood prior to extraction does not stabilize DAM in human or rat blood (Umans and Inturrisi, unpublished observations).

We describe here a method using rapid freezing of whole blood or biofluid samples, followed by solvent—solvent extraction and HPLC to resolve the compounds of interest; and demonstrate the utility of this method for measuring DAM and its metabolites in human and animal biological samples.

MATERIALS AND METHODS

Chemicals and reagents

The following compounds were obtained from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) through the Medicinal Chemistry and Technology Section of the National Institute on Drug Abuse (NIDA) (Rockville, MD, U.S.A.): *l*-α-acetylmethadol·HCl, 3,6-diacetylmorphine·HCl and 6-acetylmorphine base. Morphine sulfate was obtained from Mallinckrodt (St. Louis, MO, U.S.A.).

Methanol, acetonitrile, *n*-butanol, chloroform, isopropanol, acetone and *n*-hexane (all distilled in glass) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Toluene (scintillation grade), glacial acetic acid and concentrated (29.2%) aqueous ammonia were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). All solvents and reagents were used as received. Reagent grade sulfuric acid, hydroxylamine·HCl and both 10% and 50% aqueous sodium hydroxide (w/v) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Glycylglycine buffer (1 *M*, pH 8.55) was prepared by titrating a solution of the free base (Sigma, St. Louis, MO, U.S.A.) in glass distilled water with 50% (w/v) sodium hydroxide.

[^{3}H]DAM (diacetyl[1(*n*)-^{3}H]morphine, specific activity = 26.5 Ci/mmol) and [^{3}H]M ([1(*n*)-^{3}H]morphine, specific activity = 22 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, U.S.A.). [^{3}H]AM was synthesized by one of two methods, as follows:

(1) In a modification of the methods of Small (cf. Wright [16]) and of Lerner and Mills [17], 25 μl of a saturated aqueous solution of hydroxylamine hydrochloride was added to [^{3}H]DAM, dissolved in 100 μl of ethanol (US Industrial Chemicals, New York, NY, U.S.A.) in a siliconized glass centrifuge tube. The solution was incubated at 60°C for 0.5 h and then neutralized by the dropwise addition of concentrated ammonium hydroxide. The solution was buffered to pH 8.6 (ammonia—ammonium chloride buffer) and extracted with chloroform—isopropanol (3:1, v/v).

(2) [^{3}H]DAM was added to 10 units of butyrylcholinesterase (equine serum acylcholine acylhydrolase, E.C.3.1.1.8, Sigma), dissolved in 100 μl of normal saline and incubated at 37°C for 1 h. Protein was precipitated by addition of 1 ml of acetone. The purity of both products was assessed by HPLC, followed by fractional collection of the column effluent and liquid scintillation counting using a 1% Liquifluor (New England Nuclear, Boston, MA, U.S.A.) in toluene cocktail in a Model LS3100 counter (Beckman Instruments, Irvine, CA, U.S.A.). Counting efficiency was determined using [^{3}H]toluene as an internal standard. Either method provides [^{3}H]AM of > 96% radiochemical purity in > 80% yield.

Stock solutions

LAAM, DAM, AM and M stock solutions at a concentration of 1 mg/ml were prepared in methanol and stored at −20°C. Calibration standards were prepared by diluting the stock solution with methanol—acetonitrile (20:80, v/v).

Sample preparation from biofluids

A flow sheet outlining the procedure is given in Fig. 2. Whole blood samples (up to 1.5 ml) are collected in 12-ml siliconized (Prosil 28, PCR, Gainesville, FL, U.S.A.) centrifuge tubes, fitted with PTFE-lined screw caps. The samples are rapidly frozen in a dry-ice—acetone bath and stored at −20°C. To each tube, while maintained at dry-ice temperature, is added 0.10 ml of a 4.0 μg/ml solution of the internal standard and 1.0 ml of the glycylglycine buffer. The sample is extracted with 5 ml of toluene—*n*-butanol (7:3, v/v) for 20 min on an automatic reciprocating shaker and centrifuged for 10 min

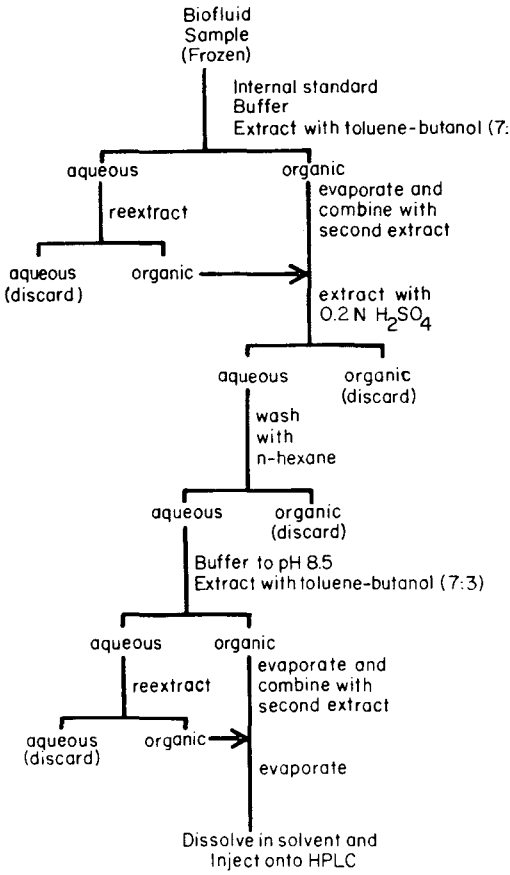


Fig. 2. Flow sheet outlining the stabilization and extraction procedures for DAM and its metabolites from blood.

at 500 *g*. The frozen sample pellet thaws during this first room temperature extraction. The toluene—butanol phase (upper) is transferred to a clean, siliconized 15-ml screw-top centrifuge tube and evaporated to dryness at 35°C (Vortex Evaporator, Model 3-2200, Buchler, Fort Lee, NJ, U.S.A.). The sample is reextracted with 5 ml of toluene—butanol, which is then added to the dried residue of the first extract. The organic phase is extracted with 2.5 ml of 0.2 *N* sulfuric acid by shaking for 10 min. After centrifugation for 7 min the toluene—butanol layer (upper) is discarded. The acid phase is washed with 5 ml of *n*-hexane and the pH adjusted to 8.5 with the addition of 0.5 ml of glycylglycine buffer, followed by 0.195 ml of 10% sodium hydroxide. This aqueous phase is extracted twice with 5 ml of toluene—butanol by shaking for 10 min. After centrifugation for 7 min, each portion of toluene—butanol is transferred to a 12-ml siliconized conical centrifuge tube and evaporated to dryness as above. The sample extract is reconstituted in 260 μl of methanol, and 200 μl are injected into the HPLC system.

Chromatographic conditions

The analysis is performed on a Varian Model 8500 liquid chromatograph (Varian, Sunnyvale, CA, U.S.A.) equipped with a displacement syringe pump, a UV—visible variable-wavelength detector (Varichrom Model VUV-10) and a Rheodyne Model 7125 syringe-loading sample injector (Rheodyne, Berkeley, CA, U.S.A.). The column is a 30 cm × 4 mm I.D. Varian Micropak containing 5-μm LiChrosorb Si-60. Chromatograms are recorded on a Varian Model A-25 dual-channel chart recorder set at 1 mV and 2 mV. The mobile phase is acetonitrile—methanol—solution A—solution B (75:25:0.040:0.216). Solution A is prepared by mixing concentrated aqueous ammonia and methanol (1:2, v/v) and solution B by mixing glacial acetic acid and methanol (1:1, v/v). The flow-rate is 80 ml/h and the column and detector are maintained at 30°C. The column effluent is monitored at 218 nm using a recorder scale that varies from 8—32 mA and a chart speed of 10 in./h.

Calibration curves and quantitation

Standard calibration curves are established by adding DAM, AM, M and LAAM to drug-free blood, plasma or normal saline and proceeding as described above. Quantitation is performed by drawing the baseline and measuring the peak heights of the compounds of interest. The peak height ratio (standard/LAAM) is calculated. A standard curve is constructed by plotting the peak height ratio against the amount added. Each calibration curve is constructed from at least triplicate determinations of five points.

Human and animal studies

Samples were taken from humans and rats following DAM administration and from in vitro incubations of DAM with rat blood. Blood was collected from three male cancer patients for 2—17 min following the intravenous administration of 4, 5 or 14 mg of DAM. Three male Sprague—Dawley rats weighing 300—350 g were prepared by cannulation of the right femoral artery and vein. Blood (0.5—1.0 ml) was collected just prior to and from 10 to 180 min after the intravenous infusion of DAM at 1.0 mg/kg/h for 3 h. Drug-free whole, heparinized blood was obtained by cardiac puncture from four rats and preincubated for 10—20 min in a 37°C water bath. DAM (1 μg/ml) was added to each sample, which was mixed thoroughly and returned to the water bath. Aliquots were taken at 0, 15, 30, 60 and 120 sec.

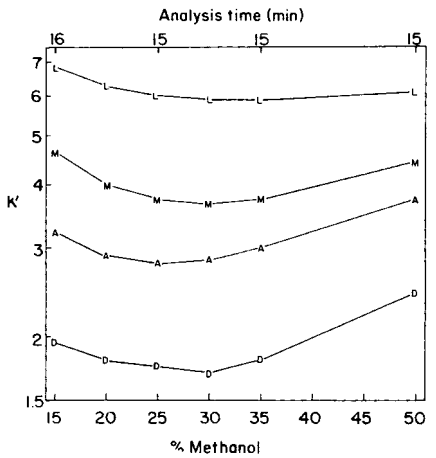
RESULTS AND DISCUSSION

Determination of chromatographic conditions

DAM and its metabolites are weak bases, which are partially ionized in aqueous solution. Separation by reversed-phase HPLC requires that ionization be suppressed by raising the mobile phase pH, which would unfortunately result in both DAM hydrolysis and column degradation [18]. The UV cut-off of paired-ion chromatographic reagents [19] would not allow detection at short wavelengths and would result in a less sensitive assay. By analogy to other aromatic, weakly basic alcohols and their esters, DAM and its metabolites are amenable to separation by normal-phase chromatography [20]. Ace-

tonitrile—methanol, buffered to apparent pH 7 with acetic acid and ammonium hydroxide provides a mobile phase with a low UV cut-off that fixes the extent of ionization of the compounds of interest.

Fig. 3 shows the effect of changing the concentrations of acetonitrile and methanol in the mobile phase on the column capacity factor (k') and analysis time. The four compounds are adequately resolved with a mobile phase containing up to 30% methanol, while at 50% methanol the resolution between AM and M begins to decrease. Since the k' value of LAAM is relatively insensitive to changes in percent methanol, the analysis time changes little over the range of 15—30% methanol.



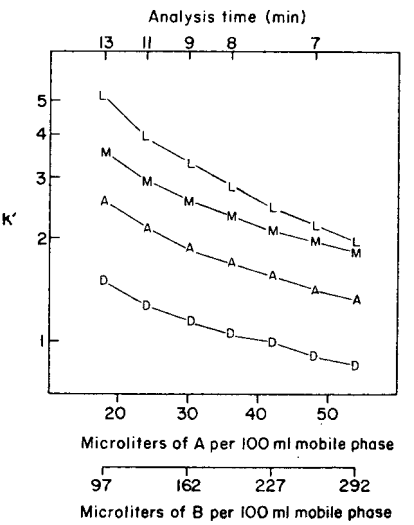


Fig. 3. Effect of increasing the percentage of methanol in the mobile phase (acetonitrile—methanol) from 15 to 50% on the column capacity factor (k') of each compound of interest. The buffer strength was kept constant at 20 μl solution A and 108 μl solution B per 100 ml of mobile phase. The time required for a complete analysis is given on the upper abscissa. Traces: L = LAAM; M = M; A = AM; D = DAM.

Fig. 4. Effect of increasing the buffer strength [μl of A and B per 100 ml of mobile phase, acetonitrile—methanol (70:30, v/v)], while maintaining the apparent pH constant at 7, on the column capacity factor (k') of each compound of interest. The time required for a complete analysis is given on the upper abscissa. Traces: L = LAAM; M = M; A = AM; D = DAM.

The variation of k' and analysis time with buffer strength is shown in Fig. 4. With the apparent pH maintained at neutrality, the analysis time decreases with increasing buffer strength. At high buffer strengths, the resolution between M and LAAM is lost. To provide adequate resolution and a short analysis time, we chose a mobile phase containing 25% methanol and 40 μl of solution A per 100 ml for routine use.

Column efficiency

The effect of flow-rate on column efficiency, as determined by the height equivalent to a theoretical plate (HETP), was investigated. As expected, Fig. 5

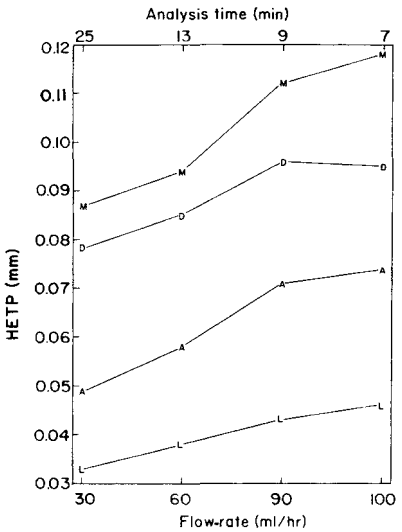


Fig. 5. Effect of the flow-rate of the mobile phase, acetonitrile—methanol (80:20, v/v) with 36 µl A and 194 µl B per 100 ml of mobile phase, on column efficiency as measured by the height equivalent to a theoretical plate (HETP). The time required for a complete analysis is given on the upper abscissa. Traces: M = M; D = DAM; A = AM; L = LAAM.

shows that the HETP is smallest at the lowest flow-rates. However, while the HETP is largest for M, it does not exceed 0.12 mm over the range of flow-rates tested. For a convenient analysis time we chose a flow-rate of 80 ml/h.

UV detection

The absorbance of DAM and its metabolites increases significantly in the far UV. A wavelength of 218 nm was selected as a compromise between maximum sensitivity and acceptable noise. At a column and detector temperature of 30°C, the retention time of each compound is quite stable. The coefficients of variation (C.V.) for retention times from eleven consecutive extracted samples are shown in Table I. This reproducibility can permit the blind collection of peak fractions when the sample concentration falls below the limits of sensitivity for UV detection. These fractions may be subsequently quantitated using a sensitive morphine radioimmunoassay [21] which also recognizes AM and DAM (studies in progress).

TABLE I

PRECISION VALUES FOR DAM AND ITS METABOLITES

Compound	Mean retention time (min)	C.V. (%)	Recovery		C.V. (%) for determination of	
			Mean	C.V. (%)	25 ng	300 ng
DAM	5.7	0.2	87.8	6.3	3.9	2.0
AM	8.1	0.3	94.3	5.8	5.1	3.4
M	10.0	0.5	92.8	8.7	3.5	2.9
LAAM	13.8	0.2	28.5	7.3	—	—

Determination of radiolabeled samples

The availability of tritiated forms of DAM and its metabolites led us to adapt the HPLC system we have described so as to be able to collect fractions containing the radiolabeled compounds of interest for analysis by liquid scintillation counting. The location and recovery of the compounds of interest are facilitated by adding 1 μg of each as unlabeled carrier such that the expected contribution of the radioisotope to the detector response will be less than 5%. The recovery of radiolabeled drug from the column is 93% (C.V. = 5%) for all three compounds and is independent of radioisotope amount from 0.0006 to 0.02 μCi. The sensitivity of this approach is therefore a function of the specific activity of the radiolabeled drug and the average background. We can easily determine 0.01 ng of [^{3}H] DAM, [^{3}H] AM or [^{3}H] M using this method with extraction recovery assessed by comparison of the cold carrier detector response to absolute standard curves for the three compounds of interest.

Extraction recovery and calibration curves

The optimal conditions for the simultaneous extraction of DAM and its metabolites are particularly dependent on pH and partition conditions. DAM is rapidly converted to AM at pH 9 or greater. The uniform recovery of M depends on forming the sulfate rather than the hydrochloride salt in the second step of the extraction. The recovery of the compounds of interest is independent of concentration from 12.5 to 500 ng/ml. After correction for aliquot losses, the mean recoveries and their coefficients of variation are shown in Table I.

While extraction from blood yields a small and variable unidentified peak eluting between the solvent front and DAM, the extraction yields a sample which is free of peaks that might interfere with the quantitation of the compounds of interest (Fig. 6).

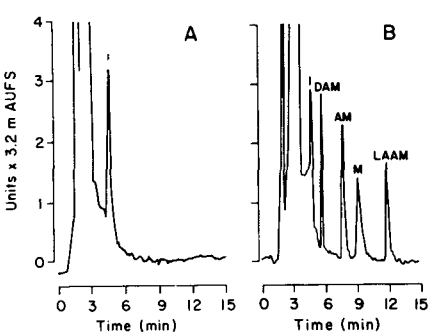


Fig. 6. Chromatograms of the extract of control human blood (A) and calibration standards recovered from human blood (B). To 0.5 ml of control human blood were added DAM (100 ng), its metabolites AM and M at 75 ng each and 400 ng of LAAM, the internal standard. The extract was reconstituted in 260 μl of methanol and 200 μl were injected. Peak 1 is unidentified. Chromatographic conditions are as described in Materials and methods.

Fig. 7 shows that, using the UV detector, linear standard calibration curves can be constructed from 12.5 to 200 ng. Although not shown in Fig. 7, linear curves may be constructed up to 4000 ng. The precision of determination of extracted 25 and 300 ng calibration standards is given in Table I. With a sample volume of 1 ml and this calibration curve the lower limit of sensitivity is 12.5 ng/ml. The effective sensitivity may be increased by using sample volumes up to 1.5 ml. The lower limit of detection (signal-to-noise ratio of 2) is 6 ng for all three compounds.

While the rapid freezing and mildly alkaline extraction procedures permit only minimal (11.2 ± 0.36% S.E.M., n = 12 at 150 ng DAM) DAM hydrolysis, the extent of hydrolysis is routinely monitored in each extraction and the DAM and AM standards are extracted separately to avoid the systematic underestimation of AM.

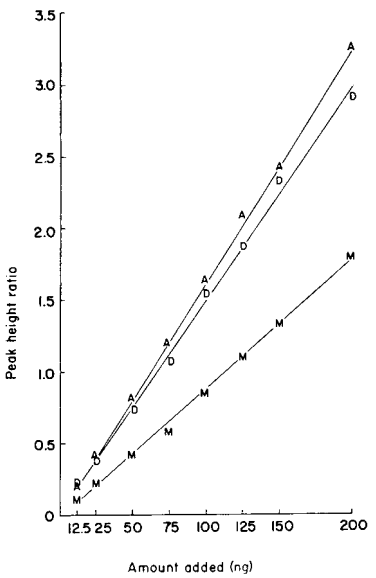


Fig. 7. Standard calibration curves for DAM (D) and metabolites M and AM (A) recovered from control human blood. Superimposible curves were derived from normal saline. Each point represents the mean of triplicate determinations.

Potential interference

As shown in Table II, DAM and its metabolites are resolved from commonly used or abused drugs and their metabolites. Only hydrocodone and quinine were not adequately resolved when injected together with morphine and analyzed with the routine system described in Materials and methods. However, if samples were suspected to contain either of these compounds, the mobile phase could be modified to resolve them from all three compounds of interest. The relatively low pH of the extracted sample also serves to selectively diminish the recovery of many of these compounds. Thus, this method may be used to analyze samples from subjects taking the drugs listed in Table II.

TABLE II

RESOLUTION OF HEROIN AND METABOLITES FROM SELECTED DRUGS AND THEIR METABOLITES

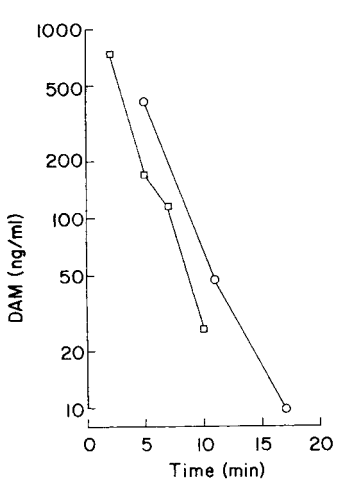
Drug	Column capacity factor (k')
Naloxone	0.57
Heroin (DAM)	0.97
Acetylcodeine	1.46
6-Acetylmorphine (AM)	1.66
Codeine	1.81
Naltrexone	1.89
Morphine (M)	2.17
Hydrocodone	2.24
Quinine	2.28
Oxycodone	2.38
Hydromorphone	2.48
Meperidine	2.61
Propoxyphene	2.85
Oxymorphone	2.85
Norpropoxyphenamide	2.85
l-α-Acetylmethadol (LAAM)	3.16
Dinoracetylmethadol	3.57
Cocaine	3.58
Dinormethadol	3.73
Methadol	4.04
Amphetamine	4.07
Normeperidine	4.13
Normethadol	4.14
Pentazocine	4.24
Methadone	5.07
Levorphanol	5.13
Acetaminophen	> 5
Caffeine	> 5

Human and animal studies

Following the rapid intravenous (i.v.) administration of DAM to two cancer patients, Fig. 8 shows the rapid ($t_{½}$ = 1.7, 2.2 min) exponential disappearance of DAM from blood. This disappearance is more rapid than that of DAM from blood in vitro (unpublished observations) and may be due to both distribution and biotransformation, in blood and extravascularly. While neither AM nor M was detected in these patients, both metabolites have been observed following the administration of 14 mg DAM (i.v.) to a highly narcotic-tolerant patient (Fig. 9). This is the first report of the detection of DAM in human blood and confirms the inadequacy of the analytical methods used in prior studies [22].

Following the termination of a 3-h infusion of DAM to three rats, no DAM was detected in any blood samples. Based on the lower limit of detection of 6 ng we can estimate the minimum steady state blood DAM clearance for the rat from the equation:

$$\text{Clearance} = \frac{k_0}{C_{ss}}$$



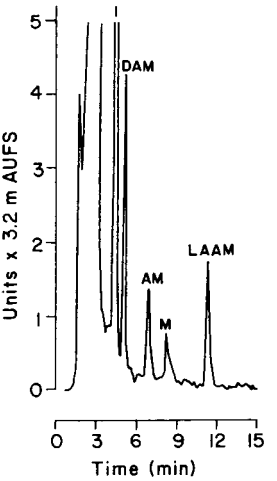


Fig. 8. Blood levels of DAM following the i.v. injection of DAM, 5 mg (○) and 4 mg (□), to two cancer patients.

Fig. 9. Chromatogram of a blood sample collected from a cancer patient 11.25 min after the i.v. administration of 14 mg DAM. Peak 1 is unidentified. Extraction and chromatographic conditions are as described in Materials and methods.

where k_0 is the zero order DAM infusion rate (1.0 mg/kg/min) and C_{ss} is the steady state blood DAM concentration (assumed to be 6 ng/ml). This calculation indicates that in the rat the blood DAM clearance must exceed 2800 ml/min/kg. The elimination of AM and M following these infusions is displayed in Fig. 10. The rapid clearance of i.v. DAM in the rat is substantiated by a study of DAM hydrolysis and reciprocal AM formation by rat blood, in vitro (Fig. 11). Under the conditions used, DAM was hydrolyzed with a mean half-life of 23 sec.

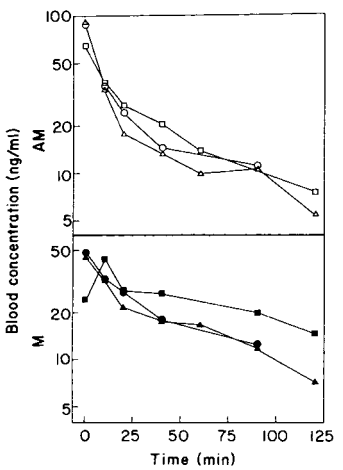


Fig. 10. Blood levels of AM (upper panel) and M (lower panel) following a continuous infusion of DAM at 1 mg/kg/h to three rats. Zero time represents the cessation of drug infusion. No DAM was detected in any samples. Extraction and chromatographic conditions are as described in Materials and methods.

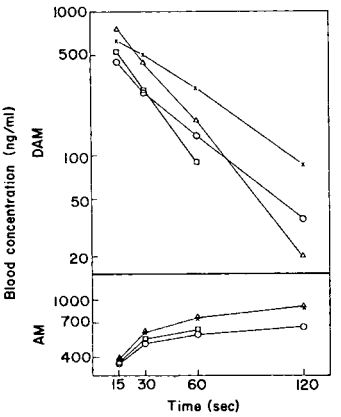


Fig. 11. Disappearance of DAM (upper panel) and the reciprocal formation of AM (lower panel) during the incubation of DAM (1 μg/ml) with rat blood (n = 4) at 37°C, in vitro. Extraction and chromatographic conditions are as described in Materials and methods.

We have developed an HPLC method for the quantitative determination of DAM and its metabolites, following their stabilization in, and extraction from whole blood. The system can be used to determine the pharmacokinetics of DAM or either of its metabolites in man and laboratory animals.

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CHROMBIO. 1457

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF THE ANTINEOPLASTIC AGENT TRICYCLIC NUCLEOSIDE 5′-PHOSPHATE AND ITS DISPOSITION IN RABBIT

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SUMMARY

Anion-exchange and reversed-phase high-performance liquid chromatographic procedures are described for the assay of the antineoplastic agent tricyclic nucleoside 5′-phosphate (TCNP) and its metabolite tricyclic nucleoside (TCN) in biological fluids. Disposition of TCNP has been studied in rabbit. TCNP is eliminated from blood and plasma with a biologic half-life of about 7.5 h. Apparent volume of distribution is 43.2 l/m^2 and total body plasma TCNP clearance is 67.8 ml/min/m^2. TCNP is hydrolyzed by plasma and probably other tissues to TCN which is present in blood and plasma at about one-tenth the concentration of TCNP. There is no accumulation of TCNP or TCN in blood or plasma over 2 days of administration. In 24 h 2.4% of a dose of TCNP is excreted in bile of a rabbit with a cannulated bile duct as unchanged TCNP and 30.7% as TCN. TCN is excreted in bile at an initial concentration half the maximum solubility of TCN in rabbit bile. Excretion of TCNP and TCN over 24 h in the urine of a rabbit with a cannulated bile duct is 1.5% and 5.2% of the dose, respectively.

INTRODUCTION

Tricyclic nucleoside 5′-phosphate [6-amino-4-methyl-8-(β-D-ribofuranosyl)-pyrrolo(4,3,2-*de*)pyrimido(4,5-*c*)pyridazine-5′-phosphate] (Fig. 1) is an antitumor agent with activity against several animal tumor model systems including L1210 and P-388 murine leukemias, CD8F1 mammary carcinoma and the human MX-1 mammary tumor xenograft [1, 2]. Tricyclic nucleoside 5′-phosphate is structurally related to certain naturally occurring 7-deazapurine nucleosides with antitumor activity. Tricyclic nucleoside 5′-phosphate is currently undergoing clinical evaluation as an antitumor agent in humans. As a preliminary step to studies in humans we developed sensitive high-performance liquid chromatographic (HPLC) assay procedures for tricyclic nucleoside

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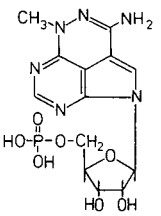

Fig. 1. Structure of tricyclic nucleoside 5′-phosphate.

5′-phosphate and its dephosphorylated metabolite tricyclic nucleoside in biological fluids. The disposition of tricyclic nucleoside 5′-phosphate in rabbit has been studied.

EXPERIMENTAL

Drugs

Tricyclic nucleoside 5′-phosphate (NSC 280594) and tricyclic nucleoside (NSC 154020) were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. Aminopyrine and 4-nitropyridine were obtained from Aldrich, Milwaukee, WI, U.S.A.

Animal and patient studies

Male New Zealand white rabbits weighing between 2 and 3 kg were injected intravenously with tricyclic nucleoside 5′-phosphate at a dose of 100 mg/m^2 (5.5 mg/kg) dissolved in 0.9% NaCl adjusted to pH 7.0 with 1 *N* NaOH. Injection was over a 1-min period into a peripheral ear vein using a vein infusion set with winged adapter (Miniset, Travenol, Deerfield, IL, U.S.A.). Tricyclic nucleoside 5′-phosphate was administered on two consecutive days in order to study possible accumulation of drug. Blood was collected at different times from a peripheral ear vein of the other ear into heparinized tubes. Plasma from a portion of the blood was collected immediately by centrifugation at 4°C. Plasma and blood were stored frozen at −70°C until assay. Rabbits used for biliary excretion studies were anesthetized with pentobarbital and a polyethylene cannula (PE 160, Intramedic, Clay Adams, Parsippany, NJ, U.S.A.) was inserted in the bile duct. Animals were allowed to recover for 3 h before giving tricyclic nucleoside 5′-phosphate and bile was collected over a 24 h period from the exteriorized bile cannula. Urine was collected using a pediatric Foley balloon catheter (French Size 10, Bard, Murray Hill, NJ, U.S.A.) inserted into the bladder through the urethra. Separate groups of rabbits were used for blood pharmacokinetic studies and biliary and urinary excretion studies.

Preparation of samples

A 1-ml volume of blood or plasma or 0.1 ml urine or bile was mixed with 4 ml ice-cold 0.5 *N* perchloric acid containing as internal standard 1 μg 4-nitropyridine for ion-exchange HPLC or 10 μg aminopyrine for reversed-phase HPLC. Protein was removed by centrifuging at 1000 *g* for 10 min at 4°C and

the supernatant applied to a 3-ml disposable octadecylsilane-bonded silica gel extraction column (J.T. Baker, Phillipsburg, NJ, U.S.A.). The column was washed with 4 × 2 ml water and adsorbed compounds eluted with 4 × 2 ml 10% water in methanol adjusted to pH 10.0 with ammonium hydroxide. Solvent was removed by evaporation under nitrogen at 30°C. The residue was dissolved in 200 μl water and 50 μl was taken for HPLC.

High-performance liquid chromatography

A Hewlett-Packard 1084B liquid chromatograph and variable-wavelength detector 798575A were used in the studies. The output from the detector was fed into a Hewlett-Packard 79850B liquid chromatograph terminal and peak areas were integrated. Two HPLC procedures were developed. Ion-exchange HPLC employed a 25-cm Partisil-10 SAX anion-exchange column, particle size 10 μm (Whatman, Clifton, NJ, U.S.A.) and a 0—100% 15-min linear gradient of 0.25 *M* KH_2PO_4, 0.5 *M* KCl, pH 4.5, in 5 m*M* KH_2PO_4, pH 3.3, at a flow-rate of 1.5 ml/min. Reversed-phase HPLC employed a 25-cm LiChrosorb RP-18, 5-μm column (Merck, Darmstadt, G.F.R.) and a 5—100% 7.5-min linear gradient of methanol in 0.1 *M* sodium acetate pH 5.3, at a flow-rate of 1.5 ml/min. Eluting compounds were detected by their absorbance at 292 nm. Reversed-phase HPLC was used to measure tricyclic nucleoside 5′-phosphate and tricyclic nucleoside in blood, plasma and bile. Anion-exchange HPLC was used to measure tricyclic nucleoside 5′-phosphate in urine. Urine contained endogenous compounds which interfered with the detection of tricyclic nucleoside at 292 nm. Tricyclic nucleoside, although not tricyclic nucleoside 5′-phosphate, is fluorescent (excitation wavelength 370 nm, emission wavelength 453 nm) and tricyclic nucleoside in urine was detected by reversed-phase HPLC using a Varian Fluorichrom fluorescence detector (Varian, Walnut Creek, CA, U.S.A.).

Pharmacokinetic analysis

Non-linear least-squares regression analysis of the data to obtain pharmacokinetic parameters employed the NONLIN pharmacokinetic program [3].

RESULTS

Octadecylsilane bonded extraction columns provided a simple and rapid way of concentrating tricyclic nucleoside 5′-phosphate and tricyclic nucleoside from biological fluids. Efficiency of extraction of tricyclic nucleoside 5′-phosphate was 54% and of tricyclic nucleoside 100%. Columns could be used up to 3 times with no loss of efficiency. Two chromatographic procedures were developed for assaying tricyclic nucleoside 5′-phosphate. A method employing anion-exchange HPLC detected tricyclic nucleoside 5′-phosphate but tricyclic nucleoside was not retained by the column and eluted in the void volume with other endogenous compounds (Fig. 2). The limit of sensitivity for detection of tricyclic nucleoside 5′-phosphate by this method was 50 ng/ml. An alternative method employed reversed-phase HPLC on an octadecylsilane-bonded column and separated tricyclic nucleoside 5′-phosphate and tricyclic nucleoside (Fig. 3). The limit of sensitivity for detection of tricyclic nucleoside 5′-phosphate

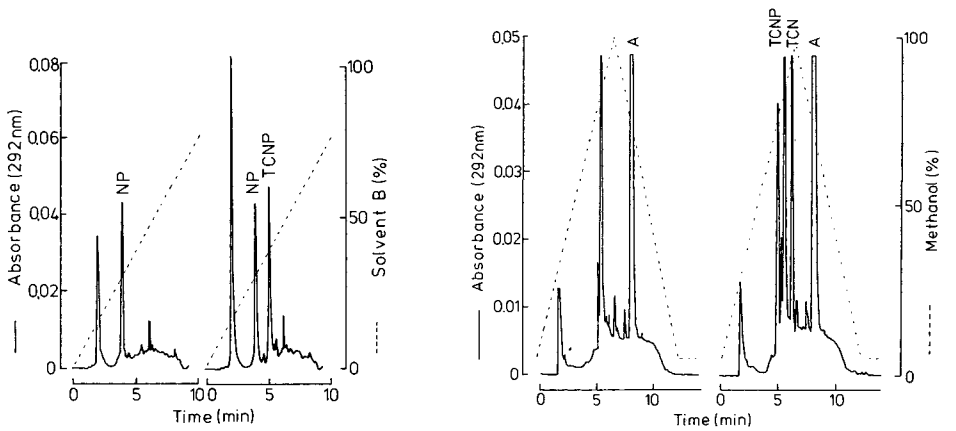

Fig. 2. Ion-exchange HPLC of human plasma to which had been added, left panel, 1 μg/ml 4-nitropyridine internal standard (NP); right panel, 1 μg/ml 4-nitropyridine internal standard and 1 μg/ml each of tricyclic nucleoside 5′-phosphate (TCNP) and tricyclic nucleoside. Tricyclic nucleoside was not retained by the column and eluted in the void volume with other endogenous compounds. Chromatographic conditions: anion-exchange HPLC on a 25-cm Partisil-10 SAX column with a 0—100% linear gradient of 0.25 *M* KH_2PO_4, 0.5 *M* KCl, pH 4.5 (solvent B) in 5 m*M* KH_2PO_4, pH 3.3 (solvent A). Flow-rate, 1.5 ml/min. Detection by absorbance at 292 nm.

Fig. 3. Reversed-phase HPLC of human plasma to which had been added, left panel, 10 μg/ml aminopyrine internal standard (A); right panel, 10 μg/ml aminopyrine internal standard and 1 μg/ml each of tricyclic nucleoside 5′-phosphate (TCNP) and tricyclic nucleoside (TCN). Chromatographic conditions: reversed-phase HPLC on a 25-cm C_{18} column with a 5—100% linear gradient of methanol in 0.1 *M* sodium acetate, pH 5.3. Flow-rate, 1.5 ml/min. Detection by absorbance at 292 nm.

in rabbit plasma was 50 ng/ml and for tricyclic nucleoside 25 ng/ml. The coefficient of variation of the method at 1 μg/ml in plasma was ± 4.3% for tricyclic nucleoside 5′-phosphate and ± 5.4% for tricyclic nucleoside. The assay was linear up to 100 μg/ml for both tricyclic nucleoside 5′-phosphate and tricyclic nucleoside.

Tricyclic nucleoside 5′-phosphate was administered to rabbits on two consecutive days at a daily dose of 100 mg/m^2. Blood and plasma concentrations of tricyclic nucleoside 5′-phosphate and tricyclic nucleoside are shown in Fig. 4. Insufficient data points were obtained to accurately define an initial phase of tricyclic nucleoside 5′-phosphate distribution in either blood or plasma and the data were fitted to a one-compartment model. Tricyclic nucleoside 5′-phosphate concentrations in whole blood decreased with a half-life of 6.6 h on day 1 and 8.4 h on day 2. Tricyclic nucleoside 5′-phosphate concentrations in plasma were generally lower than in whole blood and fell with a half-life of 8.8 h on day 1 and 6.3 h on day 2. The apparent volume of distribution of tricyclic nucleoside 5′-phosphate (mean of day 1 and 2) calculated using plasma concentrations was 43.2 l/m^2 and plasma clearance 67.8 ml/min/m^2. Tricyclic nucleoside was found as soon as 5 min after administration of tricyclic nucleoside 5′-phosphate in both blood and plasma. Concentrations of tricyclic nucleoside were approximately one-tenth the

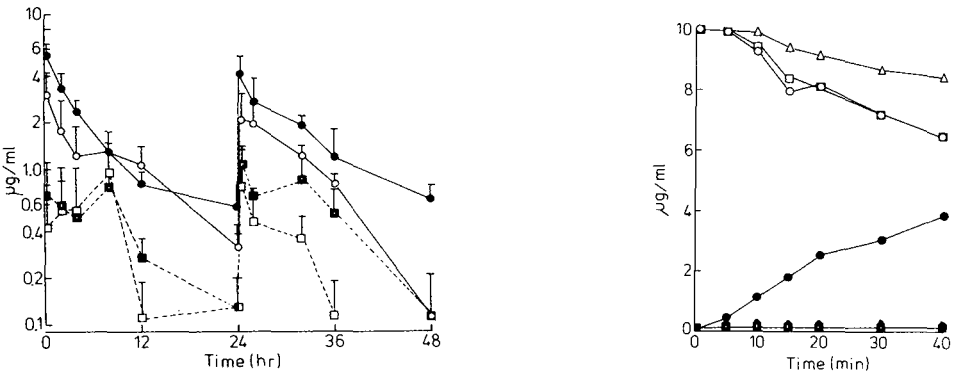

Fig. 4. Time course of tricyclic nucleoside 5′-phosphate (TCNP) and tricyclic nucleoside (TCN) in rabbit blood and plasma. TCNP, 100 μg/m², was administered at 0 and 24 h by i.v. infusion over 1 min. (●) TCNP in blood, (■) TCN in blood, (○) TCNP in plasma, (□) TCN in plasma. TCNP is shown by continuous lines, TCN by dotted lines. Each point is the mean of three rabbits. Bars are S.E. of mean.

Fig. 5. Stability of tricyclic nucleoside 5′-phosphate (TCNP) in rabbit blood and plasma at 37°C. Fresh heparinized plasma or blood was incubated with TCNP (10 μg/ml) with gentle shaking at 37°C. Open symbols are TCNP, closed symbols TCN. (◑) Plasma alone, (▲) whole blood and (◨) plasma separated from blood after incubation with TCNP.

concentration of tricyclic nucleoside 5′-phosphate and appeared to fall at a rate similar to tricyclic nucleoside 5′-phosphate, although an estimate of half-life could not be obtained from the data. There was no accumulation of tricyclic nucleoside 5′-phosphate or tricyclic nucleoside in blood or plasma over 2 days of administration.

To determine the contribution of plasma and blood cells to the conversion of tricyclic nucleoside 5′-phosphate to tricyclic nucleoside fresh heparinized rabbit plasma or blood was incubated with tricyclic nucleoside 5′-phosphate, 10 μg/ml, at 37°C (Fig. 5). Tricyclic nucleoside 5′-phosphate was destroyed by plasma with the appearance of tricyclic nucleoside. Disappearance of tricyclic nucleoside 5′-phosphate from plasma was not affected by the presence of blood cells but tricyclic nucleoside was not found in plasma separated after incubation from whole blood. Disappearance of tricyclic nucleoside 5′-phosphate assayed in whole blood was slower than disappearance assayed in plasma and no tricyclic nucleoside was detected in whole blood. Similar findings were obtained with human blood and plasma (results not shown). Cooling blood and plasma to 4°C inhibited disappearance of tricyclic nucleoside 5′-phosphate. Tricyclic nucleoside 5′-phosphate was much more rapidly destroyed by hemolyzed blood than by plasma or non-hemolyzed blood (results not shown). Care should therefore be taken not to hemolyze blood during collection for pharmacokinetic studies of tricyclic nucleoside 5′-phosphate.

Biliary and urinary excretion of tricyclic nucleoside 5′-phosphate and tricyclic nucleoside in rabbit with a cannulated bile duct is shown in Fig. 6. Small amounts of tricyclic nucleoside 5′-phosphate were found in bile during the first few hours after administration but in 24 h only 2.4% of the dose was excreted as unchanged tricyclic nucleoside 5′-phosphate. Much more tricyclic

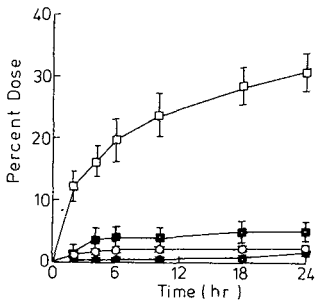

Fig. 6. Excretion of tricyclic nucleoside 5′-phosphate (TCNP) and tricyclic nucleoside (TCN) in rabbit bile and urine. TCNP (100 mg/m²) was administered to rabbit with a cannulated bile duct. Cumulative biliary excretion of (▪) TCNP and (▫) TCN. Cumulative urinary excretion of (•) TCNP and (○) TCN. Each point is the mean of three animals, bars are S.E. of mean.

nucleoside was excreted in bile, 30.7% of a dose of tricyclic nucleoside 5′-phosphate was excreted in bile in 24 h as tricyclic nucleoside. The concentration ratio of tricyclic nucleoside in bile to plasma over the first 2 h after administration of tricyclic nucleoside 5′-phosphate was approximately 230:1. The concentration of tricyclic nucleoside in bile collected over 2 h after administration of tricyclic nucleoside 5′-phosphate was (± S.E.M., n = 3) 115 ± 16.7 μg/ml. The maximum solubility of tricyclic nucleoside in rabbit bile at room temperature was determined by adding a concentrated solution of tricyclic nucleoside, 10 mg/ml, to rabbit bile to give a theoretical concentration in bile of 1 mg/ml, shaking the bile for 45 min at room temperature and removing undissolved drug by centrifugation. The maximum solubility of tricyclic nucleoside in rabbit bile was (± S.E.M., n = 3) 257 ± 35 μg/ml. Only small amounts of tricyclic nucleoside 5′-phosphate and tricyclic nucleoside were excreted in urine in 24 h, 1.5% and 5.2% of the dose administered, respectively.

DISCUSSION

Tricyclic nucleoside 5′-phosphate was synthetized as a water-soluble derivative of tricyclic nucleoside [4]. Tricyclic nucleoside 5′-phosphate and tricyclic nucleoside have similar antitumor activity [2]. The present study shows that tricyclic nucleoside 5′-phosphate is dephosphorylated in vivo by (an) enzyme(s) present in rabbit and human plasma. Cellular ecto-5′-nucleotidase may also contribute to the formation of tricyclic nucleoside from tricyclic nucleoside 5′-phosphate [5]. Human erythrocytes, which lack ecto-5′-nucleotidase [5], and rabbit erythrocytes do not contribute to the dephosphorylation of tricyclic nucleoside 5′-phosphate by whole blood. Erythrocytes take up tricyclic nucleoside almost as rapidly as it is formed from tricyclic nucleoside 5′-phosphate by plasma and in vitro no tricyclic nucleoside could be detected in plasma separated from whole blood after incubation with tricyclic nucleoside 5′-phosphate. In vivo tricyclic nucleoside is found in plasma at low concentrations suggesting that there could be saturation of the erythrocyte uptake process. This could be due to the rapid breakdown of tricyclic nucleoside

5′-phosphate to tricyclic nucleoside by tissues in addition to plasma. Some tricyclic nucleoside might be formed from tricyclic nucleoside 5′-phosphate during collection and separation of plasma. Tricyclic nucleoside is rephosphorylated by erythrocyte adenosine kinase to form tricyclic nucleoside 5′-phosphate [6]. This probably accounts for the apparently slower rate of tricyclic nucleoside 5′-phosphate disappearance from whole blood than from plasma. The fact that some destruction of tricyclic nucleoside 5′-phosphate was apparent in whole blood without appearance of tricyclic nucleoside suggests that other metabolites are formed which are not detected by the assay procedure. Schweinsberg et al. [7] have identified three oxidation products of tricyclic nucleoside in addition to tricyclic nucleoside 5′-phosphate, formed when tricyclic nucleoside is incubated with human erythrocytes. Metabolites, apart from tricyclic nucleoside, were not detected in vitro or in vivo by our assay procedures.

Tricyclic nucleoside 5′-phosphate administered to rabbit disappeared slowly from blood with a half-life of approximately 7.5 h. Tricyclic nucleoside 5′-phosphate concentrations in plasma are somewhat lower than in blood but fall at the same rate. Tricyclic nucleoside concentrations in blood and plasma are much lower than tricyclic nucleoside 5′-phosphate but fall at about the same rate. The results suggest that there might be an equilibrium between plasma and erythrocyte tricyclic nucleoside 5′-phosphate and tricyclic nucleoside. Only small amounts of tricyclic nucleoside 5′-phosphate and tricyclic nucleoside were excreted in rabbit urine, 1.5% and 5.2% of the dose in 24 h, respectively, in rabbits with a cannulated bile duct. Some tricyclic nucleoside 5′-phosphate was excreted in rabbit bile, 2.4% of the dose in 24 h, but relatively large amounts of tricyclic nucleoside, 30.7% of the dose in 24 h. Tricyclic nucleoside is relatively insoluble. The maximum solubility of tricyclic nucleoside in rabbit bile at room temperature is 257 μg/ml. This is about twice the concentration of tricyclic nucleoside seen in rabbit bile in the first 2 h after administration of tricyclic nucleoside 5′-phosphate. Although the rabbit has a gallbladder, bile did not appear to be concentrated but flowed continuously during the 24-h collection period. It is possible that under normal conditions bile is stored and concentrated in the gallbladder and if so the solubility of tricyclic nucleoside could be exceeded. Studies in dog have shown extensive excretion and crystallization of tricyclic nucleoside in the bile duct following administration of tricyclic nucleoside at doses of 5—25 mg/kg [8]. In the present study rabbits were given tricyclic nucleoside 5′-phosphate at 5.5 mg/kg. It is possible that crystallization of tricyclic nucleoside in rabbit bile might occur with higher doses of tricyclic nucleoside 5′-phosphate.

ACKNOWLEDGEMENT

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CHROMBIO. 1460

IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE NEW ANTINEOPLASTIC AGENTS BISANTRENE AND MITOXANTRONE

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SUMMARY

Bisantrene and mitoxantrone are two new anthracene derivatives which have shown significant antitumor activity against a wide variety of animal tumors and in human phase I and II clinical trials. We have developed a rapid, simple and sensitive sample cleanup procedure and high-performance liquid chromatographic (HPLC) assay for both drugs. This method uses a commercially available mini-cartridge with C_{18} reversed-phase packing to isolate the drugs from the biological matrix prior to HPLC. For both drugs the average recovery of the assay was 98 ± 6% with a coefficient of variation (C.V.) of less than 7%. Using this new method our assay sensitivity has improved to less than 10 ng/ml for bisantrene and 1 ng/ml for mitoxantrone, allowing us to document a prolonged terminal phase plasma half-life for both bisantrene and mitoxantrone. Equilibrium dialysis studies showed that both drugs are highly protein bound. Mitoxantrone appears less stable in human plasma than bisantrene. Recoveries from plasma after a 24-h incubation at 25 and 37°C were 40 and 20% for mitoxantrone and 90 and 85% for bisantrene, respectively. Addition of ascorbic acid prior to incubation of mitoxantrone in human plasma at 37°C resulted in less than a 10% decrease in the latter's concentration over a 24-h period. To maintain sample integrity, all plasma samples should be fortified with ascorbic acid and kept frozen prior to analyses.

INTRODUCTION

Bisantrene {9,10-antracenedicarboxaldehyde-bis-[(4,5-dihydro-1H-imidazol-2-yl)hydrazone] dihydrochloride, NSC 337766} and mitoxantrone {1,4-

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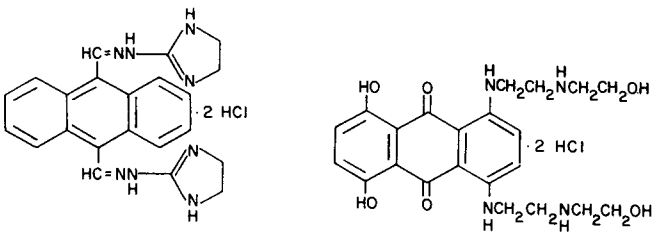
dihydroxy-5,8-bis-[2-(2-hydroxyethyl)amino ethylamino]-9,10-anthracenedione dihydrochloride, NSC 301739} (Fig. 1) are two new anthracene derivatives which have shown significant antitumor activity against a wide variety of animal tumors [1, 2] and in phase I and II clinical trials [3—6]. Our preliminary pharmacokinetic studies [7, 8] showed that bisantrene has a relatively long terminal half-life. The reported pharmacokinetic data of mitoxantrone are, however, controversial [9—11].



BISANTRENE
(NSC 337766)

MITOXANTRONE
(NSC 301739)

Fig. 1. Chemical structure of bisantrene and mitoxantrone.

A simple, precise and sensitive analytical method is essential for pharmacokinetic studies of both drugs. We have recently reported a high-performance liquid chromatography (HPLC) method for the measurement of bisantrene in biological samples [12]. In that report, we used a more extensive sample cleanup procedure, including perchloric acid precipitation of proteins and ethyl-acetate partitioning of bisantrene. Other published HPLC methods for mitoxantrone also require lengthy sample cleanup procedures, including double liquid extraction with chloroform [13] or XAD-2 column isolation [14]. Additionally, these methods have limited sensitivity (i.e., sensitivity ca. 75 ng/ml) [14] and fail to identify a terminal phase of mitoxantrone plasma elimination after the administration of a standard dose (12 mg/m^2).

In the present study, we have developed a new sample cleanup procedure and HPLC assay for both bisantrene and mitoxantrone. This method uses a commercially available mini-cartridge with C_{18} reversed-phase packing to isolate the drugs from biological matrix prior to HPLC. This system allows accurate determination of bisantrene and mitoxantrone with an analysis time of less than 10 min. Using this improved method we have studied the stability and the protein binding of the drugs and have measured the plasma concentrations of both bisantrene and mitoxantrone in patients.

EXPERIMENTAL

Materials

Bisantrene and mitoxantrone reference standards, obtained from Lederle Labs., American Cyanamid Company (Pearl River, NY, U.S.A.), were dissolved in methanol and stored at −80°C. Organic solvents were distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and filtered through a 0.45-μm fluoropore filter (Millipore, Bedford, MA, U.S.A.) prior to use. Aque-

ous solvents for HPLC use were filtered through a 0.45-μm cellulose acetate filter prior to use. Ammonium acetate (HPLC grade) was obtained from Fisher Scientific, Fair Lawn, NY, U.S.A., L-ascorbic acid was obtained from Gibco, Grand Island, NY, U.S.A., human albumin and γ-globulin were obtained from Sigma, St. Louis, MO, U.S.A. and 3 *M* methanolic HCl was obtained from Supelco, Bellefonte, PA, U.S.A.

Sample cleanup procedure

A Vac-Elut™ system equipped with Bond-Elut™ 1-ml C_{18} cartridges (Analytichem International, Harbor City, CA, U.S.A.) was used for sample cleanup. The Vac-Elut's components included a stainless-steel vacuum basin. Vacuum was applied through a 1/8-in. NTP hose fitting located on one end of the basin. A 10-place molded cover with a foam polyethylene gasket precisely fitted the top of the basin. Bond-Elut cartridges were inserted into luer fittings which were an integral part of the cover. Sample eluates were collected in test tubes which were held under the cartridges in a stainless-steel removable rack.

Plasma samples of 1—2 ml were passed through a cartridge, which was sequentially preconditioned by washing with 10 ml of methanol and 5 ml of water. After the plasma had passed through the cartridge, it was washed with 5 ml of water and 300 μl of 0.5 *M* methanolic HCl was used to elute the drug. The eluate was collected, vortexed and kept at −20°C for HPLC analysis.

HPLC apparatus

HPLC was performed with an apparatus consisting of a Model 660 solvent programmer, two Model 6000A solvent delivery systems, a Model U6K injector, a Model 440 detector (Waters Assoc., Milford, MA, U.S.A.), and a Model A-25 recorder (Varian, Palo Alto, CA, U.S.A.). A Waters Assoc. μBondapak C_{18} (30 cm × 3.9 mm I.D.; particle size 10 μm) reversed-phase column preceded by a 7 cm × 2.1 mm I.D. guard column packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was used for all analyses. The guard column packing was changed every two weeks or whenever there was significant back pressure build up.

Chromatographic conditions

Bisantrene was eluted isocratically at ambient temperature with acetonitrile—0.2 *M* ammonium acetate pH 4.0 (27:73) as solvent at a flow-rate of 2.0 ml/min. Bisantrene was detected at 436 nm using a Waters Assoc. Model 440 fixed-wavelength detector.

Mitoxantrone was eluted isocratically at ambient temperature with acetonitrile—0.2 *M* ammonium acetate pH 4.0 (25:75) as solvent at a flow-rate of 1.5 ml/min. Mitoxantrone was detected at 658 nm using a Waters Assoc. Model 440 fixed-wavelength detector.

Quantitation

Quantitation of both drugs was done by the external standard method of analysis. Plasma standard curves were obtained by plotting the resulting peak heights against the known concentration of standards added to the plasma samples.

Recovery and precision

Various amounts of standard bisantrene or mitoxantrone were added to 1 ml of human plasma at room temperature. The plasma was immediately prepared for HPLC analysis. Recovery was calculated by comparing the peak heights of the spiked samples to that of the standards. All experiments were run in triplicate.

Precision and accuracy were determined by assaying patient's plasma in triplicate on different days.

Mass spectral identification

Confirmation of bisantrene and mitoxantrone in the plasma samples was accomplished as previously described, using a Finnigan Model 3300 mass spectrometer coupled to an Incos Model 2061 data system (Finnigan Instruments, Sunnyvale, CA, U.S.A.). Both drugs were analyzed for complete spectra and then plasma samples were analyzed by selected ion monitoring [12, 15].

Stability studies

The stability of bisantrene and mitoxantrone in human plasma and in the presence of human plasma proteins (5% human albumin + 3% γ-globulin) was studied at 37 and 25°C. Samples were assayed for bisantrene or mitoxantrone concentration at various time intervals up to 24 h.

The stability of mitoxantrone in human plasma containing ascorbic acid was also studied. A volume of 100 μl of 5% L-ascorbic acid in citrate buffer (0.1 *M*, pH 3.0) was added to 1 ml of human plasma (maintained at 37°C) at the following intervals: (a) prior to the addition of mitoxantrone, or (b) 1—2 h after the addition of mitoxantrone.

Equilibrium dialysis study

The dialysis apparatus (Chemical Rubber Co., Cleveland, OH, U.S.A.) had a 2-ml capacity. A cellulose acetate membrane separated the system into two symmetrical 1-ml chambers. Two sets of apparatus were run simultaneously, one containing 1 ml bisantrene or mitoxantrone (20 μg/ml) in normal saline dialyzed against 1 ml human plasma, the other containing 1 ml bisantrene or mitoxantrone in normal saline dialyzed against 1 ml normal saline. The dialysis apparatus was kept at room temperature with gentle shaking. Aliquots were removed from both chambers at time intervals up to 24 h for the measurement of bisantrene or mitoxantrone.

RESULTS

Figs. 2 and 3 show the chromatographic profiles of standard bisantrene and mitoxantrone extracted from normal plasma and a typical 0 time patient's plasma. There were no co-eluting peaks at the chromatographic positions of bisantrene and mitoxantrone, demonstrating the clean background associated with our sample cleanup procedure for both drugs.

Excellent linearity ($r > 0.996$) was observed for the standard curves over a 10—2000 ng/ml range for bisantrene and a 1—2000 ng/ml range for mitoxantrone. The precision and recovery data for the assay are shown in Tables I

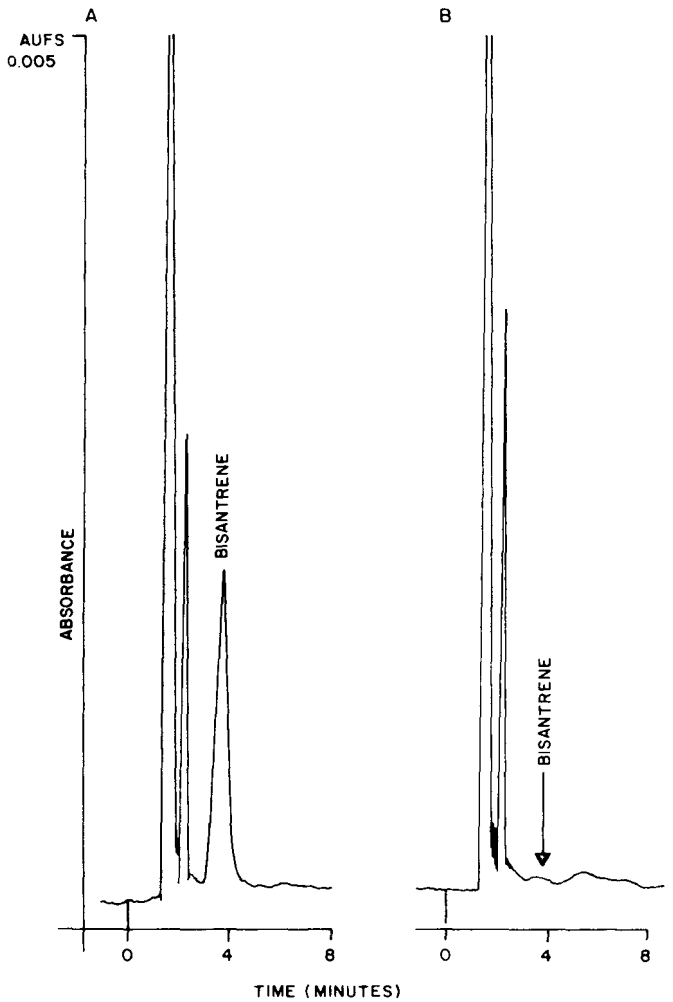


Fig. 2. Chromatographic profiles of (A) standard bisantrene (200 ng/ml) extracted from normal plasma and (B) a typical 0 time patient's plasma. Equivalent of 0.5 ml plasma was injected at 0.005 a.u.f.s.

and II. The average recovery was 98 ± 6% with a coefficient of variation (C.V.) of less than 7%. The sensitivity of the assay was 10 ng/ml for bisantrene and 1 ng/ml for mitoxantrone.

The full mass spectra of bisantrene and mitoxantrone are shown in Figs. 4 and 5. Note the prominant molecular ion at *m/e* 398. This ion and three other prominant ions (313, 228, 215) were used in the selected ion monitoring analysis of patient samples.

Fig. 6 shows the plasma disappearance curves for bisantrene and mitoxantrone following 60- and 30-min infusions, respectively, in two patients. Note that both drugs had prolonged terminal plasma half-lifes in these two patients.

Fig. 7 shows the stability of mitoxantrone and bisantrene in human plasma.

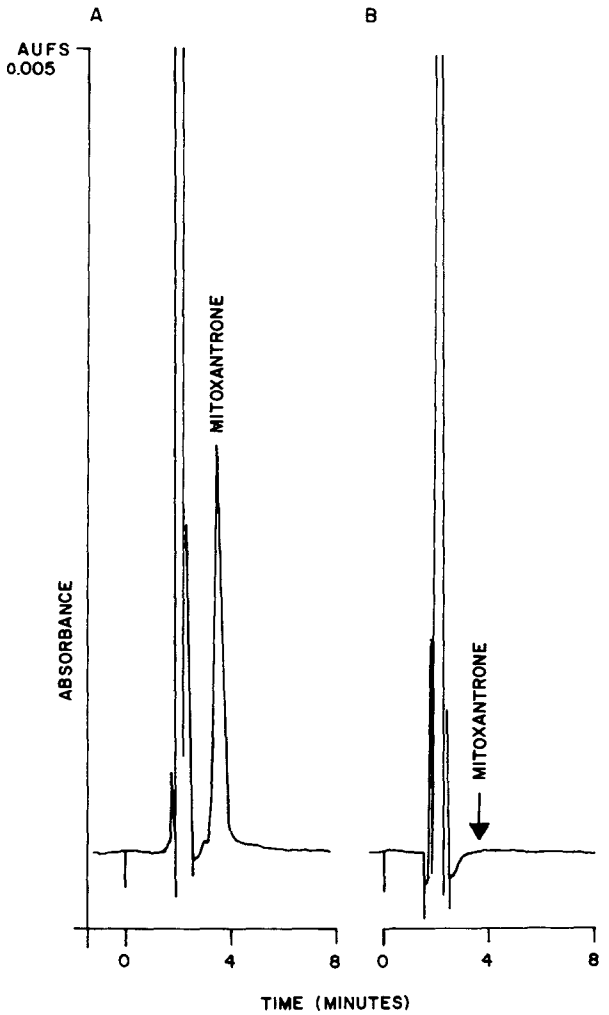


Fig. 3. Chromatographic profiles of (A) standard mitoxantrone (50 ng/ml) extracted from normal plasma and (B) a typical 0 time patient's plasma. Equivalent of 0.5 ml plasma was injected at 0.005 a.u.f.s.

Recoveries after a 24-h incubation at 25 and 37°C were 40 and 20% for mitoxantrone and 90 and 85% for bisantrene, respectively. Incubation of mitoxantrone with 5% human albumin + 3% human γ-globulin for 24 h resulted in recoveries of 80% at 25°C and 60% at 37°C.

To determine whether ascorbic acid stabilizes mitoxantrone in plasma as has been reported by Reynolds et al. [14] we incubated mitoxantrone with human plasma containing ascorbic acid. Mitoxantrone incubation for 24 h at 37°C resulted in drug recovery of greater than 90% (Fig. 8). Ascorbic acid was unable to regenerate but did prevent further loss of mitoxantrone when it was added to plasma following a 30% decrease in mitoxantrone concentration (Fig. 8).

TABLE I

RECOVERY OF BISANTRENE AND MITOXANTRONE FROM PLASMA

All experiments were run in triplicate on different days.

Amount added (ng/ml)	Recovery* (%) ($\bar{x}$ ± S.D.)	C.V. (%)
Bisantrene		
2000	105 ± 2.5	2.4
500	101 ± 5.1	5.0
100	93 ± 2.3	2.5
Mitoxantrone		
1000	105 ± 5.1	4.8
100	94 ± 2.0	2.1
10	94 ± 2.3	2.4
Average**	98 ± 5.6	5.7

*The immediate recovery of bisantrene and mitoxantrone at 25°C using sample extraction method as described in text.

**This is the average of all concentrations tested.

TABLE II

PRECISION OF ASSAY

Samples A, B, C and D are patient's plasma samples obtained after an intravenous infusion of bisantrene or mitoxantrone.

Experiment	Concentration* (ng/ml) ($\bar{x}$ ± S.D.)	C.V. (%)
Mitoxantrone		
Sample A	185 ± 10	5.4
Sample B	1.75 ± 0.12	6.8
Bisantrene		
Sample C	1560 ± 20	1.3
Sample D	114 ± 7.2	6.3

*Each patient sample concentration represents the average of three independent analyses on different days.

The results of dialysis experiments are shown in Fig. 9. Because of the instability of mitoxantrone in plasma, we placed the drug in normal saline for dialysis against plasma. After 24 h only 5% of the initial bisantrene and mitoxantrone concentrations remained in the dialysis chamber containing saline, suggesting that both drugs are highly protein bound. In contrast when both dialysis chambers contained saline, after 24 h 50% of the initial drug concentrations were detected in both chambers, suggesting that there was no significant adsorption of either drug onto the dialysis membrane.

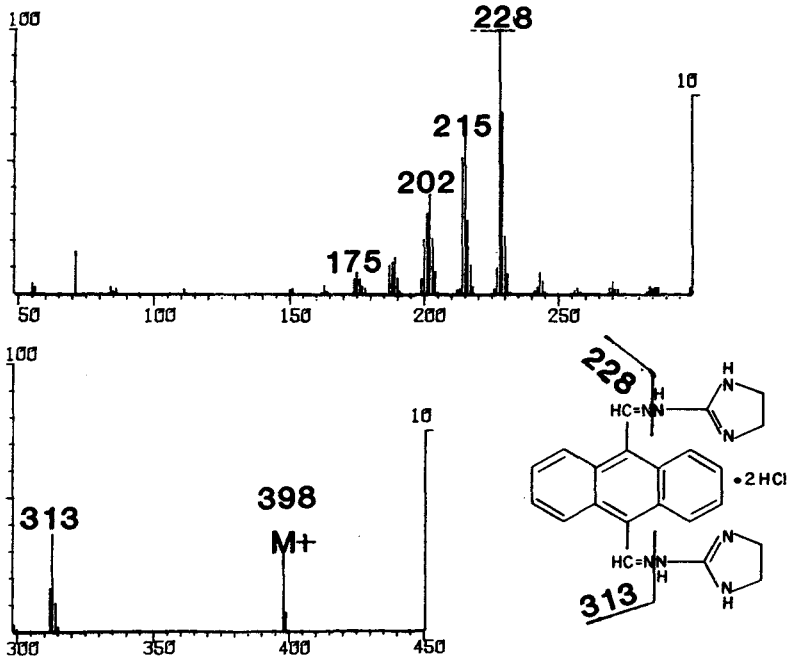


Fig. 4. Complete mass spectra of bisantrene from pure standard.

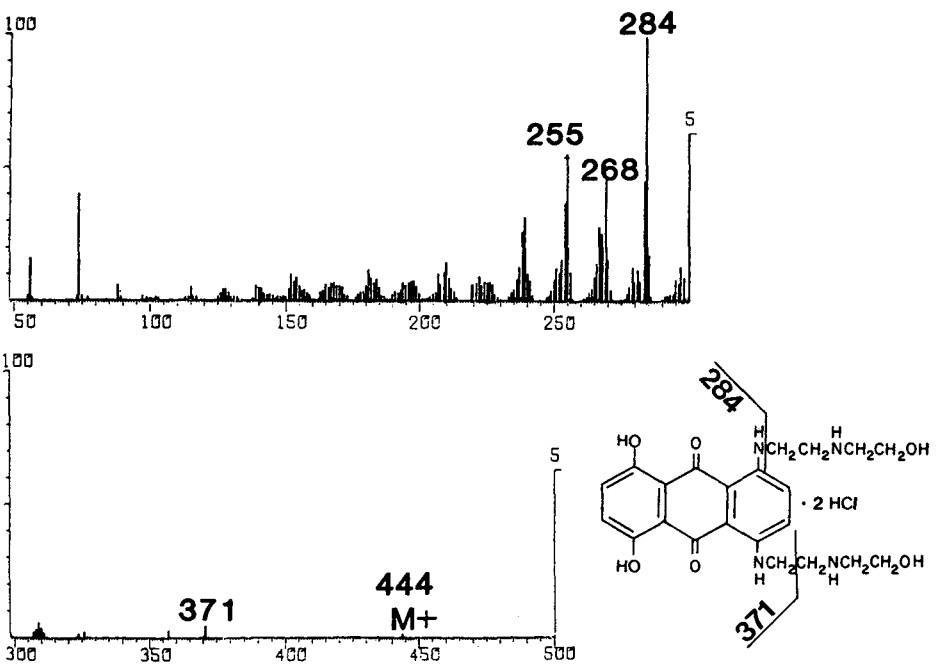


Fig. 5. Complete mass spectra of mitoxantrone from pure standard.

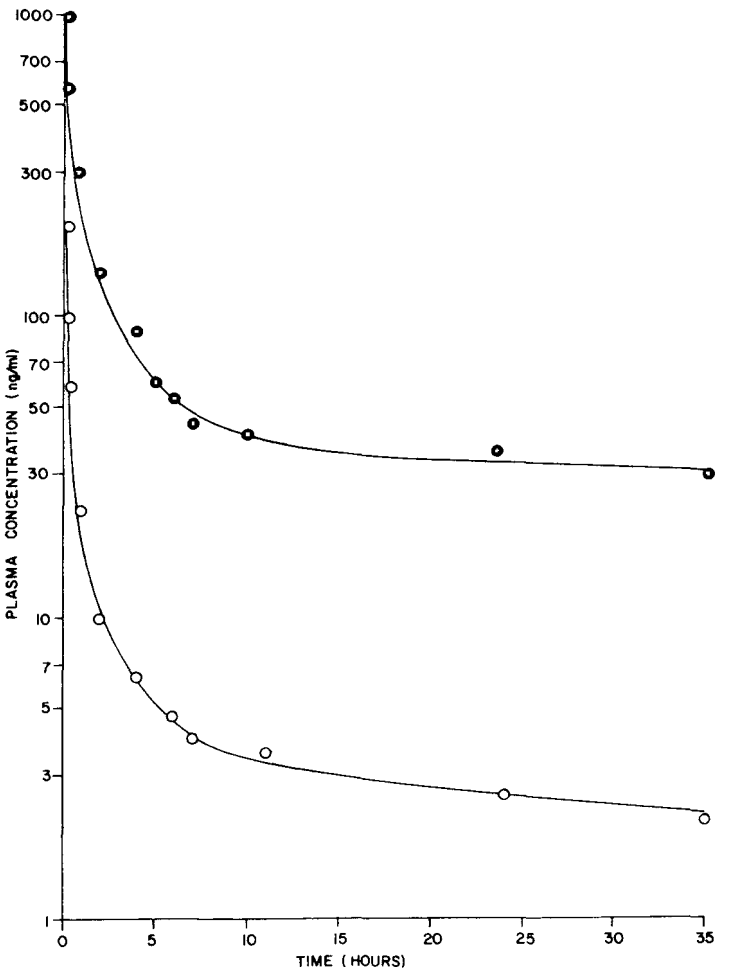


Fig. 6. Plasma disappearance curves of bisantrene (260 mg/m^2) (•) and mitoxantrone (12 mg/m^2) (○) after an intravenous infusion.

DISCUSSION

In a previous report [12], we used ethyl acetate for the extraction of bisantrene from biological fluids. Liquid extraction often resulted in a large volume of solvent, which had to be evaporated to dryness and reconstituted into a small volume for subsequent HPLC analysis. We found this process to be time consuming. Other published HPLC methods for mitoxantrone also require lengthy sample cleanup procedures, such as the double liquid extraction with chloroform described by Ostroy and Gams [13] or the XAD-2 column isolation described by Reynolds et al. [14].

Using commercially available and relatively inexpensive C_{18} mini-cartridges, we developed a simple, precise and quantitative extraction method for both bisantrene and mitoxantrone. The drug was separated from plasma constituents

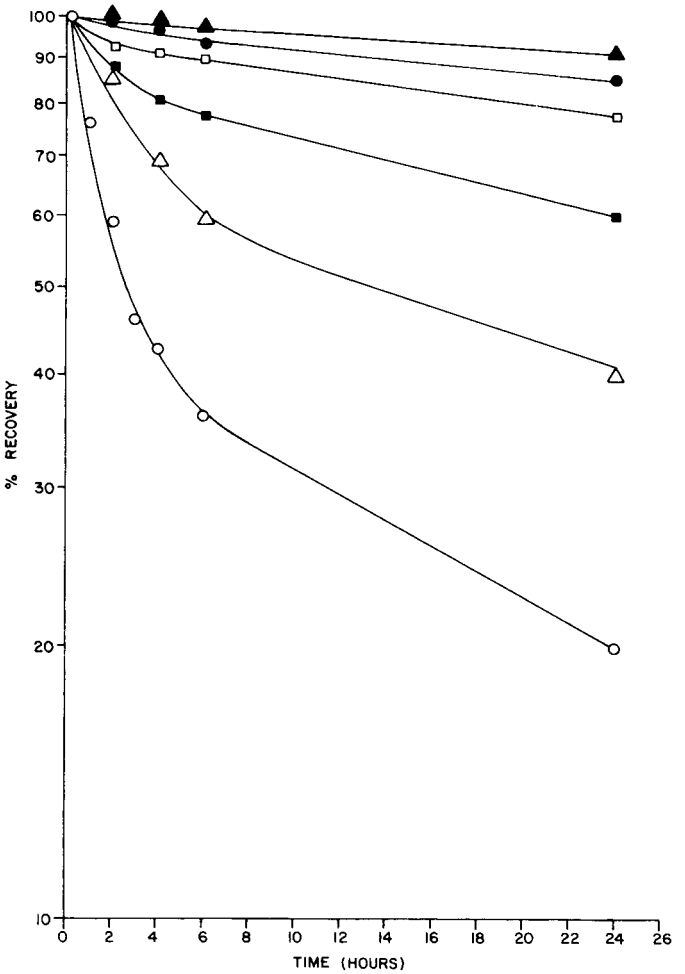


Fig. 7. Recovery of bisantrene (10 μg/ml) and mitoxantrone (10 μg/ml) in human plasma and in the presence of human plasma proteins (5% albumin + 3% γ-globulin) at 25 and 37°C. (▲) Bisantrene in plasma at 25°C, (●) bisantrene in plasma at 37°C, (△) mitoxantrone in plasma at 25°C, (○) mitoxantrone in plasma at 37°C, (□) mitoxantrone in plasma protein at 25°C, (■) mitoxantrone in plasma protein at 37°C.

by its retention on the C_{18} mini-cartridge. One advantage of the mini-cartridge is that it can be loaded with large quantities of plasma. This allows the assay of very low bisantrene and mitoxantrone drug concentrations.

Several solvents were tested for the washing and elution process in an effort to obtain optimal analytical conditions. Washing with distilled water alone resulted in a relatively clean chromatographic background (Figs. 2 and 3). Subsequent washing with acetonitrile, methylene chloride, or ethyl acetate did not improve the chromatography. Washing with methanol or chloroform resulted in poor drug recoveries (i.e., <50%). The 0.5 *M* methanolic HCl is a

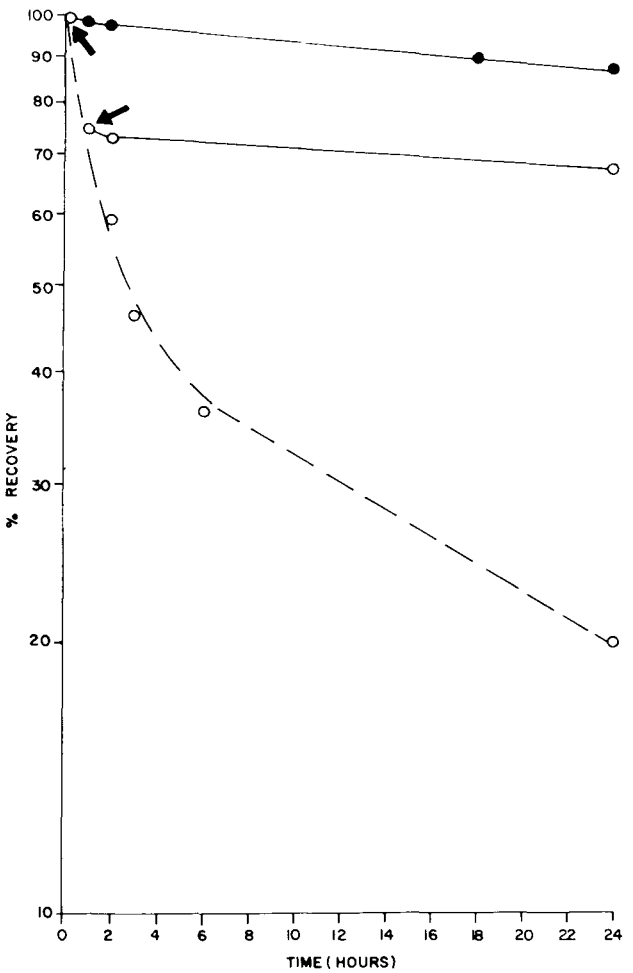


Fig. 8. Effect of ascorbic acid on the recovery of mitoxantrone in plasma at 37°C. To 1 ml of plasma containing mitoxantrone (10 μg/ml), 100 μl of 5% ascorbic acid in citrate buffer (0.1 *M*, pH 3.0) was added at the time as indicated by the arrow point. (○ — — — ○) Mitoxantrone in plasma without ascorbic acid, (●——●) ascorbic acid was added at time 0, (○——○) ascorbic acid was added at 1 h after incubation.

unique solvent chosen to elute the drug because we were able to accomplish a 98% recovery (Table I) by eluting with only 300 μl methanolic HCl. This is a valuable saving of time and organic solvents as compared to other procedures [12—14] and results in a much better recovery, precision and sensitivity.

Reynolds et al. [14] have described a similar method which utilizes a XAD-2 column for the extraction of mitoxantrone. However, packing the XAD-2 column is tedious, time consuming, and may not be uniform which can lead to variable recoveries and imprecision. Furthermore, the 254-nm detection wavelength used for their assay may not be optimal. Detection in the UV range

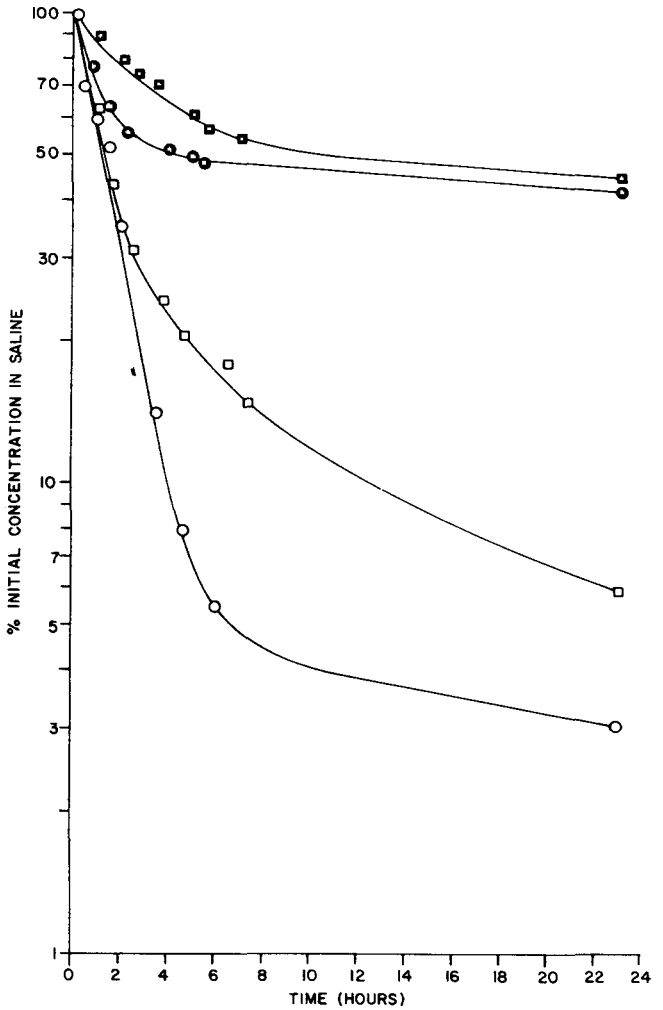


Fig. 9. Equilibrium dialysis of bisantrene and mitoxantrone at room temperature. Bisantrene or mitoxantrone (20 μg/ml) was added to 1 ml of normal saline to dialyze against 1 ml of normal saline or human plasma. Samples from both chambers were taken simultaneously at time intervals and assayed for bisantrene and mitoxantrone. (•) Bisantrene, saline vs. saline; (○) bisantrene, saline vs. plasma; (▪) mitoxantrone, saline vs. saline; (▫) mitoxantrone, saline vs. plasma.

will usually encounter a great deal of interfering peaks from biological samples. This will hinder the sensitivity of the assay and could lead to misidentification if mass spectrometry is not utilized for peak confirmation. Their reported sensitivity was 75 ng/ml which is not adequate to describe the plasma pharmacokinetics of mitoxantrone.

Mitoxantrone can also be detected at 546 nm as described by Ostroy and Gams [13], but detection at this wavelength is considerably less sensitive than the known λ_{max} of 658 nm. We detected mitoxantrone at 658 nm which has improved detection sensitivity markedly with no interfering peaks when we used the present rapid and precise sample cleanup procedure. The sensitivity

of our assay is below 1 ng/ml which has allowed us to document a prolonged terminal phase plasma half-life for this drug.

Mitoxantrone appears less stable in human plasma than bisantrene. Instability may result from chemical degradation or irreversible interaction of mitoxantrone with plasma proteins. The results of our stability studies of mitoxantrone in human plasma proteins (Fig. 7) suggest that its instability is related to more than just its interaction with plasma proteins. Reynolds et al. [14] have reported a similar instability of mitoxantrone in plasma. They suggested that this instability may result from an oxidative process. We have confirmed that ascorbic acid can stabilize, but not reverse prior loss of mitoxantrone from plasma samples (Fig. 8). Therefore, to maintain sample integrity, all plasma samples should be fortified with ascorbic acid and kept frozen prior to HPLC analyses of mitoxantrone.

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ANALYSIS OF 6-MERCAPTOPURINE IN HUMAN PLASMA WITH A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD INCLUDING POST-COLUMN DERIVATIZATION AND FLUORIMETRIC DETECTION

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SUMMARY

A relatively simple assay with improved reliability and sensitivity for measuring levels of 6-mercaptopurine in human plasma is presented. After extraction of the compound and the added internal standard with phenyl mercury acetate, samples were separated by ion-pair reversed-phase high-performance liquid chromatography. On-line the analytes were oxidized to fluorescent products and detected in a flow-fluorimeter. The within-day coefficient of variation was 3.8% at a concentration of 25 ng/ml. The lower detection limit was 2 ng/ml when 1.0 ml of plasma was used. Mercaptopurine concentration versus time curves of two subjects after a single oral dose of azathioprine are shown.

INTRODUCTION

Since 1953 the 6-thiopurine anti-metabolite 6-mercaptopurine (6-MP) has been in use for the treatment of leukemia. In 1963 azathioprine (AZA), a

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derivative of 6-MP, became available. AZA is used as an immunosuppressive agent in patients who have received organ transplants and for the treatment of autoimmune disorders such as systemic lupus erythematosis, rheumatoid arthritis, idiopathic thrombocytopenic purpura and autoimmune hemolytic anaemia.

Concerning the mode of action of these drugs there is no general agreement. Metabolites of the thiopurines are known to inhibit purine biosynthesis [1—3] and the synthesis of DNA and RNA [1, 3]. The incorporation of thiopurine nucleotides into DNA and RNA of treated mouse lymphoma cells has been shown [4]. It has been suggested that not all the effects of AZA are to be attributed to its major metabolite 6-MP. Other metabolites are thought to have some additional effects on the immune system [5—9].

Little is known about the relationship between the pharmacokinetics of these drugs and their biological effects. Although it is likely that biological effects correlate better with intracellular thiopurine metabolite concentrations than with the plasma concentration of unmetabolized 6-MP [1], it is to be expected that useful information can be obtained from studies of AUC (concentration × time) versus biological effects. The assay of 6-MP plasma concentrations can also be useful for the study of interactions of other drugs with this agent and possibly for dosage adjustments in patients with impaired organ function.

Several methods for measuring plasma levels of 6-MP and AZA have been described of which high-performance liquid chromatography (HPLC) combined with UV detection [10—13] and methods using fluorimetric detection [14, 15] seemed most practical. However, the former lacked sensitivity while the latter were not specific enough. Tidd and Dedhar [4] were the first to combine the advantages of HPLC specificity with the sensitivity of fluorimetric detection in developing a method for determining intracellular levels of thiopurine nucleotides. They performed pre-column oxidation of samples which were separated on an anion-exchange column using a buffer gradient.

The present method is based on separation by ion-pair reversed-phase HPLC, on-line oxidation to fluorescent products and fluorimetric detection of the analytes.

MATERIALS AND METHODS

Chemicals

All aqueous solutions were prepared with double-distilled water. 6-MP and 6-thioguanine (TG; used as internal standard) were obtained from Wellcome (The Wellcome Foundation Ltd., London, Great Britain). Dithioerythritol (DTE) was obtained from Sigma (St. Louis, MO, U.S.A.), sodium octane sulphonate from Serva (Heidelberg, G.F.R.) and phenyl mercury acetate (PMA) from BDH (Poole, Great Britain). All other chemicals were purchased from E. Merck (Darmstadt, G.F.R.). All chemicals were of analytical reagent grade.

Stock solution of 6-MP (1 mg/ml) was prepared in methanol, that of TG by dissolving a 40-mg tablet of TG (Lanvis®) in 250 ml of methanol (160 μg of TG per ml). Standard aqueous solutions of 6-MP (1 μg/ml) and TG (4 μg/ml) were prepared from these stock solutions.

Apparatus

The eluent was delivered by a Kipp 9208 HPLC pump (Kipp Analytica, Emmen, The Netherlands). Samples were injected with a Rheodyne 7120 (Berkeley, CA, U.S.A.) injection valve equipped with a 1-ml loop. Autoanalysis equipment from Technicon Instruments was employed. The detector was an Amin-co-Bowman spectrophotofluorimeter fitted with a 30-μl flow-cell. Excitation and emission wavelengths were 295 nm and 380 nm, respectively. For scanning the fluorescence spectra a standard quartz glass cuvette was used.

Chromatographic system

Separations were performed on LiChrosorb 10 RP-18 obtained as pre-packed columns (250 × 4.6 mm) from Chrompack (Middelburg, The Netherlands). The mobile phase was water–isopropanol (97:3) containing 13.80 g of $NaH_2PO_4 \cdot H_2O$, 0.2 ml of H_3PO_4 (85%), 60 mg of DTE and 500 mg of sodium octane sulphonate per liter. The pH of the eluent was between 3.6 and 3.7. The system was operated at ambient temperature with a flow-rate of 1.5 ml/min. The effluent from the column was fed directly into an automatic system for the oxidation of 6-MP and TG to their fluorescent purine 6-sulphonate derivatives [15] and subsequent detection. Fig. 1 shows the arrangement of this auto-analyzer system.

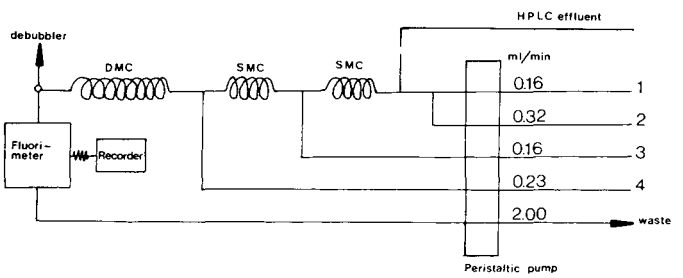

Fig. 1. Diagram of the autoanalyzer system used to produce the purine 6-sulphonates from 6-MP and TG after their separation by HPLC. SMC = single mixing coil; DMC = double mixing coil; 1 = 8 m*M* K_2CrO_4 in 0.5 *N* HCl; 2 = air; 3 = 1.6% $Na_2S_2O_5$; 4 = 4 *M* NH_4OH.

Sample preparation

To 1.0 ml of plasma in a glass-stoppered tube (5 ml) were added 25 μl of the internal standard solution (100 ng of TG), 0.1 ml of a 0.4 *N* sodium hydroxide solution, 1 ml of ethyl acetate containing 0.3% of PMA and 3 ml of diethyl ether. This mixture was shaken on a tumble-mixer for 10 min and centrifuged for 5 min. The overstanding organic layer was then transferred to another tube and 0.5 ml of a 0.1 *N* hydrochloric acid solution was added. This mixture was whirlmixed for 2 min and centrifuged for 5 min. Almost all of the organic layer was then removed by suction, and the remainder was evaporated under a gentle stream of nitrogen at room temperature during 15 min. To the residue 10 μl of an aqueous solution of 3 mg/ml DTE was added, and after mixing it was injected into the chromatographic system.

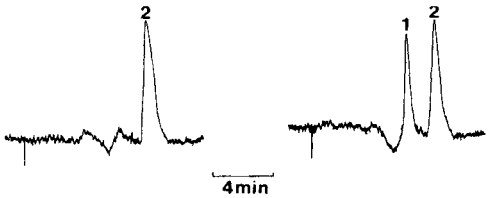

Fig. 2. Chromatograms of plasma samples. Left: blank plasma with internal standard. Right: plasma sample containing 25 ng/ml 6-MP. Peaks: 1 = 6-MP; 2 = internal standard (TG).

RESULTS

Representative chromatograms of plasma samples are shown in Fig. 2. The dip in the baseline just before the 6-MP peak is caused by the rather large volume of dilute hydrochloric acid that is injected into the liquid chromatograph, causing a temporary decrease in the background fluorescence of ammonium hydroxide.

The average of seven calibration plots made during a period of three weeks is described by the equation $y = 0.0231x + 0.0979$, the standard deviation of the slope being 0.0029. The coefficient of correlation ranged from 0.9850 to 0.9994 with a mean of 0.9955. The within-day coefficient of variation, calculated from eleven measurements of a plasma sample spiked with 25 ng of 6-MP, was 3.8%. In the same experiment the recovery was 76.8 ± 3.8% for 6-MP and 63.5 ± 3.2% for the internal standard TG. In view of the peak height to noise ratio (Fig. 2) it can be stated that the lower detection limit of 6-MP in our assay is less than 2 ng/ml when 1 ml of plasma is used.

Fig. 3 presents the 6-MP concentration–time curves of two subjects who received a single oral dose of 275 mg of azathioprine. Plasma samples of 1.0 ml were used for analysis; each dot represents the mean of duplicate determinations.

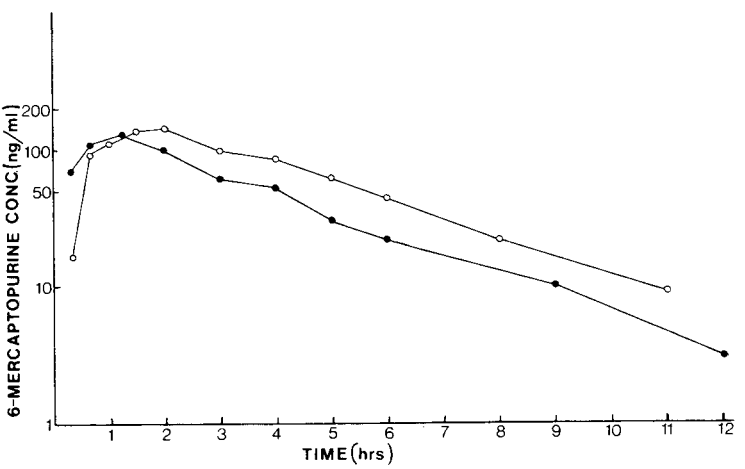

Fig. 3. Semilogarithmic plot of plasma 6-MP concentration vs. time of two subjects after oral administration of 275 mg of azathioprine.

DISCUSSION

The extraction method used is a modification of the method described by Maddocks [15]. Because an HPLC separation is included in our method it was possible to sacrifice some extraction specificity for a higher recovery. In alkalinised plasma the thiol group of thiopurines reacts with the mercury atom of PMA

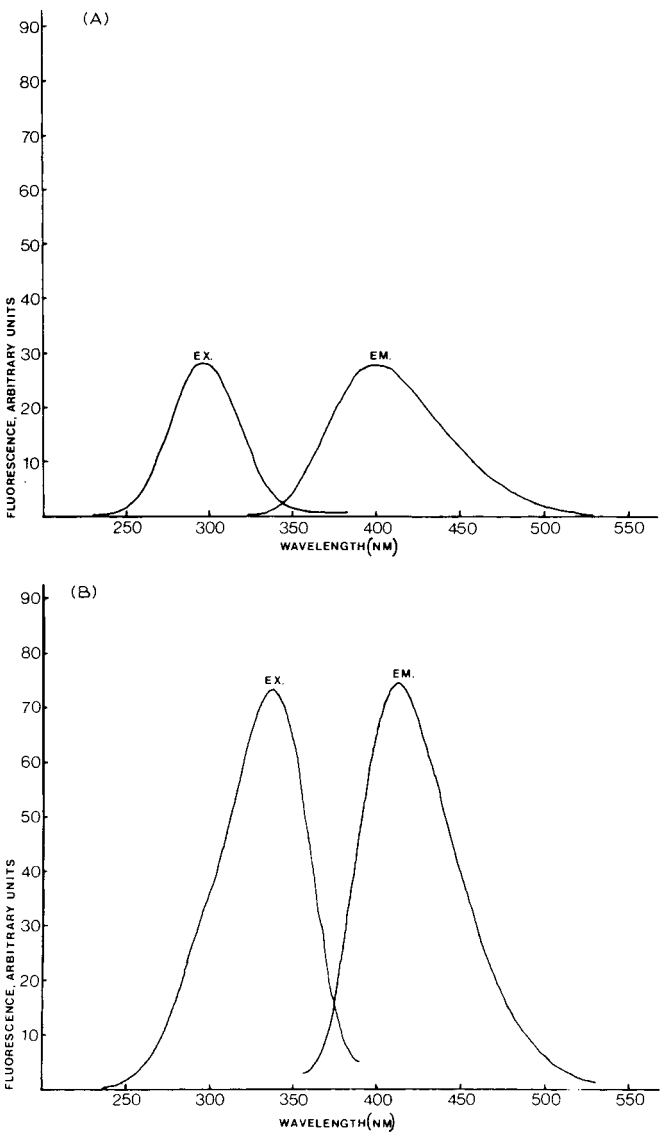

Fig. 4. Fluorescence spectra of oxidized 6-MP (A) and TG (B) in 1 *M* NH_4OH; concentration 5 μg/ml for both compounds and the same instrument setting for both spectra. EX: excitation spectra, maximum at 295 nm for 6-MP and 335 nm for TG. EM: emission spectra, maximum at 405 nm for 6-MP and 410 nm for TG.

to form a complex which can be extracted into organic solvents. When the organic layer is shaken with dilute hydrochloric acid the complex dissociates and the thiopurines are extracted back into the aqueous phase. The method can also be made suitable for the determination of AZA, as this compound can be converted to 6-MP by alkaline hydrolysis. Using this assay, the amount of AZA present in the plasma can be calculated by subtracting the concentration of 6-MP in non-hydrolyzed samples from that of hydrolyzed samples [15].

In order to achieve resolution between 6-MP and TG on the LiChrosorb 10 RP-18 column the addition of octane sulphonate to the eluent was needed. Octane sulphonate increases the capacity factor of TG due to ion-pair formation with the primary amino group of this molecule [16]. Therefore the resolution between 6-MP and TG was also dependent on the pH of the eluent resulting in an increased resolution at a lower pH.

It was found necessary to add DTE to the eluent in order to obtain a linear relationship between injected amount of 6-MP and observed peak height. Furthermore, DTE caused an improvement of peak shape. DTE was synthesized and described for the first time by Cleland [17]. It was presented as a protective agent to keep thiol groups in the reduced state and to reduce disulfides quantitatively. Bailey et al. [18] used DTE during the extraction of 6-MP to prevent decomposition, and Ding and Benet [12] observed improved peak heights when DTE was added during extraction. We never observed any improvement in the recovery of both compounds by adding DTE during the extraction of spiked plasma samples. Therefore we concluded that no significant decomposition of 6-MP or TG occurred during our extraction procedure. Furthermore, DTE was not added during the extraction because it would convert unmetabolized AZA to 6-MP and because DTE could interfere with the PMA extraction. Furthermore, we concluded that an additional effect of DTE in our assay was to prevent adsorption of 6-MP to metal surfaces.

In the post-column manifold the thiopurines were oxidized to their fluorescent purine 6-sulphonates according to the mechanism described by Finkel [14] and modified by Maddocks [15], who applied it in a manual method for 6-MP. By varying all parameters involved in the reaction it was established that the conversion took place quantitatively under our conditions. It appeared that the alkali used was responsible for a background signal. We used ammonium hydroxide instead of an alkali metal hydroxide because, using the same concentration, it reduced the noise level without loss of signal intensity.

The excitation and emission spectra of oxidized 6-MP and TG are shown in Fig. 4A and B, respectively. Because the fluorescence spectrum of the background signal showed an excitation maximum of 325 nm and an emission maximum of 410 nm, the emission wavelength was set at 380 nm instead of 405 nm which is the emission maximum of oxidized 6-MP. Thus some of the response of 6-MP was sacrificed in order to obtain a better signal-to-noise ratio.

In view of the results shown our assay can be considered a reliable and sensitive method for measuring levels of 6-MP in human plasma.

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SEPARATION AND QUANTITATION OF *cis*- AND *trans*-THIOTHIXENE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A high-performance liquid chromatographic procedure is described for the separation of *cis* and *trans* isomers of thiothixene, a thioxanthene derivative used as an antipsychotic agent. A radial compression module (RCM-100) was used with both silica and cyanopropyl cartridges. A fixed-wavelength UV detector (254 nm) was used in these studies for quantitation. Mesoridazine is used as an internal standard because of its separation characteristics and reproducible quantitation. C_{18} Sep-Pak cartridges are used for biological sample clean-up. Plasma samples from patients treated with thiothixene (Navane) were assayed for *cis* and *trans*-thiothixene. No *trans*-thiothixene was detectable and *cis*-thiothixene concentrations ranged from 0 to 22.5 ng/ml.

INTRODUCTION

Thiothixene (Navane®; N,N-dimethyl-9-[3-(4-methyl-1-piperazinyl)-propylidene]-thioxanthene-2-sulfonamide) is a low-dose antipsychotic agent of the thioxanthene class. It is now recognized that monitoring plasma levels of anti-

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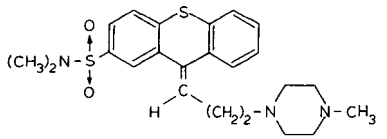
psychotic drugs and their metabolites in some cases aids the evaluation of the clinical response to the drug. A very sensitive method is required in view of the low concentrations found in plasma samples (4—20 ng/ml). Further, thiothixene (TTX) exists in two isomeric forms, *cis* (I) and *trans* (II), the *cis* being



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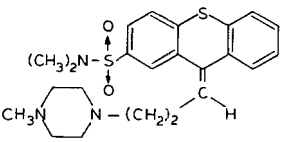

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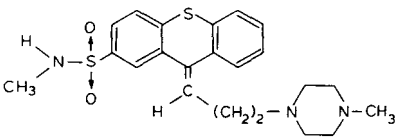
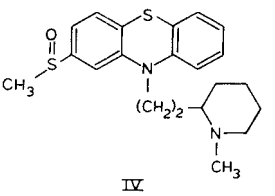


III



IV

the active antipsychotic and the *trans* being inactive. It is believed that differences in clinical response between patients treated with TTX may be due to different concentrations of the *cis* and *trans* forms of the drug produced by isomerization of the *cis* form in vivo. In order to verify this hypothesis the analytical methods should be capable of separating the *cis* and *trans* forms. In addition, N-desmethylthiothixene (III) is a major metabolite [1] and there are no published reports for its quantitation.

Previous methods of analyses used spectrofluorometry [2] and gas chromatography—mass spectrometry (GC—MS) [3, 4]. The spectrofluorometric method is sensitive but not specific since it cannot distinguish between the *cis* and *trans* isomers and is susceptible to interference by metabolites. Hobbs et al. [3] used a GC—MS method with electron impact ionization and selected ion monitoring. C^2H_3-thiothixene was used as internal standard and they monitored the base peak at *m/z* 113 for TTX and *m/z* 116 for the internal standard. This method also did not distinguish the *cis* and *trans* isomers.

Bombardt and Friedel [4] improved the sensitivity of the CG—MS procedure by using methane chemical ionization and monitoring the [M + 1] ion of TTX (*m/z* 444) and the C^2H_3-internal standard (*m/z* 447). They described a GC procedure to separate the *cis* and *trans* isomers and demonstrated that patients show varying capacities to convert the active *cis* isomer to the relatively inactive *trans* form. Drawbacks of this procedure include the expense and expertise needed for the routine assay of clinical samples by GC—MS and the inconsistency of *cis* and *trans* separation by GC due to thermal rearrangement.

The purpose of this investigation was to develop a means of measuring *cis*-TTX in human plasma that is simple, sensitive and specific. We decided to

develop a high-performance liquid chromatographic (HPLC) method for the separation and quantitation of *cis*- and *trans*-TTX and validate the results by the more specific GC—MS method. The only LC methods reported in the literature for TTX were for quantitation in pharmaceutical formulations [5]. Li Wan Po and Irwin [6] reported the separation of *cis—trans* isomers of tricyclic neuroleptics, fluopenthixol, clopenthiol and chlorprothixene but could not separate TTX isomers. In our present work, one aim was to compare different LC columns, solvent phases and detector conditions for the quantitation of *cis*-TTX in plasma samples. In addition, we developed an extraction method and found a suitable internal standard, mesoridazine (MSZ, IV).

MATERIALS AND METHODS

Reagents

Ethyl acetate, hexane, isopropanol, methanol and acetonitrile were distilled in glass, LC-grade (Burdick and Jackson, Muskegon, MI, U.S.A.). Triethylamine was 99% pure (Aldrich, Milwaukee, WI, U.S.A.). Ammonium hydroxide was ACS reagent grade 28%. All water used was deionized reagent quality. All other chemicals were analytical grade.

2 *N* NaOH was diluted from stock 10 *N* NaOH (Fisher Certified, Fisher Scientific, Pittsburgh, PA, U.S.A.). 0.05 *N* NaOH was diluted from 2 *N* NaOH. 0.6 *M* NaH_2PO_4 was prepared by dissolving 8.28 g of NaH_2PO_4 in 100 ml of water. 8.5% H_3PO_4 was prepared by diluting 85% phosphoric acid.

All HPLC mobile phases were filtered through 0.22-μm membrane filters and degassed under vacuum. Mobile phases containing 0.03 *M* aqueous NaH_2PO_4 were adjusted to the desired pH using 8.5% H_3PO_4 or 2 *N* NaOH.

Standards

Cis- and *trans*-TTX and N-desmethyl-TTX were obtained as the free bases (Charles Pfizer, New York, NY, U.S.A.). A 50-mg mesoridazine tablet (Serentil, Sandoz, Hanover, NJ, U.S.A.) was dissolved in 50 ml 0.5 *N* HCl, then the solution was adjusted to pH 9 with 10 *N* NaOH and extracted five times with 20 ml of hexane—isopropanol (9:1). A solution of approximately 100 μg/ml was prepared by diluting 10 ml to 50 ml with methanol.

Separate stock solutions of 1 mg/ml of *cis*- and *trans*-TTX and N-desmethyl-TTX were each made in methanol. These were diluted with methanol to 10 μg/ml.

A solution containing the internal standard, mesoridazine, and "carrier" *cis*-thiothixene was prepared for addition to all standard and unknown plasma samples. This solution was prepared by mixing 0.20 ml of the approximately 100 μg/ml MSZ solution, 1.6 ml of the 10 mg/ml *cis*-TTX solution and 38.2 ml of deionized water. This solution contained 400 ng/ml of *cis*-TTX and approximately 500 ng/ml of MSZ.

Cis-TTX plasma standards of 5, 10 and 20 ng/ml were prepared in drug-free blood bank plasma. Aqueous *cis*-TTX standards of 5, 10, 20, 50 and 100 ng/ml were prepared by dilution of 1 mg/ml methanolic standards with deionized water.

Collection of specimen

Blood was collected into 10-ml green-stoppered Venoject tubes containing sodium heparin (Terumo Medical, Elkton, MD, U.S.A.). The specimens were centrifuged and the plasma layer removed as soon as possible. The plasma was stored at −20°C and protected from light until analyzed.

Special apparatus

C_{18} Sep-Pak cartridges were supplied by Waters Assoc., Milford, MA, U.S.A. Multifit glass syringes (10 ml) were obtained from Beckton-Dickinson, Oxnard, CA, U.S.A.

Instrumentation

The HPLC system used was a Waters Assoc. Model 20A consisting of a U6K injection loop, a Model 6000A positive displacement solvent delivery system, a Model 440 254-nm UV detector and a Model RCM-100 radial compression module for chromatography cartridges. A 10 cm × 5 mm (10 μm) Radial-Pak silica cartridge and a 10 cm × 8 mm (10 μm) Radial-Pak Nitrile CN cartridge (both Waters Assoc.) were used. A C_{18} Guard-Pak (Waters Assoc.) pre-column insert was used with the Nitrile CN HPLC cartridge. A stainless-steel 50 cm × 4.6 I.D. pre-column filled with HC Pellosil (Whatman, Clifton, NJ, U.S.A.) high-capacity silica gel bonded to 30—38 μm glass bead was used with the silica HPLC cartridge. An Omni Scribe Model B5117-2 strip chart recorder (Houston Instruments, Austin, TX, U.S.A.) was used to record detector response.

A Model 4000 (Finnigan MAT, Sunnyvale, CA, U.S.A.) GC—MS system was used for identification of thiothixene from the HPLC fractions. A 0.6-m glass column packed with 1.5% SP-2100 was used at 230°C with a helium flow of 35 ml/min. The base peak ion at *m/z* 113 was monitored to identify thiothixene.

C_{18} Sep-Pak extraction

The use of C_{18} Sep-Pak cartridges for biological sample clean-up was reported by Narasimhachari [7]. The Sep-Pak method has now been adopted with some modification for the extraction of TTX from plasma samples.

C_{18} Sep-Pak cartridges were activated prior to use by passing 2 ml of methanol followed by 5 ml of deionized water at a flow-rate of 5 ml/min. Two ml of plasma standards (20 ng/ml), 100 μl of internal standard MSZ and carrier TTX solution were mixed with 2 *N* NaOH (0.5 ml) and passed through separate Sep-Pak cartridges followed by 3 ml of 0.05 *N* NaOH as a wash. The Sep-Pak cartridges were then eluted twice in 4 ml of different organic solvents: ethyl acetate, hexane, hexane—ethyl acetate (1:1), hexane—isopropanol (1:1), hexane—isopropanol (4:1), methanol and isopropanol. All solvents contained 0.1% triethylamine. The eluates were collected in 15-ml centrifuge tubes and centrifuged at 1000 *g*. The lower aqueous layer was removed and discarded. The organic solvents were evaporated at 40°C under nitrogen. Finally, the sides of the tubes were washed with 0.5 ml of extraction solvent, vortexed and dried. The residue in each tube was dissolved in 40 μl of LC mobile phase and 50% of this was injected into the LC system. From the peak height ratio of extracted sample and direct standard injection the extraction efficiency for thiothixene

was calculated for each solvent system. Thirteen control plasma samples containing no thiothixene were analyzed after adding a standard MSZ—TTX mixture using the Sep-Pak method to calculate the extraction efficiency for each compound and to evaluate the reproducibility of peak height ratio of TTX and the internal standard, MSZ.

Cyanopropyl stationary phase

LC system: A 10-μm radial compression module (RCM) silica cartridge was used in the earlier studies. In view of poor stability of silica columns at alkaline pH a cyanopropyl cartridge was evaluated as a reversed phase for the separation of *cis*- and *trans*-thiothixene, desmethylthiothixene and the internal standard. A mobile phase consisting of methanol—acetonitrile—0.03 *M* NaH_2PO_4—triethylamine (400:50:50:1.0) adjusted to pH 7.45 with phosphoric acid gave complete separation of all the four compounds.

Specificity

The LC peak corresponding to the retention time of *cis*-thiothixene was recycled through the chromatographic system five times when one symmetrical peak was always obtained. The fraction corresponding to this peak was collected and evaporated under nitrogen. The residue was dissolved in methanol and used for GC—MS identification.

Duplicate plasma samples were run by GC—MS—selected ion monitoring technique for quantitation of *cis*-TTX. For this purpose 2 ml of plasma sample containing 200 ng of d_3-thiothixene was processed by the C_{18} Sep-Pak procedure, using 10 ml of hexane—isopropanol (9:1) as eluting solvent. The solvent was evaporated under nitrogen, the residue redissolved in 20 μl ehtyl acetate and 2 μl injected into the GC—MS system. The ionizer temperature was 270°C and separator temperature 260°C. Electron ionization at 70 eV and ionizing current 0.45 mA was used. Ions *m/z* 113 and 116 were monitored for sample and internal standard, respectively. It was found necessary to saturate the column at least by three injections containing 1 μg/ml of standard *cis*-TTX solutions before using the system for quantitation.

Calculations of unknowns

Plasma samples were obtained from patients on standard antipsychotic TTX therapy and were drawn during initiation of therapy, steady-state and withdrawal of medication.

One hundred fifty plasma TTX samples have been assayed using the standard addition method, the C_{18} Sep-Pak extraction and either silica normal-phase or cyanopropyl reversed-phase LC. UV absorbance at 254 nm was used to detect *cis*-TTX and the internal standard, MSZ. Levels of *cis*-TTX were calculated from a peak height ratio of *cis*-TTX to MSZ versus plasma standard concentration graph.

RESULTS

Sample extraction

Previous to the use of the C_{18} Sep-Pak cartridges, various solvent mixtures

were tried for the extraction of thiothixene. Of the solvents we used, hexane—isopropanol (4:1) was found to be most satisfactory. Solvent extractions produced dirtier chromatograms than did C_{18} Sep-Pak extraction especially when used with a silica stationary phase for chromatography. A three-step solvent extraction reduced interferences but also reduced extraction efficiency, critical for low therapeutic concentrations of thiothixene. The extraction efficiencies for different solvent systems for *cis*-thiothixene from plasma standards are shown in Table I.

TABLE I

EXTRACTION EFFICIENCIES FOR DIFFERENT SOLVENT SYSTEMS FOR *cis*-TTX FROM PLASMA STANDARDS

Solvent		Extraction efficiency (%)
Hexane		37
Hexane—ethyl acetate	(1:1)	11
Hexane—isopropanol	(9:1)	40
Hexane—isopropanol	(8:2)	70
Hexane—isopropanol	(7:3)	62
Hexane—isopropanol	(6:4)	76
Hexane—isopropanol	(5:5)	57

For C_{18} Sep-Pak extraction, ethyl acetate was found to be the best solvent of those we tried. Table II shows the solvent system and the corresponding extraction efficiency. C_{18} Sep-Pak extraction gave consistently cleaner chromatograms than one-step hexane—isopropanol (4:1) solvent extraction and also saves time compared to the solvent extraction method. The Sep-Pak cartridges can be re-used when washed with 5 ml of methanol followed by 5 ml of water.

TABLE II

PERCENT RECOVERY OF *cis*-TTX FOR DIFFERENT SOLVENT SYSTEMS FROM C_{18} SEP-PAK CARTRIDGE

Solvent		Extraction efficiency (%)
Ethyl acetate		62
Hexane—ethyl acetate	(1:1)	40.5
Hexane—isopropanol	(1:1)	55
Hexane—isopropanol	(4:1)	50
Methanol		0
Isopropanol		45

We found variation in extraction efficiency using the Sep-Pak procedure. The extraction of 13 blank plasma samples using the standard addition—internal standard method gave mean extraction efficiencies of 65% and 63% for *cis*-TTX and MSZ, respectively. The standard deviations were 11.2% and 12.2% for *cis*-TTX and MSZ extractions, respectively. However, the peak height ratio of *cis*-TTX and MSZ was 0.331 with a standard deviation of 0.008. Thus, the presence of internal standard is necessary to correct for the variation in the extraction.

Chromatography

We investigated the separation of *cis*- and *trans*-thiothixene on 10-μm silica columns (μPorasil, Waters Assoc.) and silica cartridge (5 mm I.D.). Fig. 1 shows typical chromatograms of a plasma based standard and a patient plasma standard. We found that the silica column was not stable with the hexane—isopropanol—methanol—ammonium hydroxide (400:400:200:1) mobile phase that we used. Silica is soluble in low-molecular-weight alcohols at alkaline pH. Column instability prompted us to try reversed-phase columns. C_{18} Reversed-phase chromatography of these compounds yielded broad peaks. We found that the cyanopropyl reversed-phase columns gave good chromatography of thiothixene and mesoridazine.

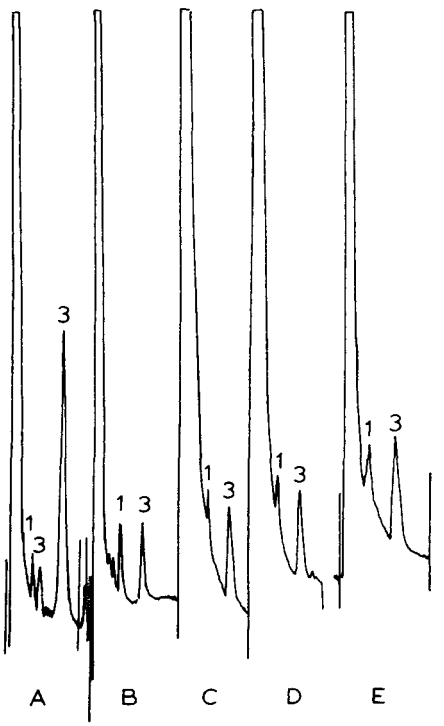


Fig. 1. HPLC separation on RCM silica cartridge of (1) *cis*-TTX (I), (2) *trans*-TTX (II), (3) MSZ (IV) internal standard. (A) Pure standards; (B) plasma standard 20 ng/ml extracted after standard addition; (C and D) drug-free blank plasma extracted after standard addition; (E) patient plasma extracted after standard addition (plasma concentration 4 ng/ml).

We found two satisfactory mobile phase systems for use with the 10-μm cyanopropyl reversed-phase radial compression cartridge. The first, methanol—acetonitrile—0.03 *M* NaH_2PO_4—triethylamine (650:100:250:1.0) pH 3.7, gave us chromatograms similar to the silica phase. There was a large solvent front so that this system is compatible with C_{18} Sep-Pak extraction but not with one-step solvent extraction. The second mobile phase of methanol—acetonitrile—0.03 *M* NaH_2PO_4—triethylamine (400:50:50:1.0) pH 7.45 gave much better

separation of *cis*-TTX, *trans*-TTX, N-desmethyl-TTX and MSZ. The compounds of interest were better separated from the "solvent front" so that C_{18} Sep-Pak or solvent extraction could be used. Fig. 2 shows chromatograms of extracted plasma standard and extracted patient plasma samples. A standard curve of peak height ratio of *cis*-TTX to MSZ versus standard *cis*-TTX concentration was used to calculate unknown *cis*-TTX concentrations in patient plasma samples.

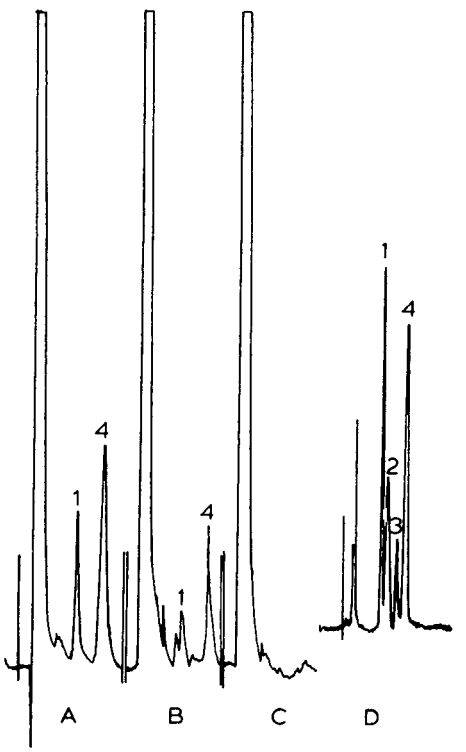


Fig. 2. HPLC separation on RCM-CN-Nitrile cartridge of (1) *cis*-TTX (I), (2) *trans*-TTX (II), (3) nor-TTX (III) and (4) MSZ (IV). Extraction by Sep-Pak method. (A) Plasma standard 20 ng/ml; (B) patient plasma (3.5 ng/ml); (C) drug-free plasma blank; (D) pure standards.

The linear regression equation of the line gave a slope of 0.019, intercept of 0.324 and r of 0.998. The coefficient of variation of the peak height ratio of 21 extracted plasma standards used to prepare this standard curve was 4.8%. Plasma blanks were run under identical conditions and no interference from any endogenous compounds were observed (Fig. 2). Extracts of plasma samples containing d_3-thiothixene were run on HPLC, the LC peaks for the *cis* form were collected, and after evaporation were quantitated by GC—MS—SIM. A complete mass spectrum of the LC peak was also obtained on GC—MS and also by direct solid probe inlet (Fig. 3). The spectrum did not show any detectable contamination.

Retention data for *cis*- and *trans*-TTX, desmethyl-TTX and internal standard using a cyanopropyl reversed-phase column are presented in Table III.

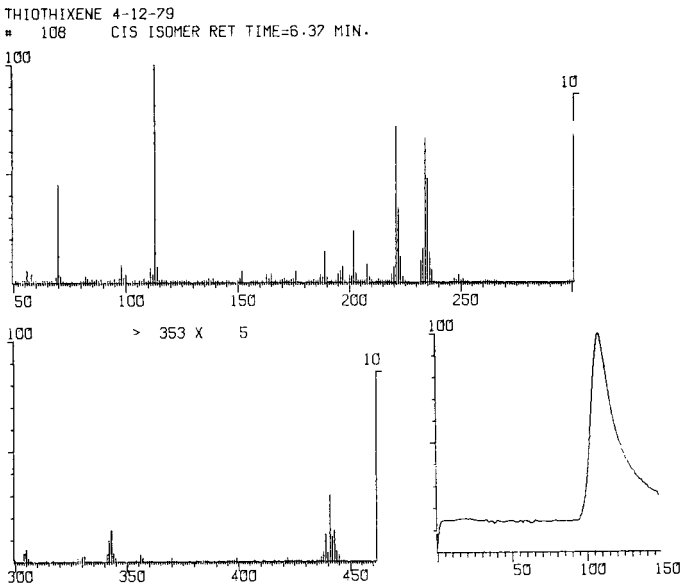


Fig. 3. Mass spectrum and total ion chromatogram (lower right) of HPLC fraction containing *cis*-TTX.

TABLE III

RETENTION DATA ON CN NITRILE RADIAL COMPRESSION CARTRIDGE (8 mm I.D.) WITH MOBILE PHASE OF METHANOL—ACETONITRILE—0.03 *M* AQUEOUS NaH_2PO_4—TRIETHYLAMINE (400:50:50:1) pH 7.45; FLOW-RATE 1.5 ml/min

Compound	Retention time (min)	k'
cis-TTX	7.0	3.7
trans-TTX	8.1	4.4
N-Desmethyl-TTX	9.6	5.4
Mesoridazine	11.0	6.3

Plasma cis-thiothixene concentrations

The 150 plasma samples assayed for *cis*-TTX had levels ranging from 0 to 22.5 ng/ml. The mean of all levels was 4.3 ng/ml. No *trans*-TTX was noted in these plasma samples.

DISCUSSION

We report the determination of *cis*-thiothixene in human plasma specimens by HPLC. Mesoridazine is used as internal standard and the addition of a constant amount of *cis*-TTX as a "carrier" increases the sensitivity of the assay. *Cis*-TTX and MSZ can be extracted from alkalinized plasma by either solvent extraction using hexane—isopropanol (4:1) or by the C_{18} Sep-Pak method. We

have found the C_{18} Sep-Pak extraction to reduce interferences and the time required to prepare the specimen for LC. We are routinely using the Sep-Pak procedure and cyanopropyl reversed-phase chromatography to measure *cis*-TTX levels during clinical trials with patients diagnosed as schizophrenic or borderline schizophrenic.

Bombardt and Friedel [4] reported finding varying proportions of *trans*-TTX by GC—MS in plasma of patients receiving *cis*-TTX. In contrast, we have not found *trans*-TTX in the plasma samples we have analyzed. This HPLC procedure clearly separates the isomers whereas GC gives poor separation. During the course of this investigation we found that standard solutions of *cis*-TTX showed contamination by *trans*-TTX after 1 or 2 weeks, the height of the *trans* peak increasing with time. We therefore studied the effect of UV irradiation on solutions of pure *cis*-TTX, in methanol or ethyl acetate. Results of such irradiation by a 15-W UV lamp (TLC scanner) for 15 min is shown in Fig. 4. From these results it is our view that the presence of *trans*-TTX in plasma has to be interpreted as probably artifactual unless adequate precautions are taken in the storage and handling of the samples.

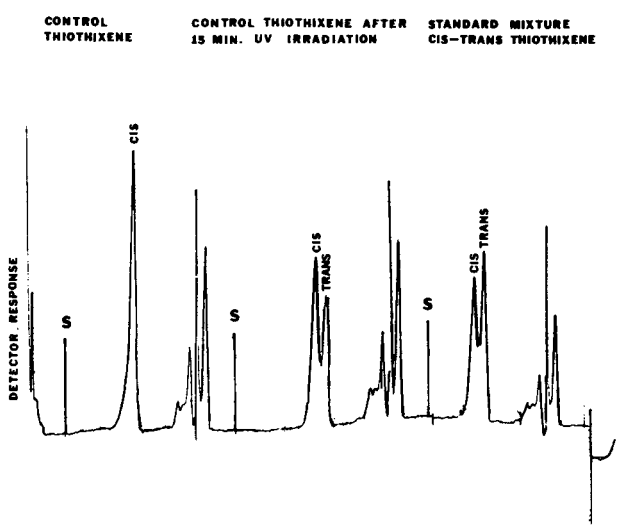


Fig. 4. The effect of UV irradiation on *cis*-TTX. (Left) standard *cis*-TTX; (center) *cis*-TTX after 15 min irradiation; (right) standard *cis—trans* mixture.

The sensitivity of the HPLC assay can be improved by measuring UV absorbance at 229 nm, the UV absorbance maximum for *cis*-TTX. Absorbance is roughly double at 229 nm compared to 254 nm. The use of a variable-wavelength detector at 229 nm would allow the measurement of thiothixene without the standard addition procedure. We have carried out experiments on detection limits for *cis*-TTX using the Beckman Model 150 fixed-wavelength UV detector using a cadmium lamp (229 nm) (Beckman Instruments, Berkeley, CA, U.S.A.). In our future studies we intend using this more sensitive wavelength for TTX quantitation. Hobbs et al. [3] used a GC—MS procedure to

measure *cis*-TTX in plasma of patients taking TTX. They reported a range of peak TTX concentrations of 10.0—22.5 ng/ml which correspond to a dose range of 15—60 mg per day which adequately controlled the symptoms of fifteen chronic schizophrenic patients. The plasma levels we determined were drawn at various times after drug administration. The range of *cis*-TTX plasma concentrations we found was 0—22.5 ng/ml which compares favorably with the reported concentrations [3].

N-Desmethyl-TTX was reported as a metabolite of TTX by Hobbs [1]. N-Desmethyl metabolites of phenothiazines and tricyclic antidepressants have been found to be active agents similar to their parent compounds. We have been able to separate and detect N-desmethyl-TTX on the cyanopropyl reversed-phase column. Simultaneous measurement of *cis*-TTX and N-desmethyl-TTX is possible and may yield additional information in the study of plasma levels and clinical response.

The HPLC procedure described in this paper coupled withthe Sep-Pak method for sample clean-up and the use of a closely related internal standard will be highly useful for routine monitoring of plasma *cis*-TTX levels. This simple, rapid analytical procedure can therefore be used for pharmacokinetic studies, studies in the metabolic differences between responders and non-responders and also for quickly checking compliance among patients. We have recently extended this study for monitoring saliva levels of TTX and found the HPLC method suitable for measuring saliva levels.

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SENSITIVE ASSAY FOR THE TRICYCLIC ANTIDEPRESSANT Ro 11-2465 IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic assay, suitable for pharmacokinetic studies, has been developed for the new tricyclic antidepressant Ro 11-2465, at present under clinical investigation. For concentrations above 0.5 ng/ml, the method involves a simple extraction at basic pH with an organic solvent followed by direct chromatography of this extract on a silica gel column using fluorescence detection. For concentrations below 0.5 ng/ml, an extensive clean-up procedure is required. In both procedures, however, evaporation of the extract and reconstitution of the residue is avoided. The detection limit, using 1 ml of plasma, is about 0.1 ng/ml. This sensitivity is sufficient for following single-dose kinetics of Ro 11-2465 in man.

INTRODUCTION

Tricyclic drugs such as imipramine and clomipramine (Fig. 1) are widely used as antidepressants. Pharmacokinetic and pharmacological studies have prompted the development of several analytical procedures for monitoring the plasma and urine levels of these drugs. However, the concentrations of these drugs in biological fluids are extremely low and the development of a suitable method is difficult. Many papers using thin-layer chromatography, gas chromatography, mass spectrometry and high-performance liquid chromatography (HPLC) have been published [1], but not all of these procedures are sensitive enough for single-dose pharmacokinetics. During the last few years, the use of HPLC has become increasingly important for these compounds [2]. Imipramine was given orally to volunteers in doses of 25 mg and plasma levels of less than 10 ng/ml were reported [3]. In a pharmacokinetic study with

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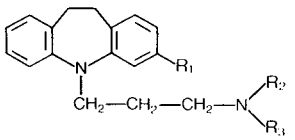
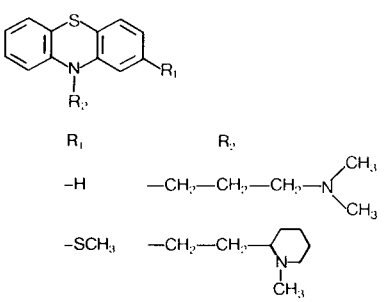


	R_1	R_2	R_3
Imipramine	-H	$-CH_3$	$-CH_3$
Clomipramine	-Cl	$-CH_3$	$-CH_3$
Ro 11-2465 *)	-CN	$-CH_3$	$-CH_3$
N-desmethyl compound of Ro 11-2465	-CN	$-CH_3$	-H
N-bisdesmethyl compound of Ro 11-2465	-CN	-H	-H

Internal standards



	R_1	R_2
Promazine	-H	$-CH_2-CH_2-CH_2-N(CH_3)(CH_3)$
Thioridazine	$-SCH_3$	$-CH_2-CH_2-$(N-CH_3 piperidine)

Fig. 1. Chemical formulae of the relevant compounds. *Ro 11-2465 = 5-[3-(dimethylamino)-propyl]-10,11-dihydro-5H-dibenz[*b,f*]azepine-3-carbonitrile (USP 4'138'482).

clomipramine, the dose was 50 mg orally and the plasma levels in healthy subjects were less than 30 ng/ml [4].

The imipramine derivative Ro 11-2465 (Fig. 1) is a potent and selective inhibitor of serotonin uptake and is currently under development as an antidepressant drug. Due to its potency, the highest dose given to volunteers for pharmacokinetic studies was 4 mg, orally. Since this compound is similar to imipramine and clomipramine, the expected plasma levels are below about 5 ng/ml. Preliminary experiments with thin-layer and gas chromatography were not successful. An HPLC procedure using UV detection has been reported, which is capable of determining nanogram quantities of tricyclic drugs [5]. Highly sensitive methods for imipramine using fluorescence detection have been reported [6, 7] and our own laboratory has had good experience with this detection method [8, 9]. Considerable preliminary work was necessary in order to establish the optimal HPLC and fluorescence conditions.

Losses of tricyclic drugs on glass surfaces is a well-known phenomenon [10, 11], and for this reason evaporation of extracts to dryness, followed by reconstitution of the residue, should be avoided whenever possible. Therefore, a procedure was developed in which the extracts could be chromatographed directly. Two different sample preparation procedures were used: for concentrations above 0.5 ng/ml plasma a single extraction from biological material and chromatography of the extract was suitable; for concentrations below 0.5

ng/ml, an extended sample preparation procedure with back-extraction was necessary.

EXPERIMENTAL

Materials

NaOH 1 *N*, ammonia 33% (B.P. 1953, stored at −20°C), 2% H_3PO_4, methanol (Uvasol), diisopropyl ether p.a. (freshly distilled and stored in the dark), and *n*-hexane p.a. were supplied by E. Merck, Darmstadt, G.F.R.

For the extraction, the following mixtures were used: 2.5% methanol in diisopropyl ether, and 2% isoamyl alcohol in *n*-hexane.

Ro 11-2465 was first synthesized by Dr. Dostert. The metabolites were synthesized by Dr. Joos and Dr. Hunkeler, Chemical Department of Roche, Basle, Switzerland. The internal standard promazine is commercially available and thioridazine was supplied by Sandoz, Basle, Switzerland.

Equipment and chromatography

The liquid chromatograph consisted of an Altex (Berkeley, CA, U.S.A.) pump Model 110, a Rheodyne (Berkeley, CA, U.S.A.) 71-25 high-pressure sample valve with a 300-μl loop, or a Kontron (Zürich, Switzerland) automatic sample injector MSI 660 with a 200-μl loop.

A stainless-steel column (250 × 3.2 mm), filled with LiChrosorb Si 60 (Merck), 5 μm particle size, was used. A Perkin-Elmer (Norwalk, CT, U.S.A.) fluorimeter 650-10 LC was operated with the following instrument settings: excitation wavelength 280 nm, slit 15 nm, emission wavelength 410 nm, slit 20 nm, sensitivity range 1, fine 5, PM gain norm, response slow, mode norm. To reduce the amplitude of the short-term noise of the baseline, an additional output filter with a time constant of 2 or 4 sec was used.

To control the chromatographic system, it was advantageous to connect a UV detector in series with the fluorimeter. A Uvikon 725 (Kontron) detector with a deuterium lamp was used, wavelength 242 nm, range 0.01, time constant 3. The chromatograms were obtained on a W + W recorder, Model 1200 (Kontron), UV channel 10 mV, fluorescence channel 1 mV, chart speed 5 mm/min.

The mobile phase consisted of 0.1 ml of ammonia 33%, mixed with 6 ml of methanol and adjusted to 100 ml with distilled diisopropyl ether. Using a flow-rate of 2 ml/min, the pressure was about 200—300 bar. The retention times were approx. 4 min for Ro 11-2465 and 5—6 min for the internal standard — promazine or thioridazine.

Preparation of the glassware

The adsorption effects of Ro 11-2465 on glass surfaces could be considerably reduced by treating all the glassware with alkaline methanol (0.5 ml of 14 *N* potassium hydroxide in 100 ml of methanol), followed by rinsing with pure methanol and air drying. Polypropylene tubes, rinsed with methanol and air dried, were also suitable for the assay.

Standards

Amber glass or polypropylene volumetric flasks were used to prepare the standard solutions.

The stock solution contained 5 mg of Ro 11-2465 as hydrochloride in 10 ml of methanol. Further dilutions in methanol covering the concentration range 25 μg/ml down to 15 ng/ml were obtained, starting from the stock solution. These methanolic solutions could be stored for up to three months at −20° C.

Calibration solutions for the chromatographic system and for recovery studies were prepared as follows: To 200 μl of the methanolic solutions were added 50 μl of methanol (which may contain the internal standard, see below). The volume was adjusted to 10 ml with diisopropyl ether. The concentration range was 0.625—20 ng/ml.

Plasma and urine standards were prepared by dilution of 200 μl of the appropriate methanolic standard solution with drug-free plasma or urine to 25 ml. The concentration range for plasma was 4—0.125 ng/ml and for urine 200—6.25 ng/ml. These standards were stored in polypropylene tubes in aliquots of 2.5 ml at −20° C.

Sample preparation

Plasma or urine (1 ml) was mixed with sodium hydroxide solution (1 *N*, 0.2 ml) and 2.5% methanol in diisopropyl ether (1 ml) in a polypropylene tube or a glass tube treated as described above. The tubes were rotated for 5 min (rotary tube mixer, REAX II; Heidolph Elektro AG, Keilheim, G.F.R.) and then centrifuged at 1200 *g* for 5 min. A portion of the organic extract (200 μl) was chromatographed.

Along with the unknown samples, 4—5 plasma standards were analysed, covering the expected concentration range.

This procedure was suitable for the concentration range 0.5—20 ng/ml. For higher concentrations, which have been observed in urine, the volume of the extraction mixture was increased to 2 ml or more. For plasma concentrations below 0.5 ng/ml, an extended sample preparation procedure was used as follows. Plasma (1 ml) was mixed with sodium hydroxide (1 *N*, 0.2 ml). This mixture was extracted twice with 2% isoamyl alcohol in *n*-hexane (5 ml). The combined organic phase was extracted with 2% H_3PO_4 (1 ml). After discarding the organic phase, the aqueous phase was basified with sodium hydroxide (1 *N*, 1 ml) and extracted with 2.5% methanol in diisopropyl ether (0.5 ml). A portion of this extract (200 μl) was chromatographed. This extraction procedure is described in further detail elsewhere [12].

Calibration and calculation

The plasma or urine standards analysed along with the unknown samples were used for calculation of the unknown concentrations. A calibration curve was obtained by calculation of the least-square regression of the peak heights of the plasma or urine standards versus the concentrations of Ro 11-2465. Using these curves the concentrations of the drug in the unknown samples were calculated.

Internal standard

Thioridazine (Fig. 1) was tried as internal standard. The substance dissolved in sodium hydroxide (1 *N*, 0.2 ml) was added to the samples. Due to adsorption effects or instability in alkaline solution, poor results were obtained. Preliminary experiments showed that promazine was more suitable. It could be added to the samples as an aqueous solution (50 or 100 μl). The quantity of promazine had to be chosen such that 200 μl of the extract (which is chromatographed) contained 50 ng. It is not yet known whether or not promazine fulfils the conditions for the internal standard technique [13].

RESULTS

Characteristics of the method

Selectivity. Ro 11-2465 is well separated from endogenous plasma interferences under the chromatographic conditions described (Fig. 2). In human urine and in plasma of rats treated with Ro 11-2465 two substances with retention times of about 15—17 and 21 min, respectively, were observed. The substance which elutes at 15 min is probably the N-bisdesmethyl, and that at 20 min the N-desmethyl compound of Ro 11-2465 (Figs. 1 and 3). Due to the very low quantities present in biological fluids, a definite identification of these compounds has not yet been possible. Chromatograms of standards and of extracts of human plasma spiked with Ro 11-2465 are shown in Fig. 2.

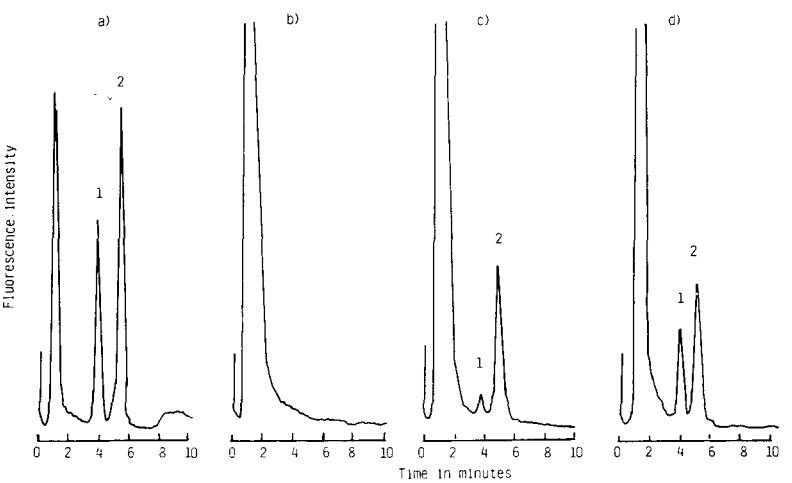


Fig. 2. (a) Chromatogram of 1 ng of Ro 11-2465 (hydrochloride) and 40 ng of thioridazine, injected in 200 μl of 2.5% methanol—diisopropyl ether. (b) Chromatogram of the extract of human blank plasma: 200 μl injected, single extraction. (c) Chromatogram of the extract of human plasma, spiked with 0.5 ng/ml Ro 11-2465 (hydrochloride) and 100 ng of thioridazine: 200 μl injected, single extraction. (d) Chromatogram of the extract of human plasma, spiked with 2 ng/ml Ro 11-2465 (hydrochloride) and 100 ng of thioridazine: 200 μl injected, single extraction. Peaks: 1 = Ro 11-2465; 2 = thioridazine. Chromatographic conditions: column 250 mm × 3.2 mm, LiChrosorb Si 60, 5 μm; mobile phase: 0.1 ml ammonia 33% + 6 ml methanol, with diisopropyl ether to 100 ml, flow-rate 2 ml/min; fluorescence detection at 280 nm/410 nm.

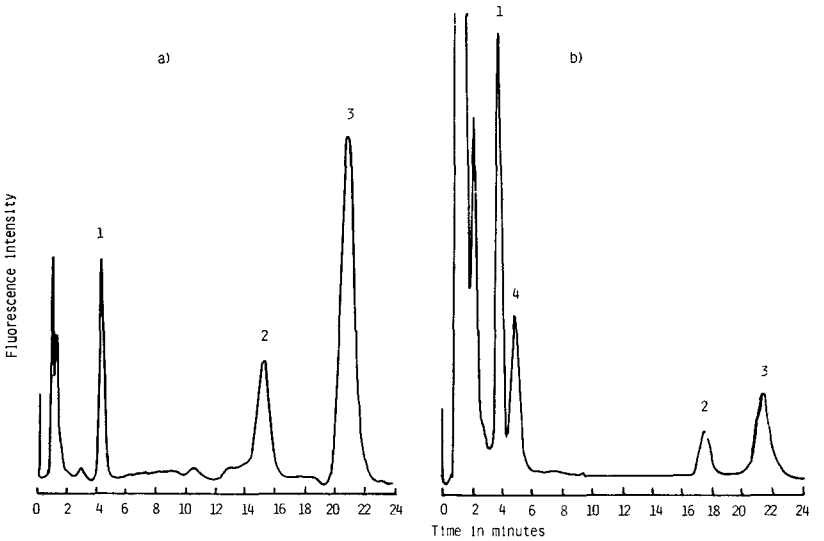


Fig. 3. (a) Chromatogram of rat plasma, collected 90 min after an oral dose of 50 mg/kg Ro 11-2465 (hydrochloride). Single extraction, 200 μl injected. (b) Chromatogram of the urine of a volunteer, collected 14—24 h following a single oral dose of 4.48 mg of Ro 11-2465 (hydrochloride); 0.5 ml of urine were mixed with 0.2 ml of 1 *N* NaOH, containing 2.5 μg of thioridazine, extracted with 5 ml of 2.5% methanol—diisopropyl ether and 200 μl of the extract were injected. Peaks: 1 = Ro 11-2465; 2 = probably the N-bisdesmethyl compound of Ro 11-2465; 3 = probably the N-desmethyl compound of Ro 11-2465; 4 = thioridazine. Chromatographic conditions as in Fig. 2.

Linearity. The response of the fluorescence detector was linear in the range 0.05—4 ng of Ro 11-2465 injected from a 200-μl extraction mixture. For most determinations, the instrument settings were chosen such that 2 ng of Ro 11-2465 (hydrochloride) gave full-scale deflection. Since the concentration of Ro 11-2465 in biological fluids is low, the linearity above 4 ng injected was not tested.

Recovery. The extraction yield (recovery) of Ro 11-2465 was above 95% for both the simple and extended sample preparation procedures.

Accuracy. The accuracy of a method may be defined as the difference between the mean value of replicate assays of the same sample and the true value. Spiked plasma samples were prepared for this purpose and the results are summarized in Table I.

Precision. The precision of a method is represented by the relative standard deviation of the mean of replicate assays of the same sample. The precision was estimated in plasma and urine by analysing the same unknown samples on different days. In this case, for plasma, a classification into two ranges was possible [14]. For concentrations from 0.8 to 4 ng/ml plasma, the relative standard deviation of the method was 6%; below 0.8 ng/ml it was 13%. These values were obtained with clinical samples, not with spiked plasma. In urine, the relative standard deviation of the method was about 9% in the concentration range 5—100 ng/ml. Accuracy and precision data from spiked samples are given in Table I and precision from clinical trials in Table II.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY OF Ro 11-2465 IN SPIKED HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found (ng/ml)	*n* replicates	Percentage of the added amount	Coefficient of variation (%)
0.30	0.33	11	110	17
0.60	0.56	14	93	12
0.90	0.89	8	99	5.6
1.20	1.20	15	100	6.3
2.40	2.37	15	99	5.5

TABLE II

PRECISION DATA FOR Ro 11-2465 FROM THE CLINICAL TRIALS

	Concentration range (ng/ml)		
	<0.8	0.8—4	5—100
Plasma	13%	6%	
Urine			9%

Detection limit. The detection limit for Ro 11-2465, defined as the amount injected giving rise to a signal-to-noise ratio of 3:1, was 0.05 ng (from a 200-μl sample). Using 1 ml of plasma and 1 ml of extraction mixture, the detection limit for the simple sample preparation procedure was 0.3 ng/ml. Using the extended clean-up procedure, a detection limit of about 0.1 ng/ml, or even lower, could be achieved for optimal instrument settings. In urine the detection limit was about 5 ng/ml; due to endogenous interferences, it would be difficult to improve this value.

Stability. Plasma samples spiked with Ro 11-2465 were stored for one year at −20°C. In the concentration range 1—5 ng/ml, no measurable degradation of the compound could be observed.

Analysis of plasma and urine samples

The method described was used to determine plasma and urine levels of Ro 11-2465 from single-dose pharmacokinetic studies. The concentrations of Ro 11-2465 in the plasma and urine of a volunteer following a single oral dose of 4.48 mg of the hydrochloride, corresponding to 4 mg of the base, are summarized in Tables III and IV, respectively. Plasma levels were detectable up to 72 h post administration of the drug, and sufficient data were obtained to allow the calculation of the pharmacokinetic parameters.

As mentioned above, two peaks were observed in the chromatograms from rat plasma after oral administration of 50 mg/kg Ro 11-2465; these probably correspond to the N-desmethyl and N-bisdesmethyl compounds of Ro 11-2465

TABLE III

PLASMA LEVELS OF A VOLUNTEER (C.C.) FOLLOWING A SINGLE ORAL DOSE OF 4.48 mg OF THE HYDROCHLORIDE OF Ro 11-2465 AS CAPSULES

Time (h) after administration	Ro 11-2465 (as hydrochloride) (ng/ml)
0.25	<0.1
1	0.18
2	1.05
3	1.79
5	2.52
8	2.11
12	1.96
24	1.30
36	0.87
48	0.57
60	0.34
72	0.12

TABLE IV

DETERMINATION OF Ro 11-2465 IN THE URINE OF A VOLUNTEER (C.C.) FOLLOWING A SINGLE ORAL DOSE OF 4.48 mg OF THE HYDROCHLORIDE AS CAPSULES

Collection period post administration (h)	Quantity of urine (ml)	Concentration of Ro 11-2465 (hydrochloride) (ng/ml)	Amount of Ro 11-2465 (hydrochloride) excreted (ng/ml)
0—2	97	<5	—
2—4	113	35.9	4.1
4—6	193	129	24.9
6—8	115	54.9	6.3
8—10	87	87.3	7.6
10—12	107	141	15.1
12—14	125	6	0.8
14—24	480	26.6	12.8
24—36	570	25.0	14.3
36—48	470	38.6	18.1
48—72	770	22.0	16.9
72—96	1295	8,6	11.1
96—120	790	<5	—

(Fig. 3a). In human urine, similar peaks were found (Fig. 3b). In human plasma, however, these metabolites were not detected after a single dose of 4.48 mg of the hydrochloride, the concentrations being below the detection limit. Possible accumulation of these metabolites could be ascertained from multiple-dose studies.

DISCUSSION

Imipramine and desimipramine have been determined in plasma by HPLC with fluorescence detection [6]. Using 2 ml of plasma, a detection limit of about 0.5 ng/ml was attained. The procedure described here has considerably higher sensitivity; with 1 ml of plasma, 0.1 ng/ml Ro 11-2465 was detectable. With the extended sample preparation procedure this limit could be lowered to 0.05 ng/ml, using 2 ml of plasma.

The application of normal-phase HPLC has several advantages. The life-time of a normal-phase column is, generally, much longer than that of a reversed-phase column: more than 1000 plasma extracts could be analysed with the same column. Furthermore, it was possible, without evaporation and back-extraction, to determine concentrations of 0.3—0.5 ng/ml of plasma following a single extraction.

The reproducibility of this procedure is better than that described in published methods [2, 4, 6, 7], especially in the concentration range 1 ng/ml and less.

The development of the present assay involved extensive experiments. Poor results obtained initially were probably due to the irreversible adsorption of Ro 11-2465 to glass and plastic surfaces. This effect is well known for other tricyclic drugs [10, 11] and may differ from one vessel to another. Stock solutions in diisopropyl ether in untreated volumetric glass flasks showed no loss for several weeks, whereas in other identical flasks large amounts of Ro 11-2465 disappeared within a few hours. Treatment of the glassware as described above reduced this effect considerably. Good results were also obtained with polypropylene tubes.

The adsorption effect of the internal standard, thioridazine, could not be reduced by using alkali-treated glassware or polypropylene tubes. For other tricyclic compounds, this phenomenon could be reduced in acidic solutions [10], but we had no success with this approach. Preliminary experiments with promazine gave promising results, although with a similar substance, chlorpromazine, irreproducible extraction yields have been reported [11].

The reproducibility of the assay for Ro 11-2465, without using an internal standard technique, is good compared to the values published for other tricyclic compounds. It is questionable whether promazine or any other internal standard would improve the precision of the method [13], and therefore little time was spent looking for other internal standards.

The high sensitivity and the good reproducibility of this assay are strongly dependent on the fluorimeter; proper calibration of the system and the quality of the xenon lamp are of fundamental importance. Furthermore, the purity of mobile phase, especially diisopropyl ether*, can influence the baseline noise of the chromatogram. Selection of suitable reagents is therefore important. Unexpected fluorescence peaks, usually a result of problems either with the fluorimeter or the chromatographic system, are sometimes encountered. The

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*Recent experiments have shown that by slight modification of the mobile phase diisopropyl ether can be replaced by *tert.*-butyl methyl ether. The baseline noise is considerably reduced with this solvent.

origin of such peaks may be readily identified using a UV detector in series with the fluorimeter. Changes in the quality of the solvents can be observed by comparing the baseline drift of both the UV and fluorescence record. This assay has been developed for single-dose kinetics of Ro 11-2465 in man following an oral dose of 4.48 mg of the hydrochloride. The sensitivity needed has been achieved, and it was possible to calculate pharmacokinetic parameters.

It is likely that this procedure can be modified for the assay of other tricyclic drugs in plasma or urine.

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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF INDOMETHACIN IN HUMAN PLASMA

PHARMACOKINETIC STUDIES AFTER SINGLE ORAL DOSE

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SUMMARY

A sensitive high-performance liquid chromatographic assay for the specific determination of indomethacin at concentrations down to 20 ng/ml in human plasma is described.

This method has been applied to investigate the disappearance of indomethacin from plasma of ten subjects following the intake of two formulations (Indocid® and generic form). An initial half-life of $1.32 \pm 0.44\ h^{-1}$ was found which is in good agreement with other findings, but the terminal phase was much longer ($13.6 \pm 6.9\ h^{-1}$) than previously reported. There is no difference between the two galenic forms ($p < 0.001$).

INTRODUCTION

Indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indol-3-acetic acid] has analgesic, anti-inflammatory and antipyretic actions. It is used to relieve the painful symptoms of ankylosing spondylitis and osteoarthritis and to relieve the pain and swelling in gout and rheumatoid arthritis.

Plasma concentration curves of indomethacin with time vary considerably after the same oral dose in different patients [1]. There is thus a definite need for pharmacokinetic investigations with indomethacin in humans as a function of dose and galenic forms, using specific and sensitive methods of analysis. Various methods have been used for its determination based on thin-layer chromatography [2], gas chromatography (GC) with electron-capture detection after derivatization [3—5], radioimmunoassay [6] and fluorimetry [7, 8]. Most of these methods are rather unspecific (fluorimetry), time-consuming or not suitable for routine analysis.

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Recently, several methods for the determination of indomethacin by high-performance liquid chromatography (HPLC) have been reported [9—13] but they are not sufficiently sensitive (detection limit 0.1 μg/ml [9] for determining plasma concentrations of the drug, after the oral administration of therapeutic doses to humans, in the slow terminal elimination phase (plasma concentration about 20—50 ng/ml at 24 h after dosing).

This paper describes an HPLC procedure for the determination of very low concentrations of indomethacin in plasma, involving deproteinization and one-step extraction with ethyl acetate at pH 3.

EXPERIMENTAL

Chemicals

Indomethacin (lot KB 104) was generously supplied by LFPG (Marly, France). Phenylbutazone, used as internal standard, was kindly furnished by Ciba-Geigy (Basel, Switzerland) (lot AN 7311/5). Acetonitrile for UV was obtained from Fisons (Loughborough, Great Britain). Ethyl acetate was obtained from Carlo-Erba (Milan, Italy). Citrate buffer (pH 3), anhydrous sodium sulfate and acetic acid were obtained from Merck (Darmstadt, G.F.R.). All other chemicals used were analytical or LC grade.

Vessels

All glassware was washed twice with re-distilled water and methanol and dried overnight at 100°C before use.

Chromatography

The HPLC system consisted of a solvent delivery system (Altex-Chromatem 380; Touzart et Matignon, Paris, France) and a 50-μl fixed-volume loop injector (Rheodyne 7010, Berkeley, CA, U.S.A.). A 25 × 0.46 cm I.D. reversed-phase column was packed with 10-μm Partisil ODS-2 (Whatman, Clifton, NJ, U.S.A.), and was fitted with a 6 × 0.46 cm I.D. precolumn packed with Co-Pell ODS (C_{18} pellicular 37—50 μm, Whatman). The downflow slurry packing technique with slamming process [14] using a constant-pressure pneumatic amplifier pump (Haskel, Burbank, CA, U.S.A.) was used to prepare the column. Slurry solvent was *n*-butanol and isooctane was used as pumping fluid (pressure 350 bars). A variable-wavelength UV detector (Pye-Unicam, Cambridge, Great Britain) was used at 250 nm. A recorder (Linear 1201, Linear Inc., Irvine, CA, U.S.A.) was linked to the detector and a chart speed of 20 cm/h was used.

The mobile phase for isocratic chromatography was a mixture of acetonitrile and 0.1 *M* acetic acid (60:40, v/v). The chromatographic system was operated at ambient temperature at a flow-rate of 1.8 ml/min (linear velocity = 0.5 cm/sec) and a pressure of 70 bars. The mobile phase was degassed by ultrasonic treatment and by a helium stream during the determination. After use, the column was washed for 10 min with water and 30 min with methanol (2 ml/min) to prolong its life.

In vivo study

Plasma was collected by venous puncture in heparinized vials from ten

healthy volunteers (six males and four females, 25.7 ± 2.3 years) who had first been randomized and then received a single oral dose of indomethacin (75 mg, about 1 mg/kg) in two formulations (Indocid® or generic indomethacin, LFPG). At least one week intervened between the administration of any two formulations to any subject. Subjects were fasted for 12 h before administration. Repeated blood samples were obtained during the following 34 h (0, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 34 h) and were centrifuged within 10 min at 1200 *g* (4°C) to obtain plasma (stored at −30°C until analysis).

Assay in plasma

One milliliter of patient plasma and 100 μl of a phenylbutazone solution (10 μg/ml, prepared daily from a stock solution of 1 mg/ml in methanol) were mixed with 1 ml of acetonitrile (Vortex). After 10 min, precipitated protein was removed by centrifugation (10 min, 1200 *g*, 4°C). One milliliter of citrate buffer (pH 3) was added to 1 ml of the supernatant in a 20-ml culture tube with PTFE-lined caps (Prolabo, Paris, France) and extracted twice with 10 ml of ethyl acetate in a rotary shaker (Cenco, Breda, The Netherlands) for 15 min. The organic phase was transferred to a clean evaporating tube with Pasteur pipet. Ethyl acetate was evaporated at 40°C under a nitrogen stream. The residue was taken up with 100 μl of the mobile phase and 50-μl aliquots were injected into the system.

Plasma standard curves were prepared from a solution of indomethacin (10 μg/ml, prepared daily from a stock solution of 1 mg/ml in methanol and stored at 4°C in darkness up to one month) by serial dilutions with drug-free human plasma (0.02—1 μg/ml).

Calculations

Concentrations of indomethacin were determined from standard curves of peak height versus concentration. Linear regression analysis and interpolation were performed with a microcalculator (HP 97, Hewlett-Packard, Palo Alto, CA, U.S.A.).

Pharmacokinetic data and statistical analyses were performed with a 48 K Apple II computer (Apple Inc., Cupertino, CA, U.S.A.) using an interactive graphic package for pharmacokinetic analysis with a feathering method (LEFERCALC) [15].

RESULTS

Statistical validation of the method

The linearity of the method was evaluated in plasma in the concentration range of 0.02—1 μg/ml. The daily standard curve was obtained using the analytical procedure. The data are best described by a linear equation $Y = 1.076\ X - 0.003$ where X is concentration of indomethacin in μg/ml, and Y is peak height ratio of indomethacin to phenylbutazone. A mean correlation coefficient of 0.9963 ± 0.0028 was obtained, indicating a high degree of linearity (n=10) ($p < 0.001$).

The recovery of indomethacin from plasma was determined by comparing the ratio of indomethacin to phenylbutazone (internal standard) peak heights

in spiked plasma specimens (indomethacin 1 μg/ml, phenylbutazone 1 μg/ml), to the ratio in spiked plasma with only 1 μg/ml of phenylbutazone (1 μg of indomethacin added just before injection) (Table I).

TABLE I

RECOVERY OF INDOMETHACIN FROM PLASMA AND REPRODUCIBILITY

Amount added to plasma = 1 μg/ml.

Experiment No.	$(I/P)^{*}_{ref}$	$(I/P)^{**}_{extr}$	Yield (%)
1	0.946	0.792	83.7
2	0.864	0.706	81.7
3	0.970	0.741	76.4
4	0.867	0.789	91.0
5	0.930	0.815	87.6
6	0.985	0.821	83.4
7	0.904	0.704	77.9
8	0.988	0.791	80.1
9	0.940	0.725	77.1
10	0.976	0.821	84.1
Mean	0.937	0.768	82.3
S.D.	± 0.046	± 0.049	± 4.7
C.V. (%)	4.3	6.4	5.7

*$(I/P)_{ref}$ = peak height ratio of indomethacin to phenylbutazone; phenylbutazone added just before injection.

**$(I/P)_{extr}$ = peak height ratio of indomethacin to phenylbutazone after complete extraction.

Recovery (mean ± S.D.) was 82.3 ± 5.7% (n=10). Reproducibility was calculated in ten calibration curves in the range 0.02, 0.05, 0.1, 0.25, 0.5 μg/ml. The statistical analysis of indomethacin peak height versus phenylbutazone peak height gave correct results (C.V. = 6.4% at 1 μg/ml, about 20% at very low concentration 0.05 μg/ml) (Table II).

In the present analytical conditions, the minimum concentration that could be accurately measured was about 20 ng/ml (signal-to-noise ratio = 5) with a 1-ml plasma sample. Higher sensitivity (about 10 ng/ml) may be possible by increasing the plasma volume.

A chromatogram of blank and patient plasma 3 h after administration (volunteer who had received 75 mg of indomethacin) is shown in Fig. 1. The indomethacin concentration is about 1.5 μg/ml (plasma sample 1 ml, a.u.f.s. 0.16).

A major metabolite of indomethacin, deschlorobenzoyl indomethacin, is well separated from indomethacin and phenylbutazone (retention time = 5.5 min for indomethacin and 3.5 min for the deschlorobenzoyl metabolite).

Table III presents those drugs which were tested and found not to interfere with these assays (10 μg/ml of each). This does not rule out the possibility that metabolites of these drugs may interfere with these assays.

TABLE II

REPRODUCIBILITY OF INDOMETHACIN DETERMINATION IN PLASMA

Peak height ratio indomethacin/phenylbutazone (internal standard) = 0.5 μg/ml.

	Concentration (ng/ml)					Correlation coefficient
	20	50	100	250	500	
	0.1243	0.2142	0.4712	1.0148	2.0133	0.9923
	0.1212	0.2535	0.4870	1.135	2.145	0.9988
	0.0724	0.1960	0.5040	0.984	2.015	0.9971
	0.1556	0.3251	0.6934	1.274	2.348	0.9987
	0.1023	0.1866	0.6193	1.368	2.388	0.9947
	0.0686	0.2264	0.4198	0.944	2.1533	0.9979
	0.110	0.325	0.666	1.118	2.0906	0.9975
	0.125	0.235	0.561	0.9679	1.810	0.9981
	0.152	0.2235	0.4148	0.9310	2.407	0.9906
	0.0843	0.220	0.622	1.053	2.0988	0.9978
Mean	0.1116	0.2406	0.5459	1.0789	2.1458	0.9963
± S.D.	0.0322	0.04828	0.1008	0.1463	0.1887	0.0028
C.V. (%)	28.8	20.1	18.3	13.6	8.8	

In vivo study

The plasma concentration kinetics of indomethacin (mean of the ten volunteers) are shown in Fig. 2. A summary of the pharmacokinetic parameters is shown in Table IV (see Discussion).

DISCUSSION

Method

The choice of 250 nm for measurement of indomethacin was based on the UV spectra in the mobile phase.

Deproteinization of plasma is essential. The use of perchloric acid (0.66 *N*) gave poor recoveries. This is probably due to the higher degree of indomethacin adsorption to plasma proteins in an aqueous perchloric acid solution at pH <1 [16].

The use of organic solvents gave greater recovery. After trials, it was found that indomethacin recovery could be improved when the plasma was deproteinized with an equal volume of acetonitrile.

The acidic nature of indomethacin required extraction at low pH and the use of an acidic mobile phase to reduce band tailing by ion suppression. Citrate buffer at pH 3 was found to be the best for extraction. Diethyl ether and chloroform gave a coextracted lipid peak at $k = 21.2$ (20 min retention time). With less polar solvents (benzene, hexane), very low recoveries were obtained ($<5\%$). Ethyl acetate resulted in the best quantitative extraction. The use of an appropriately acidic mobile phase renders the indomethacin molecule in an ionized lipophilic state, leading to retention on octadecylsilane columns.

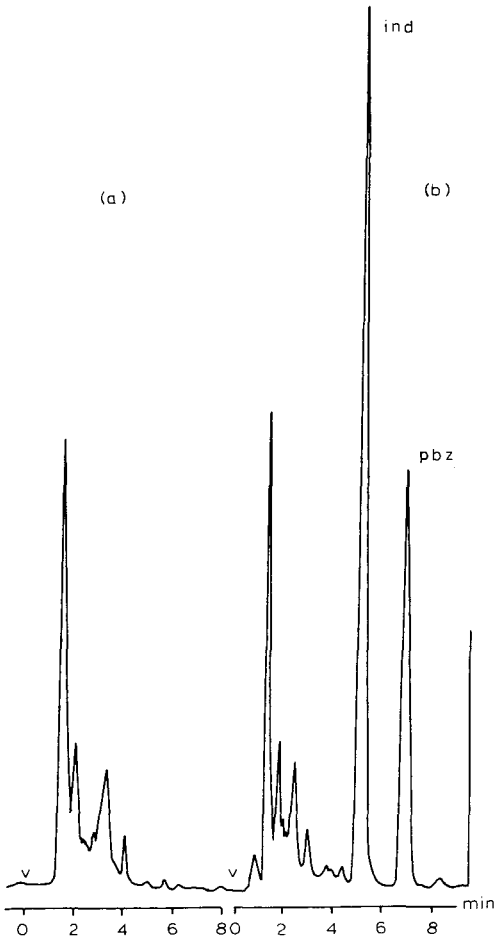

Fig. 1. (a) Chromatogram of a human blank plasma sample. (b) Chromatogram of a human volunteer plasma sample, collected 3 h after a single dose of 75 mg of Indocid®. Internal standard (phenylbutazone, pbz) = 1 μg/ml; calculated indomethacin (ind) concentration = 1.5 μg/ml.

TABLE III

SUBSTANCES CHECKED FOR INTERFERENCE

Quinine	Oxazepam
Quinidine	Clobazam
Phenobarbital	Imipramine
Secobarbital	Chlorimipramine
Meprobamate	Theophylline
Chloroquine	Salicylic acid
Promethazine	Acepromazine
Diazepam	Caffeine

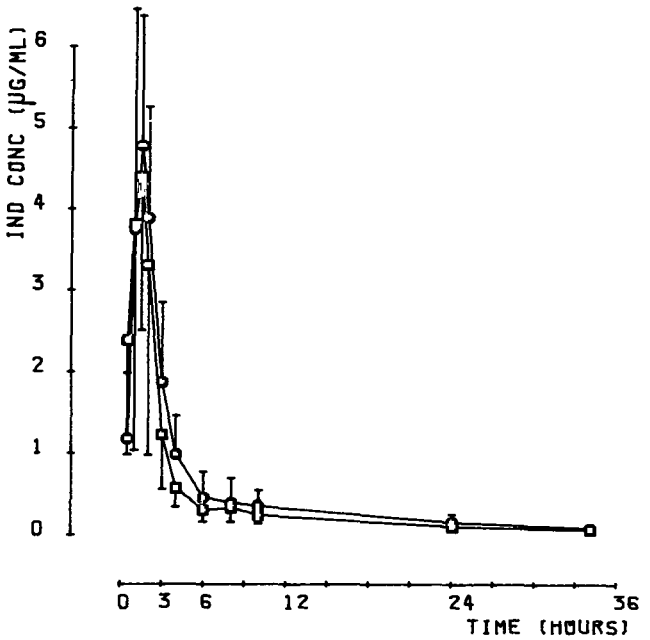

Fig. 2. Mean plasma concentration of indomethacin after a single oral dose (75 mg) (mean ± S.D., $n=10$). (□) Indocid®; (○) generic indomethacin.

TABLE IV

PHARMACOKINETIC PARAMETERS

Indomethacin given as a single oral dose (75 mg) in two formulations. Values represent mean ± S.D. for ten determinations for each formulation.

Parameters*	Indocid®	Generic indomethacin	Significativity**
t_{max} (h)	1.24 ± 0.27	1.24 ± 0.19	NS
C_{max} (µg/ml)	5.61 ± 2.98	5.48 ± 1.60	NS
$AUC_{0\to\infty}$ (µg ml^{-1} h^{-1})	15.4 ± 5.10	19.47 ± 8.33	NS
α (h^{-1})	1.32 ± 0.44	1.00 ± 0.18	NS
β (h^{-1})	0.058 ± 0.025	0.057 ± 0.0020	NS
$t_{½(\beta)}$ (h)	15.2 ± 7.3	14.0 ± 6.5	NS
A (µg/ml)	27.9 ± 2.2	25.8 ± 1.5	NS
B (µg/ml)	0.39 ± 0.03	0.62 ± 1.5	NS

*Pharmacokinetic symbols: t_{max} = time of the maximum concentration. C_{max} = maximum concentration in plasma. $AUC_{0\to\infty}$ = area under the plasma level curve extrapolated to infinity using Wagner equation: $AUC_{0\to\infty} = AUC_{0\to t} + \frac{C_t}{\beta}$, where C_t = plasma concentration at time t. A, B = coefficients of the two exponential functions. α = rate constant of distribution phase. β = rate constant of elimination phase. $t_{1/2(\beta)}$ = elimination half-life. $t_{1/2(\alpha)}$ = distribution half-life.

**Analysis of variance, t-test for paired data, Westlake, Wilcoxon and Duncan tests.

The p*K*a of indomethacin is 4.5 and a mobile phase of acetonitrile–water containing acetic acid (60 : 40) with an ionic strength of 0.1 (pH 3.8) was found to be suitable.

The solvent polarity parameter P' (Rohrschneider's parameter [17]) of this mixture was about 7.56 and the solvent strength parameter for the reversed phase (Snyder's parameter [18]) was 1.24.

Under these conditions, the capacity factors k of indomethacin and phenylbutazone were 3.7 and 4.7. The selectivity factor was 1.27 and the number of plates per meter for indomethacin was 15,525.

In vivo study

The pharmacokinetics of indomethacin in humans are only partly known and some findings appear contradictory. It is usually absorbed rapidly from the gut and a proportion may undergo enterohepatic recirculation [1, 19]. Holt and Hawkins [20] found complete elimination from the human body in 5 h, while Palmer et al. [21] could detect the drug in plasma for 32 h after intake. For several authors, the disappearance of indomethacin from plasma appears to consist of a fast primary phase ($t_{1/2}$ about 90 min) and a slower secondary elimination phase. The plasma elimination half-life in the beta phase ranges from 2.6 to 11.2 h [1].

The present work also investigated the disappearance of indomethacin from the plasma of ten subjects. With two formulations (Indocid® and generic form), a single oral dose (75 mg) give a rapid increase in the plasma concentration ($t_{max} = 1.24 \pm 0.22$ h, $C_{max} = 5.54 \pm 2.3$ μg/ml) but it was impossible to distinguish clearly between a true distribution phase and an elimination phase for these decreases.

An initial plasma half-life of 1.32 ± 0.44 h^{-1} was found, which is in good agreement with other findings (about 90 min [1, 22]).

The change in the elimination rate following these initial phases can be explained by enterohepatic recirculation and/or binding to proteins or tissues, from which the drug is slowly released.

A half-life of 13.6 ± 6.9 h^{-1} was found ($n = 20$) for these phases. The plasma half-life in the terminal exponential phase was much longer than previously reported by Alvan et al. [1] for a similar dose. An analysis of variance and Student's test were applied to this parameter between the present work and the data of Alvan et al. (Table V). There are highly significant differences between the two groups ($p < 0.001$). Considerable intra- and interindividual variations of indomethacin pharmacokinetics may perhaps explain these differences. Work is in progress to confirm and explain this higher value for the half-life of the beta phase.

CONCLUSION

A reversed-phase HPLC assay is described for the measurement of plasma indomethacin with sufficient sensitivity to measure the drug in pharmacokinetic studies.

The results indicate that the plasma curve of indomethacin is biexponential with an initial rapid phase (half-life of 1.32 ± 0.44 h^{-1}) followed by a complex

TABLE V

PLASMA HALF-LIVES (BETA PHASE)

Values are expressed in h^{-1}.

Subject	Indomethacin (generic)	Indocid®
CHA	20.0	15.2
COU	9.5	8.1
EMG	10.5	15.2
GAZ	30.3	32.3
LAL	11.1	10.1
LAM	14.3	10.3
LEC	10.5	11.2
LOM	8.5	7.1
MOR	16.3	15.3
PET	7.5	8.6
Mean ± S.D.	14.0 ± 6.5	15.2 ± 7.3
Significance (analysis of variance, *t*-test)	NS	($p < 0.001$)

	Indocid®* ($n=10$)	Alvan et al. [1] ($n=13$)	Indomethacin* (generic) ($n=10$)
$t_{1/2}$ indomethacin (h^{-1})	15.2 ± 7.3	5.9 ± 1.6	14.0 ± 6.5
Significativity	$p < 0.001$		$p < 0.001$

*Present work.

slower beta phase with a half-life of 13.6 ± 6.9 h^{-1} in ten subjects and two formulations.

There is no evidence for statistical differences between these two galenic forms (analysis of variance, Student's test for paired data, Wilcoxon and Duncan test).

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF INDOMETHACIN AND ITS APPLICATION IN PHARMACOKINETICS IN HEALTHY VOLUNTEERS

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SUMMARY

A new sensitive high-performance liquid chromatographic method for indomethacin from plasma on a reversed-phase column (C_{18}) has been developed. The method involves precipitation of plasma with perchloric acid followed by diethyl ether extraction. The assay is quantitative down to 0.25 $\mu g\ ml^{-1}$ from a 200-μl aliquot of plasma with a detection limit of 0.1 $\mu g\ ml^{-1}$ and a recovery of approximately 90%.

The method was applied to single-dose studies with volunteers under various dietary restrictions. The results of these studies indicated that intrasubject variability within these regimens may be as important a factor as the intersubject variability already documented for this drug. These results have important implications in the determination of bioavailability and pharmacokinetic parameters of this drug.

INTRODUCTION

Indomethacin has been quantitatively determined in biological fluids by means of gas chromatography (GC) [1—5] using an electron-capture detector. These methods, however, require some derivatization technique which in the case of reaction with diazomethane converts a percent of any O-desmethyl-indomethacin present back to the parent compound. In addition many of the GC methods lack an internal standard or utilize an internal standard not readily available.

Thin-layer chromatographic methods [6, 7] reported may be too cumbersome and time consuming when large numbers of plasma samples are to be analyzed.

Radioisotope dilution techniques exist but require administration of radioactivity to patients [8—10] or lack specificity. The radioimmunoassay technique of Hare et al. [11] is sensitive but indomethacin glucuronide is highly

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cross reactive. The combined GC—mass fragmentographic techniques [12, 13] are sensitive but also require derivatization.

The use of reversed-phase high-performance liquid chromatography (HPLC) in the analysis of plasma levels of indomethacin has been advocated [14—16]. The procedure of Skellern and Salole [16], requiring rather large plasma samples (1 ml), provides no precision data for proper evaluation. Soldin and Gero's method [15] is based on ethylene dichloride extraction of plasma which results in 66—68% recovery of the drug. However, the coefficient of variation at 463 and 927 ng/ml was rather large (> 10%). The described procedure is simple and sensitive, requires no derivatization technique, and metabolite or concomitantly administered drugs such as salicylic acid do not interfere with the assay. This method uses the protein precipitation technique of Terweij-Groen et al. [14], with the modification of a more convenient diethyl ether extraction solvent over methylene chloride, and quantitative recovery of the drug (88—90%). The application of this procedure to single-dose pharmacokinetic studies in healthy volunteers clearly establishes the large intrasubject variability which must be seriously taken into account in future bioavailability studies.

EXPERIMENTAL

Materials

Indomethacin and mefenamic acid were generously donated by ICN Laboratories, Montreal, Canada. Diethyl ether was distilled prior to use, all chromatographic solvents were HPLC grade, and all other chemicals were analytical reagent grade.

Apparatus

A Waters Model M-45 liquid chromatographic pump (Waters Scientific, Mississauga, Canada) and a Model 7125 Rheodyne valve-loop injector fitted with a 500-μl loop were employed (Technical Marketing Associates, Calgary, Canada). An Ultrasphere ODS 250 × 4.6 mm I.D. column (particle size 5 μm) (Beckman Instruments, Toronto, Canada) was connected to a Waters Model 440 fixed-wavelength detector set at 254 nm (Waters Scientific). The mobile phase was 0.1 *M* acetic acid—acetonitrile (30:70) pumped at a flow-rate of 1 ml min^{-1}. The solvent mobile phase was degaaased by refluxing for 5 min transferred to the solvent reservoir. All chromatography was carried out at ambient temperature.

Internal standard

A stock solution of mefenamic acid of 200 μg ml^{-1} was made weekly in 50% aqueous ethanol and stored at 4°C.

Preparation of standard curve

A stock solution of indomethacin of 400 μg ml^{-1} was made weekly in 50% aqueous ethanol as previously described [1] and stored at 4°C. Serial dilutions in 50% aqueous ethanol were made daily so that 20 μl of solution would correspond to a concentration range of 8.00—0.25 μg ml^{-1} of plasma.

Extraction of samples

To a 10-ml PTFE-lined screw-capped test tube were added 200 μl of plasma and 20 μl of internal standard solution containing 4 μg of mefenamic acid. The sample was mixed for 20 sec (Vortex Genie, Fisher Scientific, Edmonton, Canada) and 1 ml of 0.3 *M* perchloric acid added. The sample was remixed for 5 min (Evapomix, Fisher Scientific); then 6 ml of freshly distilled diethyl ether was added. The tube was tightly capped and remixed as above for 10 min. After centrifugation for 5 min at room temperature at 1725 *g* (TJ6 centrifuge, Beckman Instruments) the ether layer was transferred to another screw-capped tube containing 0.5 ml of 0.2 *M* phosphate buffer pH 7.2. The sample was capped tightly and mixed for 10 min and centrifuged for 5 min as before. The upper organic layer was transferred to another screw-capped test tube containing anti-bumping granules (BDH Chemicals, Toronto, Canada) and evaporated to dryness at 55°C (Thermolyne Dri-Bath, Fisher Scientific). The tube was allowed to reach room temperature after which 500 μl of mobile phase were added and the sample was mixed for 30 sec. An aliquot of 100—200 μl was injected into the HPLC instrument.

Plasma level study

Three healthy male volunteers weighing 61 kg, 80 kg, and 55 kg, respectively, were fasted overnight and received 50 mg of indomethacin (two 25-mg tablets), with either water (250 ml) or milk (250 ml). On a third occasion after a standardized normal breakfast these same volunteers received 50 mg indomethacin with water (250 ml).

The three treatments were each given twice each in turn. During the first week indomethacin was administered with water after fasting overnight. The time between dosages was three days. During the second week indomethacin was given with milk after an overnight fast. During the third week indomethacin with water, following a normal breakfast, was administered. Blood samples (10 ml) were obtained by venipuncture and collected in heparinized tubes (Vacutainers, Becton and Dickinson, Mississauga, Canada) at 0, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.5, 6.0, and 8.0 h following each dose. The blood samples were not permitted to touch the rubber stoppers upon collection and the plasma was removed and either analyzed immediately or stored at −20°C until just prior to analysis.

Recovery study

For the determination of recovery five replicate samples at levels of 1 and 4 μg ml^{-1} for indomethacin, and 1 μg ml^{-1} for mefenamic acid, were spiked in fresh blank heparinized plasma and run through the procedure as described for extraction of samples. The absolute peak heights obtained for the extracted samples were compared with those of fresh standards of indomethacin and mefenamic acid in mobile phase.

Quantitation

Standard curves for indomethacin were constructed by chromatographing spiked plasma extracts and plotting the peak height ratios obtained for the drug to the internal standard versus the concentration of the drug. Samples from volunteers were analyzed at the same time as calibration standards.

RESULTS AND DISCUSSION

Indomethacin and the internal standard gave sharp symmetrical peaks under the described conditions given in Experimental with retention times of 4.8 and 8.10 min, respectively (peaks a and b in Fig. 1B and C).

Fig. 1A shows a chromatogram of a 200-μl extract of fresh blank plasma which was processed as described in Experimental. Fig. 1B shows a chromatogram, obtained when the method was applied to spiked plasma containing 4 μg ml^{-1} of indomethacin and 20 μg ml^{-1} of the internal standard mefenamic acid, where it is evident that no endogenous peaks interfere. Fig. 1C is a chromatogram of a 200-μl plasma sample 7 h post dose from a volunteer (80 kg) who received two 25-mg tablets of a commercial formulation of indomethacin. The sample was estimated to contain 0.31 μg ml^{-1} of indomethacin.

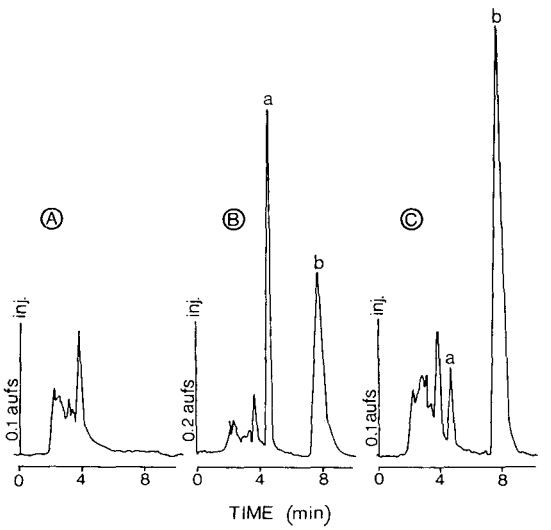

Fig. 1. Chromatograms of extracts from 200 μl of plasma. A, Blank plasma; B, plasma spiked with indomethacin (a, 4 μg ml^{-1}) and internal standard mefenamic acid (b, 20 μg ml^{-1}); C, plasma sample from a volunteer 7 h post dose (two 25-mg tablets) estimated to contain 0.31 μg indomethacin per ml of plasma.

The accuracy and precision of the assay is demonstrated in Table I, the results of which are based on six determinations for each concentration of indomethacin ranging from 0.25 to 8.00 μg ml^{-1} of plasma. The calibration curve obtained was linear from 0.25 to 8.00 μg ml^{-1} ($y = mx + b$) with a mean slope value of $m = 2.58$, an intercept of $b = 0.06$, and $r^2 = 0.99$.

Application of this method is shown in Fig. 2 for plasma concentrations over 8 h in a fasted volunteer (80 kg) who received 2 × 25 mg of indomethacin with water.

For confirmation that there was no metabolic interference under the peak due to indomethacin, pooled plasma extracts from volunteers were combined and injected into the HPLC instrument. The resulting effluent was collected at the retention time for indomethacin, the mobile phase was evaporated un-

TABLE I

ESTIMATION OF INDOMETHACIN ADDED TO PLASMA BY HPLC

$n = 6$.

Indomethacin added ($\mu g\ ml^{-1}$)	Mean peak height ratio drug/I.S.	S.D.	C.V. (%)
0.25	0.099	0.003	3.05
0.50	0.182	0.007	3.85
1.00	0.370	0.012	3.24
2.00	0.712	0.016	2.25
4.00	1.513	0.053	3.50
8.00	3.082	0.097	3.15

Mean C.V. = 3.17%

$y = 2.58x + 0.06$ ($r^2 = 0.99$)

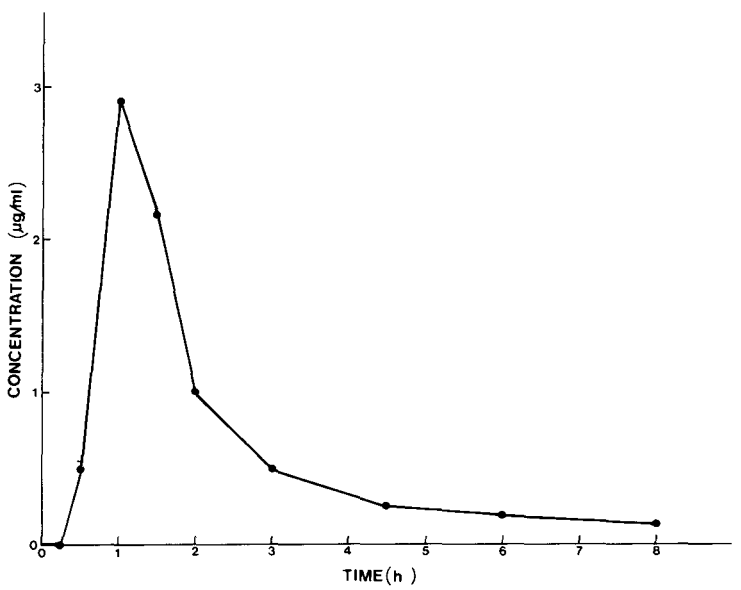

Fig. 2. Plasma concentration versus time profile for a volunteer (80 kg) who received 2 × 25 mg indomethacin with water after fasting overnight.

der vacuo at 30°C to dryness, and the residue dissolved in methanol evaporated and run by solid probe using a VG Micromass MM16F MS System (VG Micromass, Altrincham, Great Britain). The mass spectrum indicated the presence of only indomethacin.

The recovery of indomethacin and the internal standard are shown in Table II and are in the order of 89.43 ± 0.98% and 61.37 ± 0.82%, respectively.

The effect of various treatments of oral indomethacin is shown in Table III where each subject's AUC_0^8 is summarized. It is evident that there is not only

TABLE II

RECOVERY OF INDOMETHACIN AND MEFENAMIC ACID FROM PLASMA

Drug	Amount added to 200 μl plasma (μg)	Amount recovered (μg)	Mean recovery (%)	S.D. of percent recovery
Indomethacin	1	0.88	88.42	0.47
	4	3.62	90.44	1.48
			Mean 89.43 ± 0.98	
Mefenamic acid	4	2.45	61.37	0.82
			Mean 61.37 ± 0.82	

TABLE III

AUC_0^8 (μg h ml^{-1}) OF THREE VOLUNTEERS DOSED WITH INDOMETHACIN UNDER THREE DIFFERENT TREATMENTS
A = first dose; B = second dose.

Volunteer	Treatment					
	Water		Milk		Breakfast	
	A	B	A	B	A	B
1 (61 kg)	4.82	4.22	3.88	5.67	3.77	2.97
2 (80 kg)	4.85	6.03	6.07	5.18	4.54	6.36
3 (55 kg)	10.68	6.09	9.52	7.63	7.24	5.61

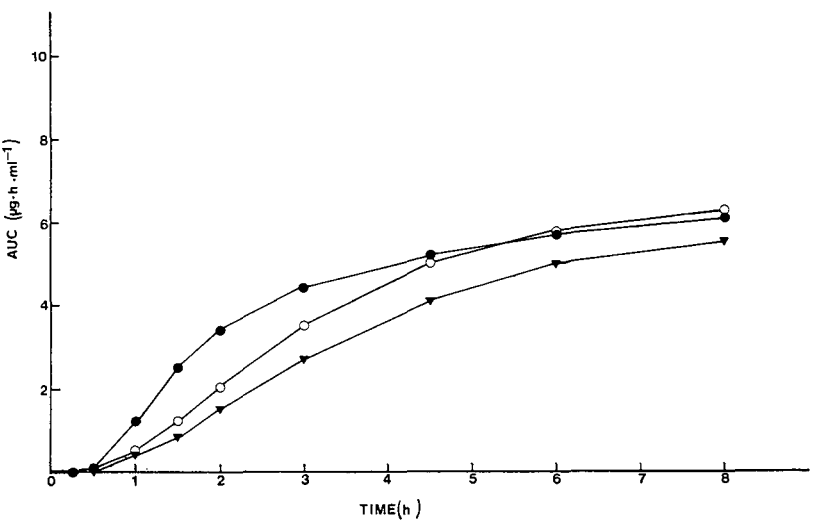

Fig. 3. Truncated mean AUC versus time profiles for three volunteers each dosed twice with indomethacin under three different conditions: overnight fast, drug administered with water (•); overnight fast, drug administered with milk (○); normal breakfast, drug administered with water (▲).

intersubject variation but also intrasubject variation. Volunteers 2 and 3 show the greatest variation, in volunteer 2 occurring with breakfast (ratio dose B/A of 140%) and in volunteer 3 occurring with water only (ratio dose B/A of 57%). Volunteer 2 exhibited the least variation with milk but the two treatments still had a B/A ratio of 85%. It can be seen that there is no observed intrasubject trend towards treatment and that, even when experimental and sampling error is accounted for, these intrasubject differences still remain. The truncated mean AUC time curves from these data are presented in Fig. 3. By plotting truncated areas under the blood level curve against time one is able to clearly observe simultaneously both the rate and extent of drug disposition. In Fig. 3 it is clear that administration to fasting subjects with water provides higher plasma levels with a shorter t_{max} as compared to the other two treatments. In addition the total bioavailability after the eight hours is practically equal for all three treatments. These observations are in agreement with those presented by Wallusch et al. [17]. In the latter study, however, each dose was given only once in each subject under each regimen, hence any intrasubject variance would not be evident. The mean values obtained, however, do simulate those findings.

In conclusion a new simple HPLC method for indomethacin has been developed and has been demonstrated to be applicable to single-dose pharmacokinetic studies of this drug in man. Our experimental results suggest that not only intersubject variability but also intrasubject variability exists for this drug. This intrasubject variability should be further investigated in a large population and must be considered in future pharmacokinetic and bioavailability studies of this drug.

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CHROMBIO. 1420

Note

Analysis of oxime-trimethylsilyl derivatives of organic acids on OV-1701 fused silica capillary column

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Recently Tanaka et al. [1] argued the statement that the diagnosis of organic aciduria by gas chromatography (GC) alone is much easier and not inferior to a diagnosis by gas chromatography—mass spectrometry (GC—MS). Essential for this GC diagnosis is the analysis of the organic acid profile on at least two packed columns with different stationary phases, e.g. SE-30 and OV-17.

However, the urinary organic acid fraction is so complex that high-resolution capillary GC should be used. Even then the analysis should be performed on two columns with stationary phases differing in polarity, to enhance the identification reliability. Up to now moderately polar capillary columns suitable for the analysis of trimethylsilyl (TMS) derivatives of organic acids were not available.

In this paper I report the excellent performance of an OV-1701 fused-silica capillary column for analyzing complex mixtures of oxime-TMS derivatives of organic acids. OV-1701 is a new stationary phase very comparable with OV-17. This is demonstrated on an organic acid profile from pooled normal urine. A tabulation of methylene units (MU) of more than 30 normally occurring compounds, separated on an OV-1701 fused-silica capillary column is given. A comparison is made between these MU values and those of the same compounds obtained by Tanaka et al. [1] on a packed OV-17 column.

MATERIALS AND METHODS

Urine samples

Urine of ten normal children was pooled (1 ml each) and creatinine was determined (4.8 mmol/l). An aliquot of 1.9 ml of urine was taken and 79.2 μg

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of 3-phenylpropionic acid (internal standard) were added. The extraction and derivatization (oxime-TMS) were carried out according to standard methods as described elsewhere [2].

Gas chromatography—mass spectrometry

The experiments were performed on a Jeol JMS D-100 GC—MS system with a JMA-0231 data system.

The GC column was a 25 m × 0.22 mm I.D. OV-1701 wall-coated fused-silica capillary column (Chrompack, Middelburg, The Netherlands). The GC column was directly introduced into the ion source. The carrier gas (helium) velocity was approximately 40 cm sec^{-1}. Samples were introduced by a modified all-glass sampling device [3] and separated using temperature programming: initial temperature 80°C, programming rate 8°C/min, final temperature 280°C. Injection port temperature was 275°C. The MS conditions were: electron energy 23 eV, emission current 0.6 mA, and source temperature 250°C. For qualitative analysis (identification) 0.5 μl was injected.

To study the column performance, 0.5 μl of the sample was injected with a split ratio of 20 : 1 and the intensity of the ion *m/z* 73, characteristic for all TMS derivatives of organic acids, was recorded. The multiplier voltage was set to −1500 V. The sensitivity was set to 1 V full scale, ten times the lowest sensitivity.

RESULTS AND DISCUSSION

Fig. 1 shows the mass chromatogram of oxime-TMS derivatives of organic acids of pooled urine of ten normal children.

The performance of the OV-1701 fused-silica capillary column is demonstrated by the inertness for TMS derivatives of organic acids for very small amounts injected. In Fig. 1 peak 11 corresponds to approximately 47 pmol of phenylpropionic acid (internal standard) injected. Compounds with a concentration of less than 1% of the internal standard, e.g. benzoic acid (peak 8) 0.6 pmol injected, show a perfect peak shape.

Table I lists the compounds and their MU values on OV-1701 fused-silica capillary column. In Table I a comparison has been made with the corresponding MU values on an OV-17 packed column as given by Tanaka et al. [1]. The MU values on OV-1701 and OV-17 are very comparable. The aliphatic acids tend to shift to higher MU values on OV-1701, whereas the aromatic organic acids shift to lower MU values on OV-1701 compared to OV-17. For urea-di-TMS there is a very noticeable difference of 1.25 MU between the two stationary phases.

The lower MU values for aromatic acid TMS derivatives on OV-1701 can possibly be ascribed to the lower content of phenyl groups in the stationary phase for OV-1701 as compared to OV-17: 7% and 50%, respectively [4].

The remarkable improvement of the performance of the OV-1701 column with respect to the OV-17 column could be ascribed to the different deactivating methods used. OV-17 columns are normally prepared by deactivating the wall with Carbowax. OV-1701 columns are prepared by deactivating the wall by a high-temperature reaction of polysiloxane with silanol groups on the

TABLE I

METHYLENE UNITS OF SOME ORGANIC ACID OXIME-TMS DERIVATIVES ON OV-1701 CAPILLARY COLUMN

GC peak in Fig. 1	Compound	MU values		
		OV-1701	OV-17*	Shift
1	α-Hydroxyisobutyric acid	11.03	10.74	0.29
2	Lactic acid	11.21	10.95	0.26
3	Glycollic acid	11.50	11.27	0.23
4	Glyoxylic acid-oxime	11.97	11.96	0.01
5	Pyruvic acid-oxime	12.08	12.11	-0.03
6	2-Methyl-3-hydroxybutyric acid	12.52	12.25	0.26
7	3-Hydroxyisovaleric acid	12.63	12.35	0.28
8	Benzoic acid	13.38	13.73	-0.45
9	Succinic acid	14.18	14.02	0.16
10	Urea	14.76	13.50	1.25
11	Phenylpropionic acid (internal standard)	15.25	—	—
12	Adipic acid	16.17	15.97	0.20
13	Methyladipic acid	16.42	16.20	0.22
14	2-Hydroxymethyl-5-carboxylfurane	16.72	—	—
15	2-Hydroxyglutaric acid	16.78	—	—
16	3-Hydroxy-3-methylglutaric acid	16.89	16.48	0.41
17	Pyroglutamic acid	17.00	16.80	0.20
18	3-Hydroxyphenylacetic acid	17.08	17.33	-0.25
19	2-Ketoglutaric acid	17.33	17.06	0.27
20	4-Hydroxyphenylacetic acid	17.53	17.66	-0.13
21	2,5-Dicarboxylfurane	17.89	—	—
22	4-Hydroxymandelic acid	18.81	18.42	0.39
23	Vanillic acid	18.93	19.08	-0.15
24	Homovanillic acid	19.09	19.32	-0.13
25	Azelaic acid	19.15	—	—
26	Citric acid	19.31	18.69	0.62
27	Isocitric acid +	19.53	18.86	0.67
	3-hydroxyphenyl-3-hydroxypropionic acid	19.53	—	—
28	Vanilmandelic acid +	20.14	—	—
	4-hydroxyphenyllactic acid	20.14	19.93	0.21
29	Palmitic acid	21.11	20.90	0.21
30	Hippuric acid (mono-TMS)	21.28		0.18
31	3-Hydroxysebacic acid	22.87	—	—
32	Oleic acid	22.87	22.87	0
33	Stearic acid	23.10	22.90	0.20
34	4-Hydroxyhippuric acid (di-TMS)	24.43	24.56	-0.13

*Ref. 1.

glass surface [5]. Probably TMS esters are hydrolyzed by the hydroxy groups of the Carbowax deactivation layer. This assumption is enforced by the fact that highly retained TMS esters on Carbowax-deactivated OV-17 capillary columns do not elute, whereas TMS esters with a short retention time elute normally.

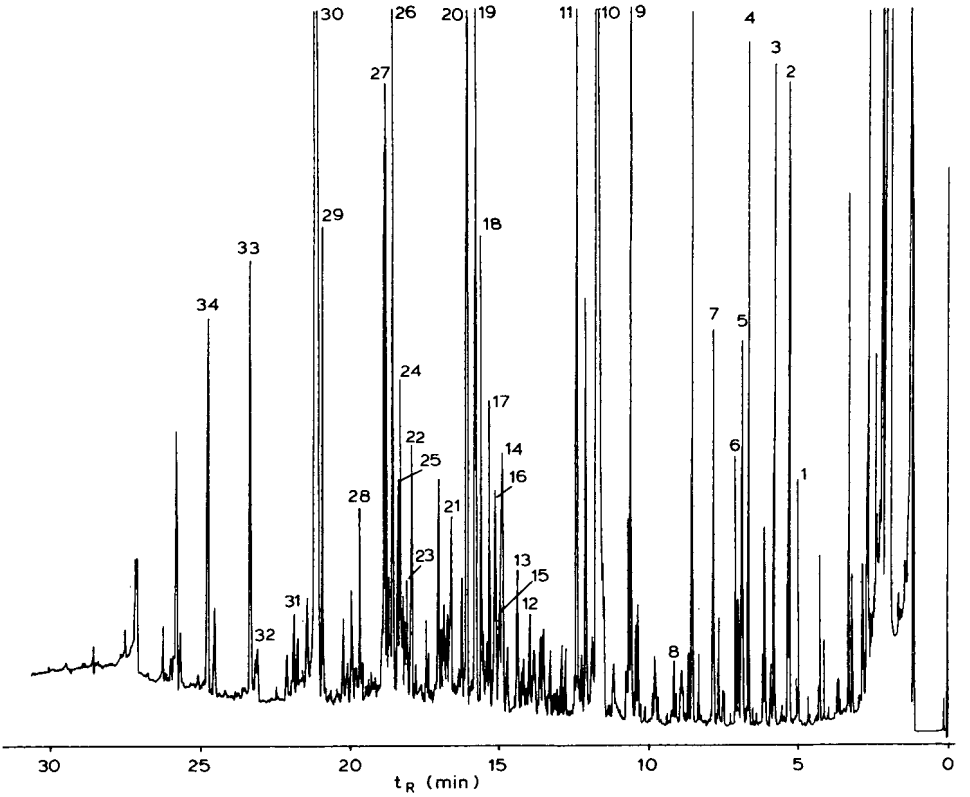

Fig. 1. Organic acid oxime-TMS derivative profile of pooled urine of ten normal children analyzed on a OV-1701 fused-silica capillary column. Peak numbers refer to Table I.

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Note

Gas chromatographic—mass spectrometric determination of aromatization of cyclohexanecarboxylic acid in guinea pig liver

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(First received May 6th, 1982; revised manuscript received July 28th, 1982)

Shikimic acid (3α,4α,5β-trihydroxy-1-cyclohexene-1-carboxylic acid) is an ubiquitous plant compound and an intermediate in the biosynthesis of essential amino acids [1, 2]. Interestingly, it has been claimed to be mutagenic and possibly carcinogenic to mice [3]. The anaerobic metabolism of shikimic acid by rat gastrointestinal microorganisms results mainly in the formation of cyclohexanecarboxylic acid [4].

Studies on the mammalian biotransformation of shikimic acid and cyclohexanecarboxylic acid have revealed that the latter is probably an essential intermediate in the metabolism of the former and that both undergo extensive aromatization [5]. That cyclohexanecarboxylic acid undergoes aromatization in mammals has long been known and efforts have been made to elucidate the properties of the enzyme(s) which catalyze the reaction(s) [6, 7]. A cell-free system from guinea pig liver was prepared which was able to convert cyclohexanecarboxylic acid to hippuric acid [8]. Later, soluble enzymes for the conversion of cyclohexanecarboxyl-coenzyme A to benzoyl-coenzyme A were isolated and some aspects of the mechanism of aromatization partially clarified [7]. Nevertheless, the information available on this subject is relatively meagre.

One of the limitations in the investigation of these enzymes has been the lack of sensitivity and specificity of the methods for quantitatively following the reaction. These include a colorimetric method [8] and a gas chromatography—radioactivity procedure [7]. The present report describes a specific and more sensitive gas chromatographic—mass spectrometric (GC—MS) method for following the enzymatic formation of hippuric acid from cyclohexanecarboxylic acid.

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EXPERIMENTAL

Materials

Adenosine triphosphate (ATP), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and bovine serum albumin (fraction IV) were purchased from Sigma (St. Louis, MO, U.S.A.); α-ketoglutaric acid (αKGA) and hippuric acid from Fluka (Buchs, Switzerland); glycine and sucrose from Koch-Light Labs. (Colnbrook, Great Britain); ethylenediaminetetraacetic acid (EDTA) and cyclohexanecarboxylic acid from E. Merck (Darmstadt, G.F.R.).

p-Methoxybenzoylglycine was prepared from *p*-methoxybenzoic acid by the method described by Sheehan and Hess [9] for the synthesis of peptides and had a m.p. of 168—170°C (literature value 171°C).

Cyclohexanecarboxylic acid was dissolved in methanol—water (1:3, v/v).

Animals

In all experiments described here, livers from Dunkin Hartly guinea pigs (Olac 1976 LTD) weighing 300—350 g were used. The animals were fed ad libitum on a standard guinea pig pelleted feed.

Preparation of whole homogenate of guinea pig liver

Animals were stunned and exsanguinated and the livers were immediately removed and chilled in 0.25 *M* sucrose containing 10 m*M* HEPES (pH 7.4) and 1 m*M* EDTA. All further procedures were carried out at 0—5°C. The livers were minced and homogenized in 4 volumes of the same buffer as described above using a Potter-Elvehjem homogenizer at 720 rpm and with two strokes of a loose-fitting PTFE pestle. This homogenized tissue mixture was used as a homogenate fraction.

Determination of protein

Protein was determined employing a Bio-Rad Protein Assay Kit (Bio-Rad Labs., Richmond, CA, U.S.A.) using bovine serum albumin as the standard.

Procedure

The incubation mixture contained 10 m*M* glycine, 5 m*M* αKGA, 5 m*M* Mg^{2+}, 0.1 m*M* ATP and 1 m*M* EDTA in 0.03 *M* phosphate buffer, pH 7.4. To this was added whole homogenate in amounts described in the text; final volume 2.9 ml. The mixture was preincubated at 37°C for 10 min in a Citenco shaker at 80 oscillations per min; then 3 μmol (100 μl solvent) of cyclohexanecarboxylic acid were added and the mixture further incubated at 37°C for periods described in the text. The reaction was terminated by the addition of 6 ml of 0.2 *M* hydrochloric acid and the flasks were placed on ice.

p-Methoxybenzoylglycine (internal standard) (1 μmol, 200 μl solvent) was then added and diethyl ether extraction was carried out using a total of five 25-ml portions. The combined ether extracts were dried over anhydrous sodium sulphate and evaporated to ca. 2 ml. The samples were then transferred to small tubes and further evaporated to ca. 0.5 ml. Following conversion of the carboxylic acids to their methyl esters by adding an ether solution of

diazomethane the samples were evaporated to dryness and finally redissolved in 200 μl methanol.

For the blank, the same procedure was carried out except that hydrochloric acid was added just before the commencement of incubation.

For calibration curves, the 3 μmol of cyclohexanecarboxylic acid was replaced by 0.05—1.4 μmol of a standard solution of hippuric acid.

Instrumentation

Analysis was performed on a Hewlett-Packard 5992A GC—MS system.

Separations were made on a OV-1 wall-coated open tubular capillary column (18 m × 0.29 mm I.D.). The chromatographic conditions were as follows: oven temperature, 160°C for 2 min then programmed 14°C/min to 260°C; injection port temperature, 245°C; and helium flow-rate, 3 ml/min. Quantitative selected ion monitoring was performed at 70 eV focusing the instrument on the ion at *m/z* 105 for methyl hippurate and *m/z* 135 for methyl *p*-methoxybenzoylglycinate.

Calculations

The concentration of hippuric acid was determined from the ratio of peak areas of methyl hippurate derived from the assay solution to that of methyl *p*-methoxybenzoylglycinate from the internal standard. Using the standard curve the mole ratio of methyl hippurate to methyl *p*-methoxybenzoylglycinate could be estimated and multiplying this number with the amount of internal standard added gives the total amount of hippuric acid in that specimen.

RESULTS AND DISCUSSION

The mass spectra of the methyl esters of hippuric acid and *p*-methoxybenzoylglycine (internal standard) are shown in Fig. 1. The molecular and base peak ions for methyl hippurate are *m/z* 193 and *m/z* 105, respectively, and for methyl *p*-methoxybenzoylglycinate, *m/z* 223 and *m/z* 135. The mass spectra indicate that the choice of molecular ions would allow for the unambiguous determination of the two compounds when using selected ion monitoring (SIM). However, these ions have relatively low intensity and the base peak ions at *m/z* 105 and *m/z* 135 were therefore chosen in order to maximize sensitivity. Fig. 2 shows the ion current profiles of a SIM chromatogram of an incubated sample containing 1 μmol of the internal standard. The retention time was 5.4 min for methyl hippurate and 7.8 min for the corresponding *p*-methoxy derivative.

The above results therefore show that only an insignificant amount of the SIM response at the chosen *m/z* values was due to the second component, and that this was eliminated by the chromatographic separation achieved. A further positive feature of the analysis is that the physical and chemical properties, including reactivity for derivatization and sensitivity for SIM, would be expected to be very similar for the two compounds. No interference from endogenous compounds was observed in our experiments.

A standard curve was constructed in which known amounts of hippuric

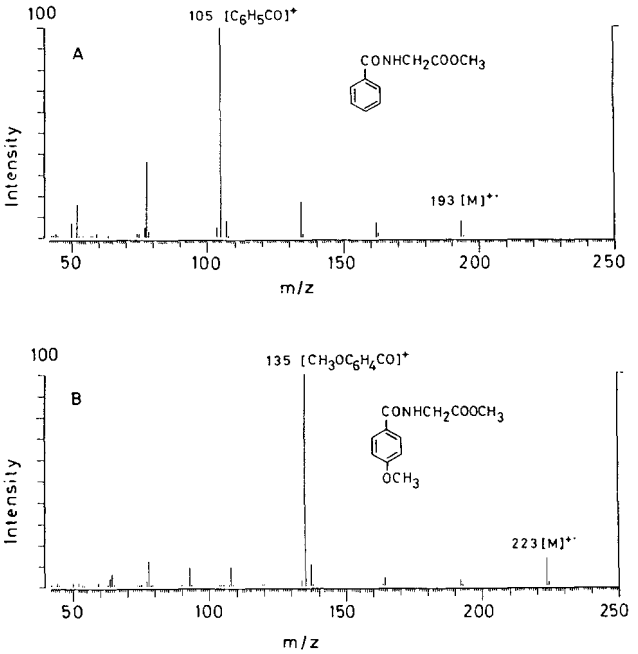

Fig. 1. Mass spectra of the methyl esters of (A) hippuric acid and (B) *p*-methoxybenzoylglycine.

acid (0.05—1.4 μmol) were added to flasks containing liver homogenate, hydrochloric acid and a fixed amount of *p*-methoxybenzoylglycine (1 μmol) and the mixture carried through the extraction and derivatizing procedure. A least-squares fit [r=0.997 ± 0.002 (S.D.)] gave a linear relationship between peak area ratios (m/z 105:135) and mole ratios. The intercept on the y axis (peak area ratio) was $0 \pm 1.37 \cdot 10^{-2}$ and the slope $0.96 \pm 2 \cdot 10^{-2}$ (n=5). This standard curve encompasses the range of values of current interest.

It is possible to measure smaller quantities of hippuric acid by constructing a standard curve using only one fifth of the given amount of internal standard. In this way we obtained lower measurement limits in the range of 10—25 pmol.

The rate of formation of hippuric acid by the homogenate of guinea pig liver was linear with time for at least 60 min (Fig. 3A). The mean activity in four different guinea pig liver homogenates was 11.5 ± 0.8 (S.D.) μmol/h/g of liver (69.4 ± 6.6 nmol/h/mg protein). This activity is somewhat higher than that observed earlier using guinea pig liver mitochondria [8]. This difference is partly due to the use of whole homogenate in the present experiments and partly due to the fact that the conditions for the enzyme reaction were optimized.

The most effective change we noted was the decrease in phosphate concentration from the earlier value of 83 m*M* [8] to 30 m*M* which resulted in a considerably greater enzyme activity (results not shown).

The formation of hippuric acid was linear with respect to protein concentration up to 8.4 mg/ml (Fig. 3B). The amount of protein used in the rate versus time studies described above was 5.6 mg/ml.

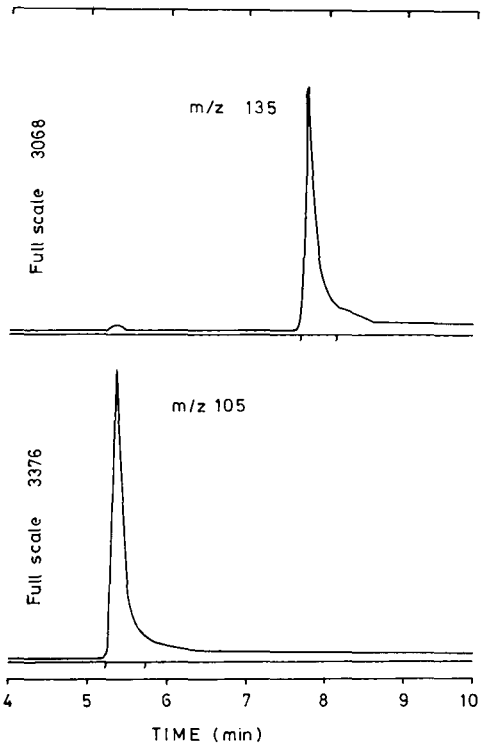

Fig. 2. Selected ion current profiles of a whole homogenate sample after incubation with cyclohexanecarboxylic acid and addition of *p*-methoxybenzoylglycine (*m/z* 135) as internal standard (1 μmol). The measured hippuric acid (*m/z* 105) concentration was 1.35 μmol.

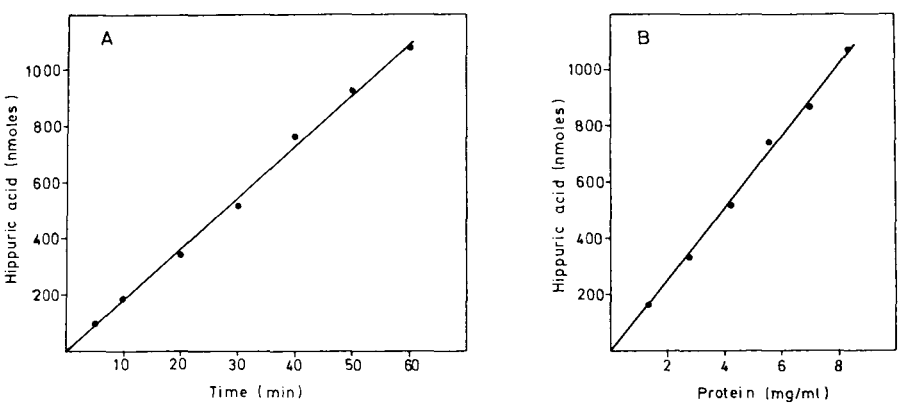

Fig. 3. Formation of hippuric acid in guinea pig homogenate as a function of time (A) and protein (B). In (A) 5.6 mg protein per ml was used and in (B) the incubation time was 40 min. Conditions otherwise were as stated in Experimental.

In conclusion, we believe that the GC—MS method described here for following the aromatization of cyclohexanecarboxylic acid has advantages over the methods reported previously [7, 8]. It shows high specificity and sensitiv-

ity and may be easily adapted to homologous substrates in order to study substrate specificity of the enzyme system. Furthermore, the method is suitable for investigation of the aromatizing activity in animal species other than the guinea pig, in tissues other than the liver and, significantly, in subcellular fractions. Such studies are of considerable interest with respect to assessing the biochemical significance of the aromatization reaction.

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Note

Determination of γ-aminobutyric acid in brain areas by high-performance liquid chromatography of dansyl derivatives with ultraviolet detection

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Many different methods for the estimation of γ-aminobutyric acid (GABA) in biological samples have been published [1—7]. Unfortunately, even though some are sensitive, most of those are expensive and time-consuming. Gas chromatography coupled with mass spectrometry [4] involves expensive instrumentation and derivatization. For gas chromatography with an electron-capture detector [2] a prior ion-exchange separation is needed. Furthermore, the experimental conditions employed for the preparation of N-trifluoroacetyl-O-hexafluoro-isopropyl derivatives must be carefully controlled since these derivatives are rapidly decomposed by traces of water in the ethyl acetate used to dissolve the dried residues.

In the present work, GABA was estimated in brain tissue by a simple and rapid dansylation reaction followed by high-performance liquid chromatography (HPLC) with UV detection. This procedure was applied to the determination of GABA levels under physiological conditions and after injection of aminooxyacetic acid (AOAA).

MATERIALS AND METHODS

Reagents and solutions

Valine, GABA and acetonitrile for liquid chromatography were obtained from Merck (Darmstadt, G.F.R.). All other chemicals and reagents were of analytical grade (Carlo Erba, Milan, Italy). Stock solutions of GABA and valine were prepared at a concentration of 1 mg/ml in 0.1 *M* hydrochloric acid and stored at 5° C.

A sodium bicarbonate solution was obtained by dissolving 0.84 g of sodium bicarbonate in 100 ml of water. Dansyl chloride stock solution was prepared

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by dissolving 100 mg in 1 ml of anhydrous acetone. The working solution was 50 μl of stock solution diluted in 4 ml of acetone.

Liquid chromatography

A Series 2/2 Perkin-Elmer liquid chromatograph with a variable-wavelength UV detector LC-75 (Perkin-Elmer, Norwalk, CT, U.S.A.) and Autocontrol system was employed. The injection valve was a Model 7125 (Rheodyne, Berkeley, CA, U.S.A.). The system was connected with a Hitachi-Perkin-Elmer Model 56 recorder. An RP-8 column (10 μm particle size; 25 cm × 4.6 mm I.D.) from Perkin-Elmer was operating at room temperature. The mobile phase was an acetonitrile–water (35:65) mixture containing 0.15% by volume H_3PO_4; the flow-rate was 1.5 ml/min. The column effluent was monitored at 254 nm.

Animals

Male Sprague-Dawley Charles River rats (150–170 g) were used. Rats were killed by microwave radiation or decapitation 2.5 min after injection of 3-mercaptopropionic acid (MPA; 100 mg/kg, intraperitoneally) dissolved in 0.154 *M* sodium hydroxide. This treatment prevents the post-mortem increase in GABA [8].

After decapitation of MPA-treated rats, the brains were quickly removed and placed on an ice-cold Petri dish; the hypothalamus, the brain stem and the striatum were dissected out, frozen on dry ice and stored at −20°C until assayed.

AOAA was injected intraperitoneally at a dose of 20 mg/kg. Injections were given in a volume of 5 ml/kg. The animals were killed by exposing their heads for 3–4 sec to high-energy microwave radiations (oven: 2.0 kW, 2.45 GHz, 75 W/cm^2; Medical Engineering Consultants, U.S.A.) [9] 2 h after injection of AOAA, and the striata were dissected out.

Analysis

Tissues were placed in 10-ml plastic tubes containing appropriate volumes of valine solution (1000 μg/g of sample) and 15 volumes of 75° ethanol. The mixture was then homogenized by a Politron and centrifuged at 1500 *g* for 10 min at 4°C. Volumes ranging from 50 to 200 μl of supernatant, depending on the expected tissue GABA concentration, were introduced into 10-ml screw-capped tubes and dried under a flow of nitrogen at 50°C.

Internal standards (IS) were prepared by adding known amounts of authentic GABA (250–1000 ng) to aliquots (50–200 μl) of supernatant pool containing appropriate volumes of valine solution and assayed in parallel with the tissue samples. The pool aliquots (50–200 μl) without added GABA served as a tissue blank (TB) or for the standards.

For derivatization 50 μl of bicarbonate solution and 100 μl of working dansyl chloride solution were added to the dried residues. The tubes were then placed on a dry block and heated for 15 min at 90°C; then 3–5 μl of this solution were injected into the chromatograph.

Calibration curve

One standard curve was constructed with increasing amounts of pure GABA

(250, 500, 1000, 1500 ng) each plus 1000 ng of valine. A second standard curve was obtained by adding the same quantities of GABA and valine to tissue extracts prepared as described before. The relationship between the peak height ratios and the amount of GABA was found to be linear over the range shown (250–1500 ng).

Recovery

Overall recovery was gauged by comparison with data obtained by HPLC analysis (assuming 100% recovery of derivatives) of the residue obtained when standard solutions of authentic compounds equal in amount to those added to striatal homogenate aliquots were taken to dryness directly. Each sample was run in duplicate.

RESULTS

Recovery after addition of GABA (200 μg/g) to homogenates was found to be 96 ± 5%. The sensitivity of the method was 1–2 ng of injected dansyl-GABA, with a signal-to-noise ratio of 2.

Amino acids that are present in the central nervous system in amounts comparable with those of GABA were tested (glutamic acid, taurine and glycine), but they did not give any peaks interfering with GABA determination. GABA concentrations were determined by the peak height ratio using valine as a marker. The amounts (μg/g) of GABA in the brain areas were calculated as follows

$$\text{GABA } (\mu g/g) = \frac{R_S \times V_H \times A_G}{(R_{IS} - R_{TB}) \times V_S \times W_T},$$

where R_S = peak height ratio of sample, R_{IS} = peak height ratio of internal standard, R_{TB} = peak height ratio of tissue blank, W_T = tissue weight (g), V_H = volume of ethanol added for homogenization, A_G = μg of GABA added to the internal standard, V_S = volume of supernatant used for derivatization.

Fig. 1 shows typical chromatograms for a reagent blank and for the tissue extract samples. In the sample chromatogram the peaks corresponding to taurine, glycine and glutamic acid overlapped the solvent front.

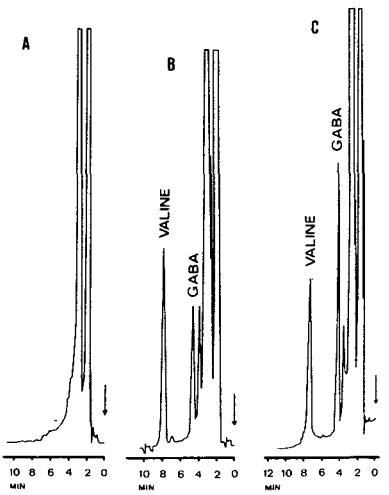
Table I shows the distribution of GABA concentration (μg/g) in different brain regions.

In Table II are the physiological levels of striatal GABA and the levels after intraperitoneal injection of AOAA (20 mg/kg). Fig. 2 shows the calibration curve obtained with pure standard and the curve obtained when known amounts of GABA were added to rat striatal extract.

The same tissue homogenate tested at three different times gave a coefficient of variation of 5.4%.

DISCUSSION

A dansylation method for GABA estimation in biological samples was developed by Neuhoff and Weise [1] based on the formation of unlabelled or



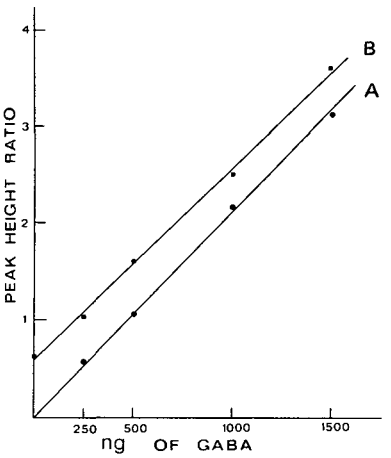


Fig. 1. (A) Chromatograms of blank reagent, (B) striatum extract with 500 μg/g valine added, and (C) the same extract after addition of 0.5 μg of GABA.

Fig. 2. Standard curves for pure GABA + valine (A) and with a striatum extract (B).

TABLE I

GABA CONCENTRATIONS IN RAT BRAIN AREAS

Animals were sacrificed by decapitation 2.5 min after injection of MPA (100 mg/kg, intraperitoneally). Values are mean ± S.E.M. of at least six determinations.

Sample	GABA (μg/g)
Brain stem	177.5 ± 9.5
Hypothalamus	513.2 ± 29.0
Striatum	238.5 ± 13.9

TABLE II

EFFECT OF INTRAPERITONEAL ADMINISTRATION OF AOAA ON STRIATUM GABA CONCENTRATIONS

Each value represents the mean ± S.E.M. of 5—7 determinations. Animals were sacrificed with high-energy microwave radiation. Striatum GABA concentrations were measured 2 h after AOAA or saline administration.

Treatment	GABA (μg/g)
Saline	181.3 ± 8.2
AOAA	502.5 ± 23.5*

*Difference from saline-treated rats $p < 0.001$, Student's t-test.

labelled dansyl derivatives, which are then separated by thin-layer chromatography. Other groups [10–12] have determined amino acids except GABA by utilizing pre-column dansyl derivatization followed by liquid chromatographic separation and detection by fluorescence. The precision, accuracy and rapidity of HPLC methodology provides an efficient means for the measurement of GABA in tissue samples. We have examined the possibility of using the pre-column dansylation technique and HPLC with UV detection.

In the UV spectrum of dansyl-GABA, recorded by means of the Autocontrol system, there are two absorbance maxima, at 220 and 287 nm. We chose to operate at 254 nm, this being the wavelength accessible also with fixed-wavelength detectors.

The precision of the method can be seen from the coefficient of variation – 5.4%. The sensitivity is satisfactory and the specificity greater than that of any other method except gas–liquid chromatography–mass spectrometry (GLC–MS).

The method is simple, reasonably rapid and has good recovery and reproducibility and apparently a high specificity. The analyses of rat brain areas gave values that are in agreement with determinations carried out by GLC–MS [4].

Treatment of rats with AOAA has been reported to elevate GABA levels markedly [13]. We also found this in the present study, in which AOAA (20 mg/kg, intraperitoneally) elevated the GABA concentration in the rat striatum about three-fold.

GABA is assumed to play an important role in the regulation of different functions, including feeding [14], heart rate [15], respiration [16], pituitary hormone release [17] and analgesia [18]. In addition, the involvement of GABA in pathological changes associated with Huntington's chorea, Parkinsonism and epilepsy adds clinical significance to the investigation of the role of GABA in the brain [19]. Thus, the availability of a new, sensitive, specific, rapid and inexpensive method for the analysis of GABA in brain areas will aid further research into the role of GABA in brain functions.

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Note

Rapid, simultaneous and sensitive determination of free hydroxyproline and proline in human serum by high-performance liquid chromatography

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Automated amino acid analysis with ninhydrin reaction gives a low sensitivity for imino acids. Fluorogenic amine reagents such as fluorescamine and *o*-phthalaldehyde yield a high fluorescence intensity with primary amino acids except imino acids [1—3]. Recently, 4-chloro-7-nitrobenzofurazan has been used for the assay of primary amino acids and imino acids [4,5]. Böhlen and Mellet [6] developed a method of determining hydroxyproline and proline using the combination of *o*-phthalaldehyde and alkaline sodium hypochlorite, which is an oxidant that converts imino acids into primary amino acids. The present paper describes a rapid, simultaneous and micro assay for free hydroxyproline and proline in human serum by high-performance liquid chromatography using the post-labeled method with *o*-phthalaldehyde and sodium hypochlorite.

EXPERIMENTAL

Reagents

Lithium citrate and the standard solution of amino acids for an amino acid autoanalyzer, *o*-phthalaldehyde of biochemical grade, and all the other reagents of analytical grade were obtained from Wako Pure Chemical Industries (Osaka, Japan). Commercial 10% sodium hypochlorite (NaOCl) was purchased from Yoneyama Yakuhin Kogyo (Osaka, Japan).

Mobile phase

One-thirtieth *M* lithium citrate solution was adjusted to pH 3.15 by perchloric acid. Lithium hydroxide (0.2 *M*) was made as a regeneration solution. These solutions were degassed under reduced pressure for 20 min.

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Reaction buffer

NaOCl solution was prepared by adding 2.0 ml of the 10% sodium hypochlorite to 0.3 *M* borate buffer; it was titrated with 4 *M* sodium hydroxide to pH 10.5, and made up to 1000 ml with distilled water for liquid chromatography. *o*-Phthalaldehyde solution was prepared as follows: 4 g of *o*-phthalaldehyde and 1 ml of 2-mercaptoethanol were dissolved in 30 ml of ethanol. After 0.3 *M* borate buffer containing 0.5 g of Brij 35 was added to the ethanol solution, the solution was adjusted to pH 10.5 by 4 *M* sodium hydroxide, and was made up to 1000 ml with distilled water for liquid chromatography.

Apparatus

The following equipment was purchased from Shimadzu (Kyoto, Japan): Model LC-3A high-performance liquid chromatograph; Model FLD-1 fluorescence detector with a mercury source lamp (excitation wavelength 360 nm; emission wavelength 450 nm); data analyzer C-R1A Chromatopac connected to the detector for identification and integration of peaks; and a 15 cm × 4 mm column filled with sulfonated polystyrene cation-exchange resin (Shimadzu Gel ISC-07/S1504, particle size, 7 μm). The column oven temperature was set at 55°C. The flow-rate of the mobile phase was set at 0.3 ml/min, yielding an operating pressure of 20 kg/cm^2. The flow-rate of the NaOCl solution and the o-phthalaldehyde solution was set at 0.25 ml/min, and the lengths of the oxidative reaction coil and the fluorogenic reaction coil were 1 m and 2 m, respectively. The reaction temperature was set at 55°C.

Sample preparation procedure

Determination of the concentration of hydroxyproline and proline was performed by the standard addition method on the basis of the peak area ratios against the internal standard. One-tenth milliliter of human serum was transferred into each of the four micro test tubes, to which the standard hydroxyproline and proline solutions were added as follows. To the first sample, 0.05 ml of distilled water was added. To the second, 0.05 ml of a solution of 10 μmol/l hydroxyproline and 100 μmol/l proline was added. To the third, 0.05 ml of a solution of 20 μmol/l hydroxyproline and 200 μmol/l proline was added. To the fourth, 0.05 ml of a solution of 40 μmol/l hydroxyproline and 400 μmol/l proline was added. Since the chemical properties of N-methylglycine (sarcosine) are similar to those of imino acids and it is rarely detected in human serum, 0.05 ml of 8% sulfosalicylic acid solution containing 1 m*M* of N-methylglycine as the internal standard was added to each sample. These samples were vortex-mixed for 30 sec and then centrifuged at 12,800 *g* for 1 min. One-hundredth of the deproteinized serum supernatant was injected into the high-performance liquid chromatograph.

RESULTS AND DISCUSSION

The NaOCl reagent dramatically attenuated the generation of fluorescence from primary amino acids (Fig. 1A and B). There was a good linear correlation between the peak area ratios and the sera containing the standard imino acids, with correlation coefficients of 0.997 and 0.999 (Fig. 2). The free hydroxy-

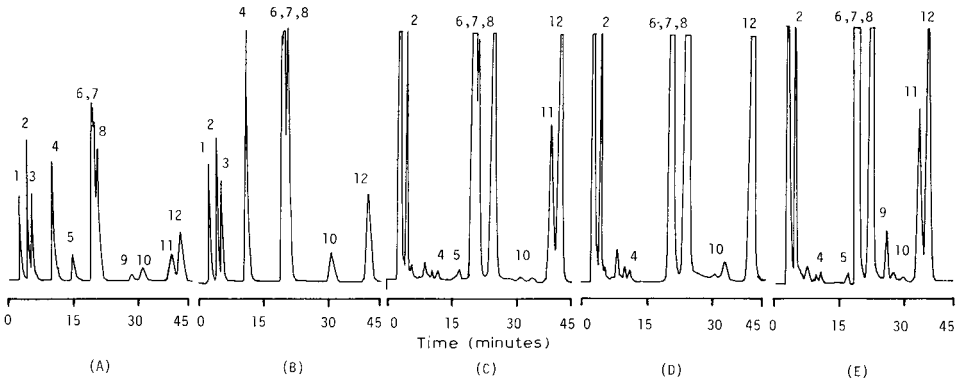

Fig. 1. Chromatograms of amino acid analysis. (A) Standard amino acids: 0.1 nmol of phosphoserine (1), taurine (2) and phosphoethanolamine (3); 0.2 nmol of aspartic acid (4), hydroxyproline (5), threonine (6), serine (7), glutamic acid (8), proline (11) and glycine (12); 0.5 nmol of N-methylglycine (9) and 0.05 nmol of L-amino adipic acid (10). (B) Standard amino acids analysed using *o*-phthalaldehyde solution without NaOCl solution. (C) Analysis of free amino acids in human serum. (D) Free amino acids in human serum analysed using *o*-phthalaldehyde solution alone. (E) Free amino acids in human serum mixed with the internal standard of N-methylglycine. The analytical condition is similar to that of (C).

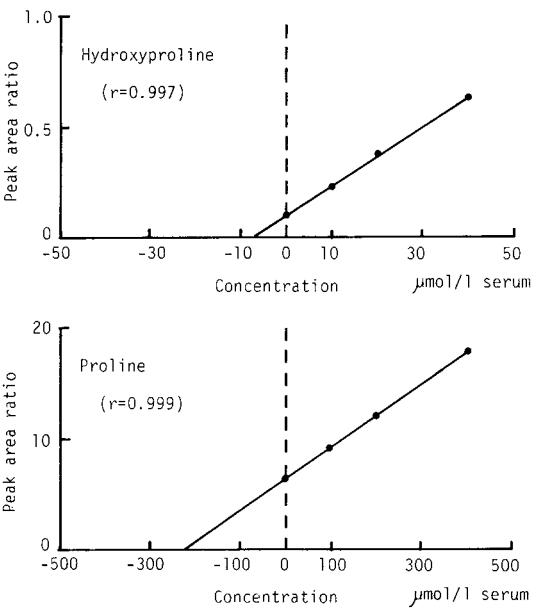

Fig. 2. Standard addition curves for determination of free hydroxyproline and proline in serum from a healthy subject. The concentrations of free hydroxyproline and proline in serum were 6.7 μmol/l and 225.3 μmol/l, respectively. The equation of the line was determined by the least-sqaures method.

proline and proline concentration in the sera from ten healthy subjects measured by the present method was 6.2 ± 1.3 μmol/l for hydroxyproline and 212.0 ± 16.0 μmol/l for proline. The limit of detection was found to be 1.0 μmol/l for hydroxyproline and 10.0 μmol/l for proline in human serum. It is

possible to determine the imino acids using 0.05 ml of serum when the method with standard calibration curves is adopted.

There have been few clinical reports on hypersarcosinemia [7], and sarcosine cannot be detected in normal human serum under the present conditions (Fig. 1C, D and E). Thus sarcosine is a convenient internal standard to assay imino acids in human serum. The present procedure is suitable for the routine determination of free hydroxyproline and proline in human serum at the picomole level by modification of Böhlen and Mellet's method [6]. This method may be useful for the detection of abnormalities in imino acid metabolism and collagen synthesis.

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Note

Reversed-phase high-performance liquid chromatography of neuropeptides related to adrenocorticotropin, including a potent adrenocorticotropin 4—9 analogue (ORG 2766)

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Neuropeotides are fragments of pituitary and brain polypeptides that influence brain functions [1]. One class of these peptides is comprised of fragments derived from adrenocorticotropic hormone (ACTH; Fig. 1). In both the pituitary gland and the brain, ACTH can be enzymatically generated from a precursor molecule of molecular weight 31,000 and further processed into α-melanocyte-stimulating

	1 5 10 15 20
HUMAN	SER-TYR-SER-MET-GLU-HIS-PHE-ARG-TRP-GLY-LYS-PRO-VAL-GLY-LYS-LYS-ARG-ARG-PRO-VAL-
PORCINE	
OVINE, BOVINE	

	21 25 30 35 39
HUMAN	LYS-VAL-TYR-PRO-ASN-GLY-ALA-GLU-ASP-GLU-SER-ALA-GLU-ALA-PHE-PRO-LEU-GLU-PHE
PORCINE	-LEU-
OVINE, BOVINE	-GLN-

Fig. 1. Structure of human ACTH and comparison with ACTH from other species.

hormone (α-MSH, $N^{\alpha 1}$-acetyl, $C^{\alpha 13}$-ACTH 1—13-amide) and corticotropin-like intermediate lobe peptide (CLIP; ACTH 18—39) [2, 3]. Accumulating evidence exists that ACTH and α-MSH as well as N-terminal fragments that are virtually devoid of classical endocrine activities, affect adaptive behaviour in laboratory animals and in man through direct actions on the central nervous system [1, 4, 5]. In addition, analogues of N-terminal fragments of ACTH, e.g. [Met$(O_2)^4$,D-Lys^8, Phe^9]ACTH 4—9 (ORG 2766), which exhibit remarkably increased

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behavioral potencies as compared to their parent peptides, have been synthesized [6, 7]. From these observations it has been postulated that endogenous ACTH can act as a precursor which is enzymatically converted into smaller, behaviourally active fragments [1]. In the investigation of this hypothesis, the application of reversed-phase high-performance liquid chromatography (HPLC) is virtually indispensable. This technique has been established as a powerful tool for the separation of complex peptide mixtures [8—11]. Resolution of various ACTH-related peptides has been described using a large variety of mobile phase combinations, including ammonium acetate and/or acetic acid [10, 12], phosphoric acid [9, 13], trifluoroacetic acid (TFA) [14–17], heptafluorobutyric acid (HFBA) [15], or triethylammonium phosphate (TEAP) [18] in the aqueous solvent. The addition of lyophilizable compounds such as ammonium acetate, TFA or HFBA in the mobile phase allows good peptide recoveries for further analysis procedures, in contrast to the use of phosphate or TEAP.

In this paper we report the resolution of ACTH 1—39 and various fragments, including peptides that have not yet been investigated, by reversed-phase HPLC with the ion-pairing reagent TFA in the mobile phase. In addition, using ammonium acetate buffers, we present the separation between numerous N-terminal fragments of ACTH as well as between an ACTH 4–9 analogue (ORG 2766) and several of its peptide metabolites that could arise upon enzymatic digestion [19].

EXPERIMENTAL

Chemicals and peptides

All chemicals were of analytical grade. Ammonium acetate and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, G.F.R.). Glacial acetic acid was supplied by Fluka (Buchs, Switzerland). Methanol of HPLC grade was purchased from Baker (Deventer, The Netherlands).

pACTH 1—39 was isolated from porcine pituitary glands by Organon International (Oss, The Netherlands). Human ACTH 18—39 (CLIP; corticotropin-like intermediate lobe peptide) was a generous gift of Dr. P.J. Lowry (St. Bartholomew's Hospital, London, Great Britain). All other peptides were synthesized, purified and kindly donated by Dr. H.M. Greven and Dr. J.M. van Nispen (Organon, Oss, The Netherlands).

Equipment

The HPLC assembly (Waters Assoc., Milford, MA, U.S.A.) consisted of two Model 6000A solvent delivery pumps, a Model 600 solvent programmer for gradient elution, a universal U6K injector and a reversed-phase μBondapak C_{18} column (300 × 3.9 mm I.D., particle size 10 μm). The column effluent was monitored continuously for UV absorbance at 210 nm (1.0 a.u.f.s.) with a Schoeffel variable-wavelength UV monitor (Model 770) equipped with an 8-μl flow-through cell. UV absorbance was recorded on a two-channel range recorder (BD9, Kipp en Zonen, Delft, The Netherlands) with a chart speed of 0.5 cm/min. Solvents were filtered and degassed using Pyrex filter holders with 0.5-μm pore diameter filters from Millipore (Bedford, MA, U.S.A.).

Chromatography

Mixtures of pACTH 1—39 and its fragments were chromatographed at ambient temperature by gradient elution with 0.09 *M* TFA, pH 1.9 (solvent A) and methanol (solvent B). Upon injection of a peptide sample, the column was eluted at 1 ml/min with a linear gradient from 60% A (40% B) to 30% A (70% B) over 25 min.

Separation of N-terminal fragments of ACTH 1—39 was performed at ambient temperature with a 30-min convex gradient (program 5) of 0.01 *M* ammonium acetate adjusted to pH 4.2 with glacial acetic acid (solvent X) and methanol containing 1.5 ml of acetic acid per liter (solvent Y); initial conditions X/Y = 95:5, final conditions X/Y = 50:50. The flow-rate was 2 ml/min.

Chromatography of an ACTH 4—9 analogue (ORG 2766) and several of its peptide metabolites was carried out at 2 ml/min with a 30-min linear gradient of 0.01 *M* ammonium acetate, pH 4.2 (solvent X) and methanol (solvent Y) as mobile phases; initial conditions X/Y = 95:5, final conditions X/Y = 65:35.

RESULTS AND DISCUSSION

The resolution of ACTH 1—39 and related peptides following RP-HPLC on a μBondapak C_{18} column with a linear gradient of 0.09 *M* TFA and methanol, is shown in Fig. 2. The chromatographic conditions are adapted to those intro-

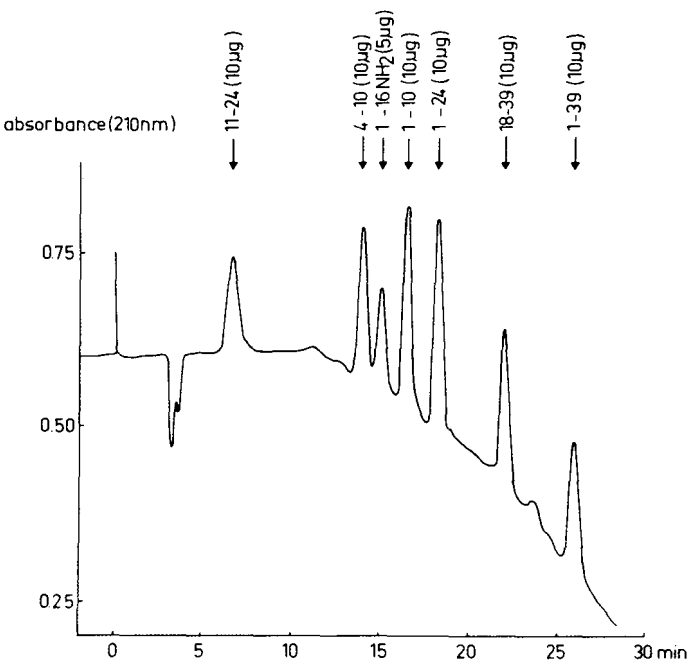

Fig. 2. Chromatography of a mixture of ACTH 1—39 and various fragments (5—10 μg of each peptide) on μBondapak C_{18}. Elution was performed with a 25-min linear gradient of 0.09 *M* TFA, pH 1.9 (A) and methanol (B). Initial conditions, A/B = 6:4; final conditions, A/B = 3:7. The flow-rate was 1 ml/min. UV absorbance was measured at 210 nm (1.0 a.u.f.s.). The abbreviations refer to ACTH 1—39 as the basic sequence (see Fig. 1).

duced by Bennett et al. [14]. The use of the hydrophilic ion-pairing reagent TFA, which is volatile and is transparent at 210 nm, can result in sharp and symmetric peptide peaks. The elution order of the peptides investigated is in agreement with that found by others [14—17]. It seems to be correlated more with the total hydrophobicity of the peptide than with the chain length. For example, $C^{\alpha 16}$-ACTH 1—16-amide has a shorter retention time than its fragment ACTH 1—10, probably because the presence of three hydrophilic lysine residues within the C-terminal sequence 11—16 (Fig. 1) more than compensates for the effect of increased chain length. Similar considerations might explain the elution of ACTH 11—24 before the shorter ACTH sequences 4—10 and 1—10. Under the conditions used in Fig. 2, no resolution was accomplished between the peptide pairs ACTH 11—24 and ACTH 4—7, ACTH 4—9 and ACTH 4—10, ACTH 1—24 and α-MSH, and no baseline separation between pACTH 1—39 and pACTH 25—39. However, by increasing the gradient time from 25 min to 50 min, baseline separation was obtained between α-MSH and ACTH 1—24 as well as between pACTH 25—39 and pACTH 1—39 (data not shown).

Using TFA in the mobile phase for HPLC analysis, the chromatographic profile of ACTH-related peptides appeared to be independent of both the amounts of peptides applied to the column and of injection volumes up to 1 ml. Decreased resolution was observed when the TFA concentration was lower-

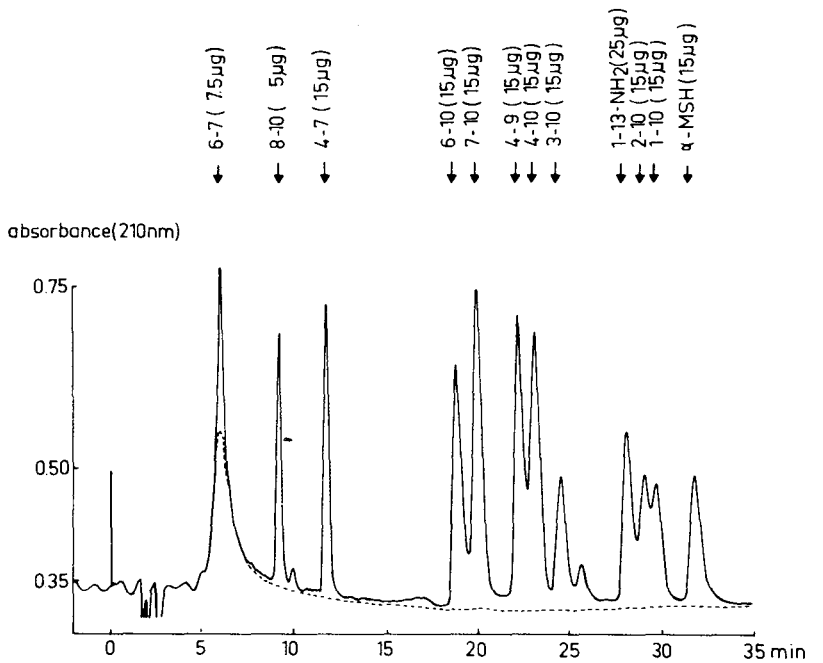

Fig. 3. Separation of a mixture of N-terminal fragments of ACTH (5—25 µg of each peptide, as indicated in the figure) on µBondapak C_{18}. Elution was carried out with a 30-min convex gradient (program 5) of 0.01 *M* ammonium acetate, pH 4.2 (X) and methanol, containing 1.5 ml/l acetic acid (Y). Initial conditions, X/Y = 95:5; final conditions, X/Y = 50:50. The flow-rate was 2 ml/min. UV absorbance was measured at 210 nm (1.0 a.u.f.s.). The baseline in the UV profile of the gradient used is shown by the interrupted line. The abbreviations refer to ACTH 1—39 as the basic sequence (see Fig. 1).

ed from 0.09 *M* to 0.009 *M*, in contrast to previously reported findings [15, 16]. During the lifetime of the columns, peak broadening and increased retention times were noticed for the peptides investigated. Nevertheless, the peak areas remained quite reproducible both on one column with time and from column to column. Extensive washing of the columns restored the original sharp peptide peaks and shorter retention times.

The separation of various N-terminal fragments of ACTH ($\leqslant$ 13 amino acid residues) by reversed-phase HPLC on μBondapak C_{18} using a convex gradient of 0.01 *M* ammonium acetate (pH 4.2) and methanol, is presented in Fig. 3. The high resolving power of the system allows satisfactory resolution of oligopeptides that differ in only one amino acid residue, e.g. ACTH 6–10 from ACTH 7—10, ACTH 4—9 from ACTH 4—10, and ACTH 2—10 from the sequences 3—10 or 1—10. Of all the peptides studied, only ACTH 5—10 and $C^{\alpha 16}$-ACTH 1—16-amide appear to coelute with ACTH 6—10 and ACTH 4—9, respectively. In general, the retention times are proportional to the peptide chain lengths, as has also been reported for β-endorphin fragments using similar HPLC systems [11]. In addition, the retention times of the peptides depend on their overall hydrophobicity as was found with the resolution of ACTH-like peptides in the TFA system. This is illustrated by the observation that the basic peptide $C^{\alpha 16}$-ACTH 1—16-amide has a lower retention than its shorter, more hydrophobic fragments ACTH 4—10, $C^{\alpha 13}$-ACTH 1—13-amide (des-$N^{\alpha 1}$-acetyl-α-MSH) or ACTH 1—10 (Fig. 3). It is noteworthy that large, basic peptides such as ACTH 1—24 and ACTH 1—39 tend to stick to reversed-phase μBondapak C_{18} columns during elution with mixtures of 0.01 *M* ammonium acetate and methanol, resulting in peak broadening and peak tailing (data not shown). In contrast, using the ion-pairing reagent TFA in the mobile phase, sharp peaks without deterioration of peak shape can be obtained for ACTH 1—24 and ACTH 1—39 (Fig. 2).

The resolution of an ACTH 4—9 analogue (ORG 2766) and its fragments on μBondapak C_{18} with a linear gradient of 0.01 *M* ammonium acetate (pH 4.2) and methanol, is depicted in Fig. 4. The fragments investigated represent peptide intermediates that could arise as radioactive metabolites following enzymatic degradation of [^{3}H-*Phe*^{7}]ORG 2766 [19]. The chromatogram shows excellent separation between these oligopeptides ranging in size from two to seven amino acid residues, sharp and symmetric peptide peaks, and retention times correlating with their chain lengths. Resolution was not achieved between the dipeptides His^6-Phe^7 and Phe^7-D-Lys^8 and between the N-terminal sequences 4—7 and 4—8. Using ammonium acetate in the mobile phase, the separation profiles of the ACTH peptides investigated were affected neither by differences in the amounts of peptide injected nor by differences in injection volumes, similar to the findings observed with the TFA system. An attendant advantage of the ammonium acetate system appeared to be that the peptide peak shapes and retention times hardly changed with increasing column lifetime, in contrast to the results obtained with TFA in the mobile phase. In the ammonium acetate system used, the addition of acetic acid to the organic solvent leads to more stable baseline recordings (compare Fig. 3 with Fig. 4). For gradient elution with the TFA system baseline flattening can be achieved by adding TFA to methanol (3.5 ml/l), although gradual increases of the gradient baseline during

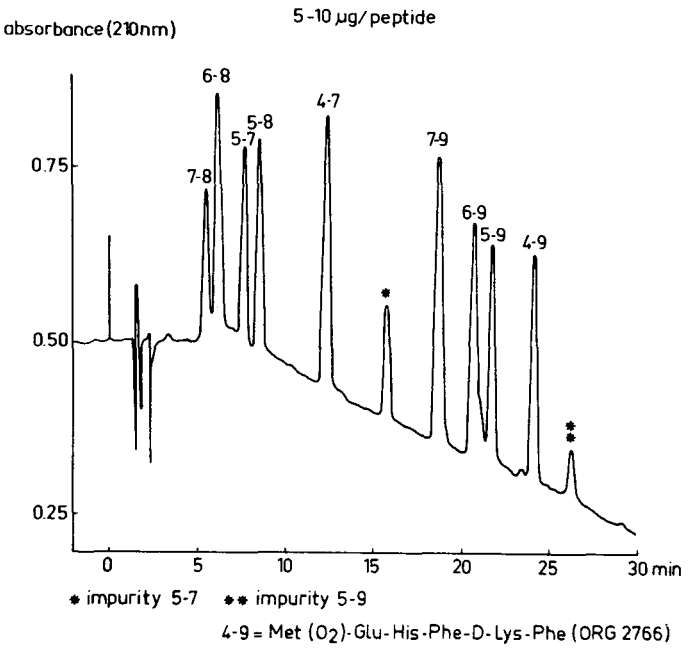

Fig. 4. Chromatography of a mixture of an ACTH 4—9 analogue (ORG 2766) and various fragments (5—10 µg of each peptide) on µBondapak C_{18}. Elution was performed with a 30-min linear gradient of 0.01 *M* ammonium acetate, pH 4.2 (X) and methanol (Y). Initial conditions, X/Y = 95:5; final conditions, X/Y = 65:35. The flow-rate was 2 ml/min. UV absorbance was measured at 210 nm (1.0 a.u.f.s.). The abbreviations refer to Met(O_2)-Glu-His-Phe-D-Lys-Phe = 4—9 (ORG 2766) as the basic structure.

the day have been noticed. Neither the resolution nor the retention times of the peptides studied were influenced by the presence of acetic acid or TFA in the organic solvent.

CONCLUSIONS

The data reported here demonstrate that reversed-phase HPLC employing either TFA or ammonium acetate in the mobile phase provides suitable resolution of ACTH-related neuropeptides. Both TFA and ammonium acetate are easily removed by sample evaporation or lyophilization, allowing good peptide recoveries. Elution systems containing the ion-pairing reagent TFA are particularly successful for the separation of larger and basic peptides such as ACTH 1—24 and ACTH 1—39, which appear to tail in ammonium acetate systems. On the other hand, using ammonium acetate buffers, high resolution can be obtained for ACTH-related oligopeptides, as has been shown for various N-terminal ACTH fragments as well as for an ACTH 4—9 analogue and its shorter fragments.

Reversed-phase HPLC with both TFA and ammonium acetate buffer systems is presently being applied to studies of ACTH 1—39 degradation by membrane preparations from rat brain. In addition, the metabolic fate of [^{3}H-*Phe*^{7}]ORG

2766 in rat plasma is under investigation using ammonium acetate in the mobile phase.

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Note

Assay of dopamine β-hydroxylase in human serum as a modification of the assay for the enzyme in rat serum by high-performance liquid chromatography

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Dopamine β-hydroxylase (DBH; 3,4-dihydroxyphenylethylamine, ascorbate : oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] catalyzes the β-hydroxylation of dopamine, the last step in the biosynthesis of norepinephrine. The enzyme is released with catecholamines into the blood stream from the peripheral sympathetic nerve endings [1], and its activity in human serum may reflect the degree of diseases accompanying changes in the level of serum DBH activity, such as hypertension and renal and neurological diseases [2], and has therefore drawn much attention from clinical and biomedical investigators.

We reported recently a sensitive method for the assay of DBH in rat serum based on the following principle. Octopamine formed enzymatically from the substrate tyramine, is separated by Dowex 50W—X4 column chromatography and oxidized with periodate to *p*-hydroxybenzaldehyde, which is then converted into a fluorescent compound with 2,2′-dithiobis(1-aminonaphthalene) (DTAN). The compound, after extraction with *n*-hexane—chloroform, is separated by normal-phase high-performance liquid chromatography (HPLC) on LiChrosorb Alox T with fluorescence detection using *n*-hexane—chloroform containing a small amount of acetic acid as mobile phase [3]. DBH activity in human serum is much higher than that in rat serum; and the optimal conditions of the reaction with the enzyme in human serum were found to be slightly different from those with the enzyme in rat serum. Thus, the reported DBH assay method has been applied to the assay of the enzyme in a minute of human serum with some modifications.

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EXPERIMENTAL

Reagents, materials and apparatus

All chemicals, water, DTAN solution, Dowex 50W—X4 column, LiChrosorb Alox T column for HPLC, high-performance liquid chromatograph, fluorescence detector and mobile phase for HPLC were the same as those used previously [3]. The chromatograph and the detector were operated in the same way as described earlier [3].

Human blood was obtained from normal volunteers (22—51 years of age). Serum was obtained by centrifugation of the blood at 1000 *g* for 10 min at 5°C. The DBH activity in serum is stable for more than one month when stored at −20°C.

Procedure

Substrate—cofactor solution comprised 10 μl each of 80 m*M* ascorbic acid, 0.3 *M* sodium fumarate, 20 μ*M* cupric sulfate and 10,000 units/ml catalase, and 20 μl each of 0.2 *M* tyramine hydrochloride, 0.15 *M* N-ethylmaleimide and 2.0 *M* acetate buffer (pH 5.0). The solution (100 μl) was placed in a 10-ml centrifuge tube containing 100 μl of water and 0.1—2 μl of serum (the sample size of 2 μl is recommended for precise sampling). The mixture was incubated at 37°C for 10 min with continual shaking. After adding 1.0 ml of 0.6 *M* trichloroacetic acid, the mixture was centrifuged at 1000 *g* for 10 min. The supernatant (1.0 ml) was poured on to a Dowex 50W—X4 column. The column was washed with three 2.0-ml portions of water, and the adsorbed amines (octopamine and tyramine) were eluted with 1.0 ml of 3 *M* ammonium hydroxide. To the eluate chilled at 0°C, 100 μl of 0.15% (w/v) sodium periodate were added. After the addition of 100 μl of 0.6% (w/v) sodium sulfite to decompose the excess periodate, the mixture was netralized with 0.3 ml of 5 *M* sulfuric acid. To the resulting solution, 2.0 ml of DTAN solution and 0.5 ml each of 0.05% (w/v) sodium sulfite and 15% (w/v) sodium hypophosphite pentahydrate were successively added. The mixture was allowed to stand at 37°C for 30 min. 2-Mercaptoethanol solution (20%, w/v; 0.5 ml) was added to stop the reaction. To the reaction mixture, 1.0 ml of *n*-hexane—chloroform (7:3, v/v) was added and the fluorescent compound was extracted with shaking for 10 min. A 20-μl volume of the upper organic layer was injected into the chromatograph. The organic layer could be used for more than 24 h when stored in the dark.

For the blank, 0.1—2 μl of serum were replaced with water and the same procedure was carried out. For the calibration curve, 100 μl of water in the procedure were replaced with 100 μl of octopamine standard solution (0.1—10 nmol per 100 μl) and the same procedure as for the blank was carried out.

The peak height in the chromatogram was used for the quantitation of octopamine.

RESULTS AND DISCUSSION

A maximum and constant activity of DBH was achieved in the presence of 2—5 m*M* ascorbic acid in the incubation mixture with observed K_m value at 0.6 m*M* and the acid at concentrations greater than 5 m*M* caused marked

inhibition of the enzyme; 4 m*M* ascorbic acid was therefore used. The amount of octopamine formed correlated well with the incubation time for the first 10 min, and then the correlation deviated slightly (Fig. 1); a 10-min incubation was thus used in the procedure. The other conditions for the enzyme reaction are optimal, and are identical to those used in the assay of DBH in rat serum [3].

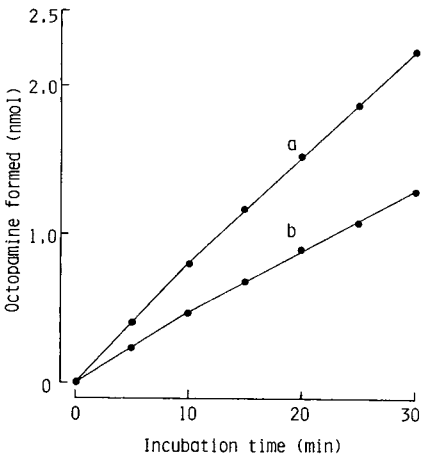

Fig. 1. Effect of incubation time on the amount of octopamine formed. Portions (2 μl) of serum were treated according to the procedure. DBH activity: (a) 40.4 and (b) 23.7 μmol of octopamine per min per liter of serum.

The calibration curve was linear. The amount of octopamine formed was proportional to the sample size of serum up to at least 5 μl. The pattern of the chromatograms obtained using the procedure was almost similar to that observed with the chromatograms for rat serum [3]; the retention times for the blank (DTAN) and octopamine were 0.8 and 3.0 min, respectively.

The recovery of octopamine added to the enzyme reaction mixture in the amounts of 0.5 and 2.0 nmol was 97 ± 2.5% (mean ± S.D., n=10 in each case). The lower limit of detection for octopamine formed enzymatically was 4 pmol per assay tube (corresponding to a DBH activity of 0.2 μmol of octopamine per min per liter of serum). This sensitivity is much higher than that obtained with the fluorimetric [4,5] and reversed-phase HPLC methods [6—8], and permits the assay of DBH in only 0.1 μl of human serum if the sampling is performed precisely, or using a water-diluted sample.

The precision of the method (serum sample of 2 μl) was established with respect to repeatability. The coefficient of variation was 3.6% for mean activity of 33.3 μmol of octopamine per min per liter of serum (n=10). DBH activities in normal sera assayed by the present method were 33.7 ± 12.5 μmol of octopamine per min per liter of serum (mean ± S.D., n=20). The data are in agreement with some previously published results [9,10].

This method is sensitive, precise and rapid, and should be useful in routine assays and in cases where only an extremely small amount of serum is obtainable.

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Note

Determination of phenylethanolamine N-methyltransferase activity in rat brain by high-performance liquid chromatography with fluorometric detection

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Phenylethanolamine N-methyltransferase (PNMT, E.C. 2.1.1.28) catalyzes the formation of adrenaline (AD) from noradrenaline (NA). This enzyme is highly localized in adrenal medulla [1, 2], but can also be detected in specific brain regions of rats by an immunohistochemical method [3] and by highly sensitive radioassay [4]. The properties of PNMT from rat brain and adrenals, and those from rat, cat and chicken brains were compared [5, 6]. All of these previous investigations on brain PNMT have been carried out by the use of radioassay.

High-performance liquid chromatography with electrochemical detection (HPLC—ElCD) provides a rapid, sensitive and accurate technique for measuring PNMT activity. Borchard et al. [7] first reported an HPLC-ElCD assay for PNMT activity using cation-exchange resin as a column support. We have also reported a highly sensitive assay for PNMT activity by high-performance reversed-phase paired-ion chromatography with electrochemical detection [8].

AD can also be measured by HPLC with fluorescence detection (FD) by the post-column trihydroxyindole reaction [9]. In this paper, we describe a highly sensitive and specific assay for PNMT activity by high-performance reversed-phase paired-ion chromatography with trihydroxyindole fluorescence detection. The reversed-phase paired-ion chromatography permitted complete separation of AD from excess NA as substrate, which cannot be achieved by cation-exchange column chromatography. We used α-methylnoradrenaline (α-methyl-NA) as an internal standard in this HPLC—FD method. Although the post-column trihydroxyindole reaction is required, this HPLC—FD method is more specific and sensitive than the HPLC—ElCD method.

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EXPERIMENTAL

Materials

L-NA bitartrate and pargyline·HCl (N-methyl-N-benzyl-2-propynylamine) were obtained from Sigma (St. Louis, MO, U.S.A.), S-adenosyl-L-methionine hydrogen sulfate (SAM) was from Boehringer (Mannheim, G.F.R.) and sodium N-heptanesulfonate (SHS) from Chromato Research (Sagamihara, Japan). α-Methyl-NA was a kind gift from Dr. J. Daly (NIH, Bethesda, MD, U.S.A.). Commercially available L-NA was kindly purified by Dr. T. Katasawa (Dainippon Pharmaceutical Co., Osaka, Japan) to remove the contaminated AD according to the method of Tullar [10] by repeated recrystallization of L-NA bitartrate. All other chemicals were of analytical grade.

Apparatus

The chromatograph used was a Shimadzu LC-3A with a trihydroxyindole reaction system and a RF-500 spectrofluorometer (Shimadzu, Kyoto, Japan). A column (30 cm × 0.4 cm I.D.) packed with Nucleosil 7 C_{18} (particle size 7.5 μm; Macherey, Nagel & Co., Düren, G.F.R.) was used for HPLC.

Procedure

Rats were killed by decapitation, the brains were removed within 30 sec and dissected on a glass plate over ice. The brain tissues were immediately homogenized in 5 volumes of 0.32 *M* sucrose by a tissue sonicator (Ohtake Works, Tokyo, Japan).

The standard incubation mixture for measurement of PNMT activity consisted of the following components in a total volume of 250 μl (final concentrations in parentheses); 10 μl of 0.01 *M* pargyline (a monoamine oxidase inhibitor) in 0.01 *M* hydrochloric acid (0.4 m*M*), 50 μl of 0.5 *M* Tris—HCl buffer, pH 8.0 (0.1 *M*), 15 μl of 0.3 m*M* SAM (18 μ*M*), 20 μl of 0.2 m*M* NA (16 μ*M*), 50 μl of brain homogenate as the enzyme, and water to make up a total volume of 250 μl. The blank reaction mixture contained no enzyme or boiled (90°C for 5 min) enzyme. An amount of 15 pmol of AD was added to another blank incubation as a standard.

Incubation was carried out at 37°C for 60 min and the reaction was stopped by the addition of 600 μl of 0.42 *M* perchloric acid containing 1.55 mg of disodium EDTA, 3.12 mg of $Na_2S_2O_5$, and 10—30 pmol of α-methyl-NA as an internal standard. After stopping the reaction, the enzyme was added to the no-enzyme blank incubation. After 10 min in an ice bath, 200 μl of 0.8 *M* potassium carbonate were added to remove excess of perchloric acid, and 1 ml of 0.5 *M* Tris—HCl buffer (pH 8.5) was added to adjust the pH to 8.0—8.5. The mixture was centrifuged at 1600 *g* for 10 min at 4°C. The clear supernatant was passed through a column (0.4 cm I.D.) containing 100 mg of acid-washed aluminium oxide. The column was washed with 2 ml of 0.05 *M* Tris—HCl buffer (pH 8.5) containing 1% EDTA, 5 ml of water, and 100 μl of 0.5 *M* hydrochloric acid. All these washing solutions were cooled in ice before use.

Adsorbed NA, AD and α-methyl-NA were eluted with 200 μl of 0.5 *M* hydrochloric acid. A 50-μl aliquot of the eluate was injected into the chromatograph. The mobile phase was 0.05 *M* sodium phosphate buffer (pH 2.6),

containing 2 m*M* SHS and 1% of acetonitrile with a flow-rate of 0.8 ml/min, at room temperature. To this column effluent, the reagents for trihydroxyindole reaction — (1) 0.2 *M* potassium phosphate buffer (pH 6.5) containing 0.05% $K_3Fe(CN)_6$ and Brij 35 (0.002 mg/ml), (2) 0.05% ascorbic acid and 0.05% $Na_2S_2O_5$, and (3) 5 *M* sodium hydroxide — were pumped sequentially at a constant flow-rate of 0.3 ml/min using the proportioning pump. Finally the fluorescent products were detected fluorometrically with activation and emission maxima of 410 nm and 510 nm (uncorrected), respectively. Under these conditions, the retention times were: solvent front, 9.2 min; NA, 16.3 min; AD, 22.0 min; and α-methyl-NA, 27.0 min.

The AD formed enzymatically by PNMT was calculated by the equation:

$$\frac{R(\mathrm{E}) - R(\mathrm{B})}{R(\mathrm{B}+\mathrm{S}) - R(\mathrm{B})} \times 15 \text{ (pmol)}$$

where R is the ratio of peak heights (peak height of AD/peak height of α-methyl-NA), R(E) being that from the enzyme incubation, R(B) from the no-enzyme or boiled-enzyme incubation (blank), and R(B + S) from the no-enzyme or boiled-enzyme plus 15 pmol AD incubation (standard).

For comparison with the HPLC—ElCD method, 50 μl of the eluate from the aluminium oxide column were injected into a liquid chromatograph (Yanaco L-2000) with an Yanaco VMD-101 electrochemical detector (Yanagimoto Co., Kyoto, Japan) and a column (25 cm × 0.4 cm I.D.) packed with Nucleosil 7 C_{18} according to the method of Trocewicz et al. [8]. The mobile phase was 0.1 *M* sodium phosphate buffer (pH 2.6) containing 5 m*M* sodium pentanesulfonate and 0.5% acetonitrile (v/v) at a flow-rate of 0.9 ml/min, and the detector potential was set at 0.6 V against a Ag/AgCl electrode.

RESULTS AND DISCUSSION

The peak height of AD showed a linear relationship with AD injected from 0.1 pmol to 100 pmol; $y = 0.87x$, where y is the relative fluorescence intensity and x is the concentration of standard adrenaline (pmol). The HPLC—FD assay for PNMT activity was developed using homogenate of rat pons plus medulla oblongata as the enzyme. Pargyline (0.4 m*M*) in the incubation mixture protected the NA substrate and enzymatically formed AD from oxidation by monoamine oxidase. The chromatographic pattern of the PNMT reaction with a homogenate of rat pons plus medulla oblongata is shown in Fig. 1. Enzymatically formed AD with 10 mg of rat pons plus medulla oblongata as enzyme during 60 min of incubation at 37°C (Fig. 1A) showed a significant increase of the peak height of AD as compared with a small amount of AD in a no-enzyme blank incubation (Fig. 1B). Fig. 1C shows blank incubation with 15 pmol of AD as a standard. In this chromatogram, only the peaks of solvent front, NA, AD, and α-methyl-NA appeared, and we could not observe any unknown peaks of interfering substances as in the HPLC—ElCD method [8]. Although time of the assay in this HPLC—FD method was longer than that in the HPLC—ElCD method due to the time for the trihydroxyindole reaction,

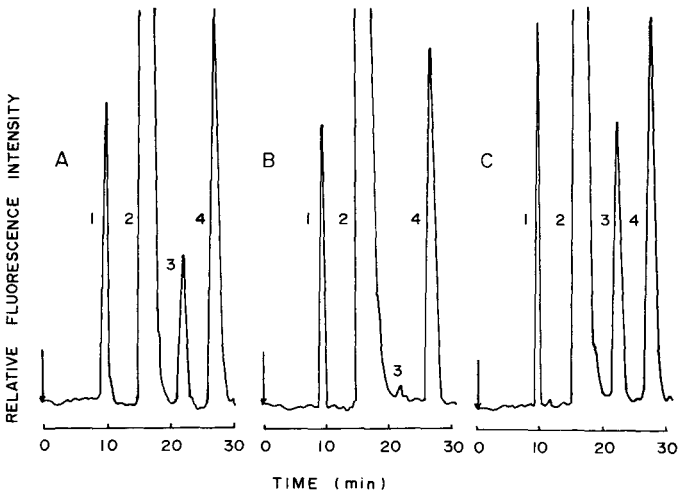
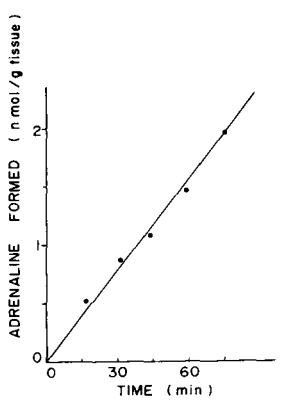


Fig. 1. Typical elution patterns of phenylethanolamine N-methyltransferase incubation mixtures with a homogenate of rat pons plus medulla oblongata as enzyme. The conditions are described in the *Procedure* section. (A) Incubation with a homogenate of 10 mg of rat pons plus medulla oblongata, 1 h incubation at 37°C. (B) Blank incubation without enzyme. (C) Another blank incubation with 15 pmol of adrenaline added as standard. Peaks: 1 = solvent front; 2 = noradrenaline; 3 = adrenaline; 4 = α-methylnoradrenaline.



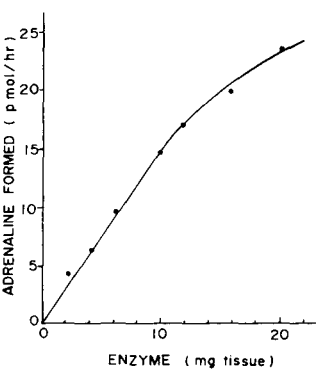


Fig. 2. Rate of adrenaline formation using a homogenate of rat pons plus medulla oblongata as enzyme at 37°C. Standard incubation system containing 10 mg of tissue was used as described in the *Procedure* section.

Fig. 3. Rate of adrenaline formation by rat brain phenylethanolamine N-methyltransferase as a function of tissue concentration. A homogenate of rat pons plus medulla oblongata was used as enzyme. Incubations were carried out for 1 h at 37°C as described in the *Procedure* section.

this assay method is more specific for catecholamines as compared with the HPLC—ElCD method.

The rate of AD formation using a homogenate of rat pons plus medulla oblongata as enzyme proceeded linearly for 75 min at 37°C, as shown in Fig. 2. A complete linearity was also observed between the amounts of ho-

mogenate from 2 mg to 10 mg tissue and enzymatically formed AD during 1 h incubation at 37°C (Fig. 3). Optimum pH was about 8.0, and this value is similar to those reported by Connet and Kirshner [11] and Yu [5]. The reproducibility of the assay with replicates of the same sample was 100 ± 10% (S.D. for five determinations). The sensitivity was 0.2 pmol AD formed enzymatically, and was higher than that of HPLC—ElCD method [8]. This blank value is mainly due to endogenous AD and contaminated AD in the NA used as substrate. Therefore, higher purification of the NA substrate to remove contaminated AD is necessary to increase the sensitivity.

We also studied kinetic properties of brain PNMT using a homogenate of pons plus medulla oblongata. The apparent K_m values calculated from the Lineweaver—Burk plots [12] by Wilkinson's program [13] were 16 ± 3 μM toward NA and 9 ± 1 μM toward SAM. These K_m values towards NA and SAM of rat brain PNMT agreed with those reported by Fuller and Hemrick [6] and with our values on the enzyme in human cerebral cortex [14].

We also found PNMT activity in rat bulbus olfactorius. Using both the HPLC—FD and HPLC—ElCD methods, we measured PNMT activity in rat bulbus olfactorius from five animals. The activities obtained by this HPLC—FD method and the HPLC—ElCD method were 363 ± 32 and 364 ± 32 pmol/h per g tissue (mean ± S.D.), respectively. The activity was comparable to that of septum or lower brain stem [8]. This suggests the presence of adrenergic terminals in rat bulbus olfactorius.

The present HPLC—FD method is the most sensitive and specific among various PNMT assay methods. Only one drawback is that it is somewhat time consuming, but this problem can be solved by using an auto-sampler for the HPLC—FD system, which can be completely automated. Therefore, this method may be useful for physiological and pharmacological studies. Vogel et al. [15] and Hobel et al. [16] reported the presence of PNMT activity in human plasma, and the activity was found to be low. This method has been found to be applicable to the assay of PNMT activity in human plasma, and therefore it may also be useful in clinical studies.

ACKNOWLEDGEMENTS

We wish to thank Shimadzu Manufacturing Co. (Kyoto, Japan) for their expert assistance in the mechanical aspects of the HPLC—FD apparatus, Dr. T. Karasawa (Dainippon Pharmaceutical Co., Osaka, Japan) for purifying commercially available samples of NA to remove contaminated AD, and Dr. J. Daly (NIH, Bethesda, MD, U.S.A.) for his kind gift of α-methyl-NA.

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Note

Enhanced high-performance liquid chromatographic resolution of hemoglobin A_{1c} at low temperatures

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Hemoglobin A_{1c} is a glycosylated hemoglobin which shows elevated levels in the hemolysates of uncontrolled diabetics [1,2], and provides a means for monitoring patient compliance.

Schnek and Schroeder [3] devised a liquid chromatographic method which was further developed by Trivelli et al. [4] and Schwartz et al. [5]. These methods had the disadvantage of being time-consuming. Faster high-performance liquid chromatographic methods (HPLC) were developed by Davis et al. [6], Cole et al. [7] and Dunn et al. [8]. While these methods are fast and provide adequate separation, they suffer from a relatively short column life; they employ minus 400 mesh Bio-Rex 70 packed in glass columns and operate at room temperature. On repeated use, column operating pressure would rise and cause either destruction of the resin or bursting of the columns. Use of coarser Bio-Rex 70 in HPLC columns at room temperature results in poorer resolution.

Our laboratory has modified the HPLC method of Davis et al. by utilizing an optimum of 200—400 mesh Bio-Rex 70 resin at column temperatures approaching 0°C. The coarse resin has prolonged the lifetime of the columns without any significant rise in the operating pressure. The low column temperature provided an excellent resolution of hemoglobin A_{1c} with a coarse resin which would otherwise give poor performance at elevated temperatures.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph consisted of two Model 110 A

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pumps, a Model 421 controller, an Hitachi 100-40 spectrophotometer with an Altex flow cell (Beckman, Fullerton, CA, U.S.A.). The output was displayed on an Altex Model C-RIA.

Temperature control of the water-jacketed glass column was achieved using a thermostatically controlled circulation pump, Polytemp Model 80 (Polyscience, Niles, IL, U.S.A.).

Reagents

Reagent grade $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, and KCN (Fisher Scientific, Springfield, NJ, U.S.A.) were used to prepare low ionic strength buffer: 4.55 g of $NaH_2PO_4 \cdot H_2O$, 1.18 of g Na_2HPO_4 and 0.64 g of KCN were dissolved in distilled water and brought up to a total volume of 1 liter. The pH was then adjusted to 6.7 at 22°C.

The high ionic strength buffer of Trivelli et al. [4] was prepared by dissolving 14.35 g of $NaH_2PO_4 \cdot H_2O$ and 6.52 g of Na_2HPO_4 in water and diluting to 1 liter. The pH was then adjusted to 6.4.

Column

A jacketed 1-cm I.D. Altex glass column (Beckman) was packed with 200—400 mesh Bio-Rex 70 (Bio-Rad Labs., Richmond, CA, U.S.A.) replacing the minus 400 mesh resin used by Davis et al. Resin height was between 8 and 9 cm. The columns were conditioned by eluting a sample of hemolyzed blood through the column and storing the column overnight, filled with high ionic strength buffer. Reconditioning of the column was carried out on each day of use.

Preparation of sample

Red blood cells were separated from plasma, washed twice in saline, and hemolyzed with 20 vol of distilled water. The hemolyzate was centrifuged for 20 min at 5000 *g* and the supernate analyzed by HPLC.

Assay

After conditioning the column, low ionic strength buffer was pumped through the column at 2 ml/min for 10 min prior to sample injection and continued until hemoglobin A_{lc} had been eluted. This was followed by pumping high ionic strength buffer at the same rate to elute unglycosylated hemoglobin. Since the retention time for hemoglobin A_{lc} varied with temperature, the shift from low to high ionic strength buffer was conducted at varying times. Experiments were performed on the day after fresh columns were packed. Since retention times and theoretical plates varied slightly from day to day, smooth curves could not be obtained from observations taken several days apart.

RESULTS AND DISCUSSION

The use of 200—400 mesh Bio-Rex 70 eliminated pressure buildup and column rupture experienced with minus 400 mesh resin. Our column operates consistently at 100—200 p.s.i. Lowering the column temperature from 37.5°C to 0°C also brought about partial separation of hemoglobins A_{1a} and A_{1b} which became more pronounced at lower temperatures (Fig. 1A and B) and effected

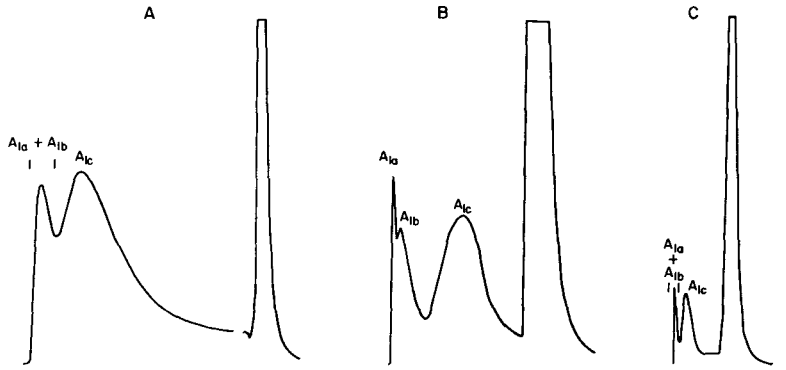

Fig. 1. Separation of hemoglobins A_{1a}, A_{1b} and A_{1c} at: (A) 37.5°C, utilizing column packed with 200—400 mesh Bio-Rex 70; (B) 0°C, utilizing column packed with 200—400 mesh Bio-Rex 70; (C) room temperature, utilizing column packed with minus 400 mesh Bio-Rex 70.

resolution of hemoglobin A_{1c} comparable to that obtained with another column packed with minus 400 mesh and used at room temperature (Fig. 1C).

Lowering the temperature of the column packed with 200—400 mesh resin also increased the retention time for hemoglobin A_{1c} from 3 to 28 min (Fig. 2). Theoretical plate numbers were calculated by measuring peak half-widths for hemoglobin A_{1c}. It was found that plate number increased sharply as the jacket temperature approached 0°C (Fig. 3). Although peak widths increased at low temperatures, longer retention times offset this negative effect as the temperature dropped. A minimum number of theoretical plates is observed at approximately 20°C in this study; two earlier studies with freshly packed columns also yielded minima in this region.

Preliminary results indicated normals average 5.65 + 0.7% of hemoglobin

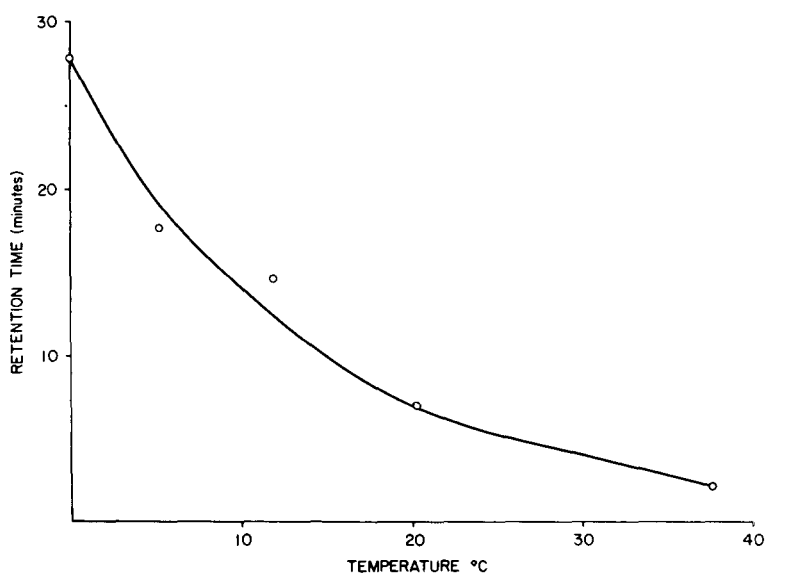

Fig. 2. Retention time of hemoglobin A_{1c} versus temperature.

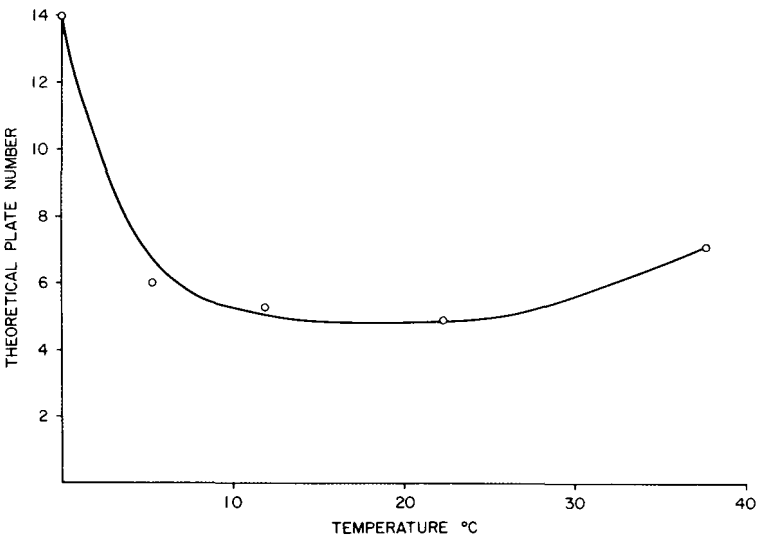

Fig. 3. Theoretical plate number versus temperature. Peak half-widths of hemoglobin A_{1c} were used to calculate plate numbers.

A_{1c} and poorly controlled diabetics average 12.4 + 0.9%. This column provides good resolution of hemoglobin A_{1c} while avoiding the high pressures associated with minus 400 mesh resin which may burst the glass column.

On the basis of our present results we make the following recommendations for the assay of hemoglobin A_{1c}: (1) In order to maintain a reliable long-lived column, we recommend substitution of 200—400 mesh Bio-Rex 70 for the minus 400 mesh resin used by Davis et al. [6]. (2) We recommend a low column temperature of 0°C in order to obtain adequate separation of hemoglobin A_{1c} with this column. This temperature may be achieved easily by immersing the column in a mixture of water and crushed ice.

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Note

Determination of coproporphyrin I and III isomers by high-performance liquid chromatography

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The coproporphyrin (CP) III is led to heme in the biosynthetic pathway, but CP I is not involved in the metabolic pathway. Therefore, a deficiency in the metabolism may be observed from the analysis of CP I and CP III.

Previously published methods for the CP isomers were a paper chromatographic method [1] and thin-layer chromatographic methods [2, 3]. But these published methods present both problems of precision and are time consuming. Battersby et al. [4] reported the separation of CP isomers (I, II, III, IV) using a high-performance liquid chromatographic (HPLC) method, but the method required a recycling system for separation of all the isomers. Recently, Englert et al. [5] reported a direct method for the isolation and measurement of porphyrins in biological samples using HPLC. Unfortunately, the method was intricate; we also examined a direct method for the determination of CP isomers in urine, but were not able to obtain adequate results. Therefore, we investigated an HPLC combined solvent extraction method, and obtained good results.

In this paper, a simple HPLC method for the separation of CP I and CP III is described. The chromatographic analysis was realized in less than 20 min without the need for temperature programming.

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EXPERIMENTAL

Reagents

Free coproporphyrin I and coproporphyrin III tetramethyl ester were purchased from Sigma (St. Louis, MO, U.S.A.). Coproporphyrin I (5 μg per vial) was dissolved in 5 ml of 0.1 *N* hydrochloric acid. Coproporphyrin III tetramethyl ester was hydrolyzed in 7 *N* hydrochloric acid overnight, and diluted with water until a concentration of 0.1 *N* hydrochloric acid was obtained; the concentration of CP III was calculated from the molecular extinction [6].

Acetonitrile was purchased from Wako Chemical (Tokyo, Japan) and all other reagents were reagent grade.

Instrumentation

The liquid chromatographic system consisted of the following components: a Model 635 High-performance liquid chromatograph (Hitachi, Tokyo, Japan); a Hitachi 3053 reversed-phase column 5 μm (150 mm × 4 mm); a Model 204S fluorescence detector (Hitachi); and a Hitachi Model 056 recorder.

Procedure

The chromatographic procedure was as follows: for the mobile phase 5 ml of acetic acid and 0.25 g of potassium dihydrogen phosphate were dissolved in water and made up to 500 ml with water. Then, 500 ml of acetonitrile were added. The solvents were degassed in an ultrasonic bath under reduced pressure (water pump) before use. The flow-rate was 1.3 ml/min. The excitation wavelength of the xenon lamp was 392 nm and the emission wavelength was measured at 610 nm. The chart speed of the dual-channel recorder was 0.5 cm/min. All chromatographic separations were performed at room temperature. The volume of the samples injected into the column was 10 μl.

Urine analysis

The method was applied to the analysis of urine levels in ten healthy volunteers. Urine (10 ml) was placed in a separating funnel, acidified with 2 ml of acetic acid and extracted with 20 ml diethyl ether until the aqueous phase showed no fluorescence under UV light. The extracts were combined and washed once with a little water. The extract was evaporated to dryness using a rotary evaporater and the CP I and CP III isomers in the residue were dissolved in 0.5 ml of 0.1 *N* hydrochloric acid. Then the 0.1 *N* hydrochloric acid solution was filtered through a membrane filter (Sartorius 0.45 μm).

RESULTS AND DISCUSSION

Fig. 1 shows the effect of acetonitrile concentration in the mobile phase on the separation of CP I and CP III. On decreasing the acetonitrile concentration, the retention times increased, and other variables were kept constant. A concentration of 50% (v/v) acetonitrile was chosen for the determination of CP I and CP III. The effect of acetic acid concentration on the separation was studied at different concentrations of 0, 0.25, 0.5, 1.0 and 4.0% (v/v).

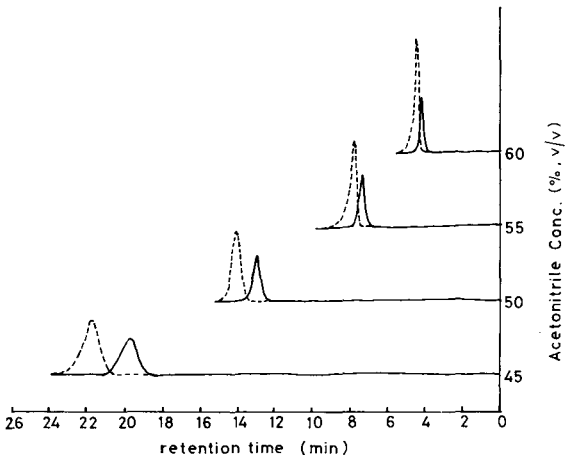

Fig. 1. Effect of acetonitrile concentration in the mobile phase on the separation and retention times of the CP isomers. Solid line: CP I; dotted line: CP III. Stationary phase: Hitachi Gel 3053 (150 × 4 mm), mobile phase: see Experimental. Flow-rate: 1.3 ml/min. Fluorophotometer: excitation wavelength 392 nm, emission wavelength 610 nm.

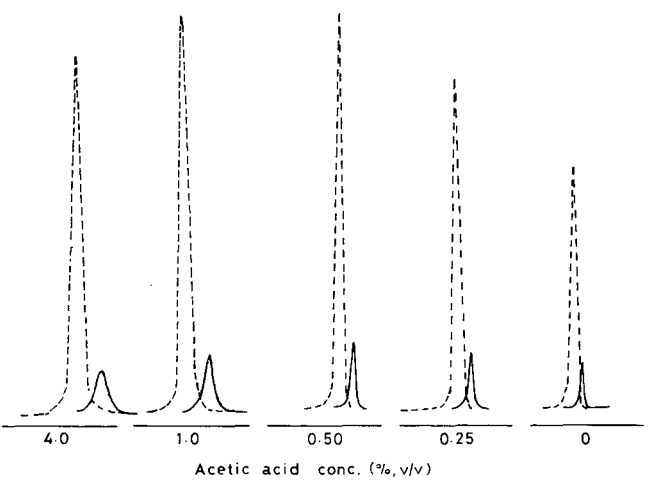

Fig. 2. Effect of acetic acid concentration in the mobile phase on the separation and retention times of the CP isomers. Solid line: CP I, dotted line: CP III. Potassium dihydrogen phosphate 0.05% (w/v) in acetonitrile—water (1:1) solution.

A good separation was obtained at a concentration of 0.5% (v/v) acetic acid (Fig. 2). Effect of the potassium dihydrogen phosphate concentration was studied at concentrations of 0, 0.025, 0.05, 0.1 and 0.2% (w/v) with both a 50% (v/v) solution of acetonitrile and 0.5% (v/v) solution of acetic acid. A good separation was obtained at a concentration of 0.025% (w/v) (Fig. 3). Therefore, to determine CP I and CP III, concentrations of 50% (v/v) acetonitrile, 0.5% (v/v) acetic acid and 0.025% (w/v) potassium dihydrogen phosphate were chosen for the mobile phase.

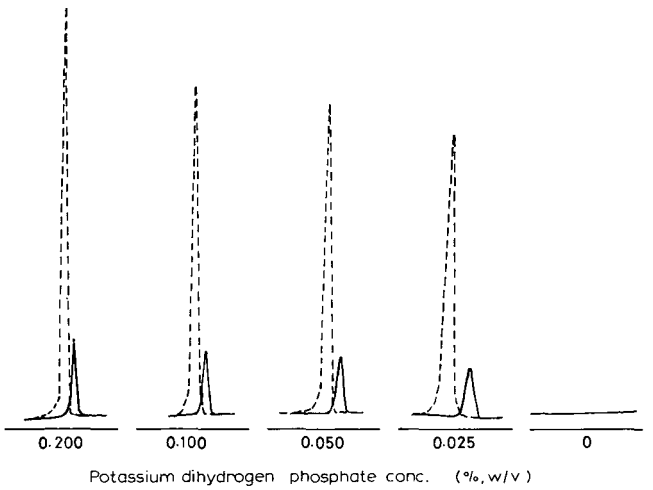

Fig. 3. Effect of potassium dihydrogen phosphate concentration in the mobile phase on the separation and retention times of the CP isomers. Solid line: CP I, dotted line: CP III. Acetic acid 0.05% in acetonitrile—water (1:1) solution.

Linearity

Under the optimal conditions, a good linear relationship between the peak heights and concentrations of CP I and CP III in the ranges 2—10 ng and 2.7—13.5 ng, respectively, is found. Sensitivity of both CP I and CP III in the described method was 0.5 ng.

Comparison of results using spectrophotometric and spectrofluorometric detection

The two detectors were connected in series directly after the column, first the photometer and secondly the fluorometer. Fluorometric detection depends on two parameters, the emission and the excitation wavelength, and yields a higher specificity than the VIS detection (Fig. 4).

Reproducibility

The reproducibilities of ten replicate analyses of CP I (200, 400, 1000 μg/l) and CP III (270, 540, 1350 μg/l) were examined. Coefficients of variation were 4.0, 2.7, 1.3% and 3.7, 2.4, 1.2%, respectively.

Application

The CP content in urine was calculated from peak heights, according to the following equation:

$$A = S \times \frac{PH_a}{PH_s} \times \frac{0.5}{0.01} \times \frac{V}{10} \times \frac{1}{1000}$$

where A = CP I or CP III concentration in the sample (μg/day); S = amount of the standard injected into column (ng); PH_a = peak height of CP I or CP III in the sample (mm); PH_s = peak height of the standard CP I or CP III (mm);

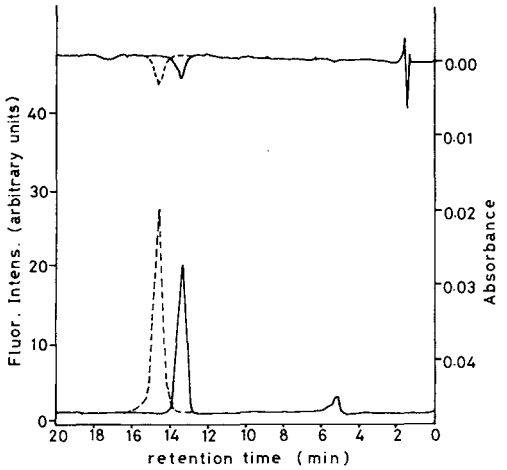

Fig. 4. Comparison of results using spectrophotometric and spectrofluorometric detection. Solid line: CP I, dotted line: CP III. Hitachi spectrophotometer 100-50 at 401 nm, 0.05 a.u.f.s.; Hitachi fluorometer 204-S, excitation wavelength 392 nm, emission wavelength 610 nm. Chromatographic conditions were as given in the text.

V = total volume of the sample (ml); 0.5 = final volume of the sample (ml); 10 = initial volume of urine (ml); 0.01 = volume of the sample injected into the column (ml); and $\frac{1}{1000}$ = conversion factor into micrograms.

Normal urine contains about 31 ± 15 μg per day CP I and about 72 ± 27 μg per day CP III. Total CP value obtained with the present method was in a good agreement with previous reports [7, 8]. CP III per total CP found in urine in ten normal individuals was 60—75%, which was in agreement with the value reported by Aziz et al. [9].

For recovery studies, distinct amounts (10 ng per 10 μl) of CP I were added to urine samples. The mean recovery of ten experiments was 85% for CP I. Thus this method might be expected to be useful in the routine analysis of CP I and CP III.

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Note

High-performance liquid chromatographic determination of nicotinic acid and its metabolites, nicotinuric acid and nicotinamide, in plasma

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Nicotinic acid has been used for the treatment of hyperlipemia. It is pharmacologically active at very low plasma concentrations of 0.1—0.4 μg/ml [1, 2]. Nevertheless, a large dose (1.5—6 g/day) of nicotinic acid is necessary to maintain the pharmacologically effective plasma concentration because of its rapid elimination from blood [3]. In order to prevent the rapid and excessive increase of plasma concentration and the subsequent side-effects, several prodrugs have been examined and used [4—7].

Though the plasma concentration of nicotinic acid is a significant index of its pharmacological effects and side-effects [1, 2], no sensitive, specific, precise, and accurate method by which such low effective concentrations can be determined has been developed. Carlson's method [8] has been widely used for monitoring plasma concentrations of nicotinic acid after administration of nicotinic acid and its prodrugs. This method is based on extraction of nicotinic acid from plasma followed by a colorimetric determination with the modified König reaction [9]. However, this method is not necessarily specific only for nicotinic acid, but also for nicotinamide and other metabolites to some extent. With this method, furthermore, a large amount of plasma (3 ml) is necessary for measuring low concentrations (less than 1 μg/ml nicotinic acid). Blank extinction equivalent to 0.5—0.8 μg/ml of nicotinic acid was obtained [1]. And this blank extinction is not ascribed to endogenous nicotinic acid or nico-

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tinamide because normal plasma contains only minute amounts of these (less than 0.05 μg/ml) [1, 10, 11].

Some other methods have been reported for the assay of nicotinic acid in plasma. With the microbiological assay, the total concentration of nitotinic acid and nicotinamide is determined [12]. Preliminary isolation of nicotinic acid by thin-layer chromatography followed by quantitative analysis is time-comsuming and losses are great [8]. The spectrophotometric assay developed by Diab [13] was not sensitive enough to assay low effective plasma concentrations of nicotinic acid. In this study, we developed a selective and sensitive high-performance liquid chromatographic (HPLC) assay method for nicotinic acid in plasma by cation-exchange chromatography.

EXPERIMENTAL

Materials

Nicotinic acid and nicotinuric acid were purchased from Kishida, Osaka, Japan, and Sigma, St. Louis, MO, U.S.A., respectively. Nicotinamide and quinaldic acid were from Wako Pure Chemicals, Osaka, Japan. All the chemicals were of reagent grade and used without further purification.

Procedures

One half milliliter of distilled water and 3 ml of acetone were added to 0.5 ml of plasma in a test tube, and agitated with a Vortex mixer. After centrifugation at 1500 *g* for 10 min, 3 ml of the supernate were transferred into a glass-stoppered test tube containing 3 ml of chloroform. The mixture was shaken for 5 min and then centrifuged at 1500 *g* for 5 min. One half milliliter of the aqueous layer was acidified with 0.1 ml of 0.1 *N* hydrochloric acid, and dried up with a centrifugal evaporator (Model RD-21, Yamato Scientific Co., Tokyo, Japan) at 60°C for 30 min. After addition of 200 μl of methanol to the residue, the solvent was evaporated in vacuo. Then, 200 μl of the internal standard (quinaldic acid) in acetone (4 μg/ml) were added. After centrifugation at 3500 *g* for 10 min, 150 μl of the supernate were taken to dryness in vacuo. The residue was dissolved in 50 μl of redistilled water and 20 μl of the solution were injected into the HPLC system. Plasma samples containing more than 2 μg/ml nicotinic acid were appropriately diluted with distilled water before being processed as above.

Chromatographic conditions

A liquid chromatograph (Hitachi 635A) equipped with a high-pressure sampling valve (635-0650, 1 μl to 2.0 ml) and multiwavelength UV detector (Hitachi 635M) was used. For the stationary phase, a cation-exchange column (Zipax SCX, 25—37 μm, 50 cm × 2.1 mm I.D., E.I. Du Pont de Nemours & Co., Wilmington, DE, U.S.A.) was used, and the column was warmed at 45°C using a constant-temperature water bath circulator. The mobile phase consisted of 0.02 *M* phosphate buffer solution [NaH_2PO_4—H_3PO_4, 65 : 35 (pH 2.6) and 78 : 22 (pH 2.8) for nicotinic acid assay and metabolites assay, respectively]. The flow-rate was 1.0 ml/min and the pressure was approximately 30 kg/cm^2.

The wavelength and absorbance units full scale were 260 nm and 0.005, respectively.

Calibration graph

Standard solutions containing 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 μg/ml nicotinic acid in distilled water were prepared. Instead of 0.5 ml of distilled water, 0.5 ml of each standard solution was added to 0.5 ml of drug-free plasma and the samples were processed as described above. The ratios of the peak height of nicotinic acid to that of quinaldic acid (internal standard) were used to construct a calibration graph.

Monitoring of plasma concentrations

The experiment was performed on a healthy female subject aged 27 years, weighing 47 kg. The basal blood sample was first taken after overnight fasting. Then 500 mg of nicotinic acid were administered orally with 100 ml of tap water. Blood samples were drawn through an indwelling venous catheter at 0.25, 0.5, 1, 2, 3, and 4 h. Food and beverages were restricted for 4 h after administration.

RESULTS AND DISCUSSION

Sample preparation

Since nicotinic acid is water-soluble at all pH values, it can not be extracted from aqueous phase by organic solvents. Therefore, it is not easy to remove the interfering endogenous substances in plasma. Deproteinization by acetone and subsequent chloroform extraction have been used to reduce such substances [8, 14]. However, the aqueous layer obtained by these procedures was not sufficiently cleaned-up for the HPLC analysis of nicotinic acid. For further clean-up, the aqueous layer was acidified to make the carboxyl group of nicotinic acid non-ionic in form and the residue was dissolved in acetone. Under this condition, the solubilities of the cationic endogenous substances which have an affinity for the cation-exchange column were restricted although nicotinic acid can be dissolved.

The steps of addition and subsequent removal of methanol before addition of the internal standard acetone solution are necessary. Without these steps, the recovery and reproducibility became poor. Although the mechanism is unknown, it is likely related to the dissolution rate of nicotinic acid in acetone.

Stationary phase

Recently, in place of ion exchange, an ion-pair reversed-phase system has been preferably used for the HPLC assay of ionic or ionizable compounds because of its high column efficiency [11,15—17]. Hengen et al. [14] described an assay method for nicotinic acid by ion-pair reversed-phase chromatography. However, we could not obtain a satisfactory result in respect of sensitivity and separation from plasma blank with this method. Cation-exchange chromatography was preferable to ion-pair reversed-phase chromatography in our study of assay of nicotinic acid in plasma. The mobile phase was examined according to the investigation by Williams et al. [18].

Selectivity

Fig. 1 shows the chromatogram of plasma sample spiked with 1 μg/ml nicotinic acid, 4 μg/ml nicotinuric acid, and 8 μg/ml nicotinamide compared to plasma blank. Nicotinic acid and internal standard were well separated from endogenous substances and metabolites (nicotinuric acid and nicotinamide). No unexpected metabolite interfered in the chromatogram obtained from plasma sample after oral administration of nicotinic acid.

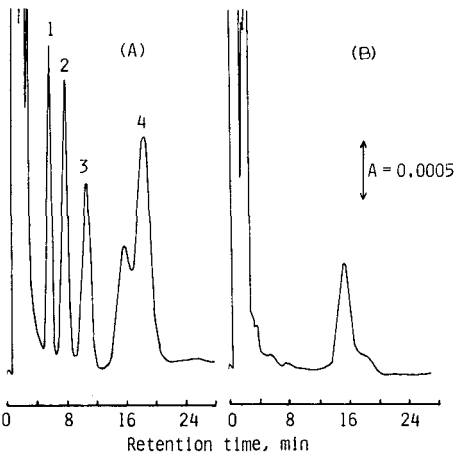

Fig. 1. High-performance liquid chromatograms of plasma sample spiked with 1 μg/ml nicotinic acid, 4 μg/ml nicotinuric acid, and 8 μg/ml nicotinamide (A), and of plasma blank (B). Mobile phase: NaH_2PO_4—H_3PO_4 (65 : 35). Peaks: 1 = nicotinic acid, 2 = internal standard, 3 = nicotinuric acid, 4 = nicotinamide.

The calibration curve of peak height ratio was linear with a correlation coefficient of 0.9997. The coefficient of variation at 0.5 μg/ml was 3.05% (n = 7).

The relative recovery of nicotinic acid from plasma sample containing 0.5 μg/ml was estimated in comparison with the assay of an aqueous solution, and 92 ± 2.8% (mean ± S.D., n = 7) was accounted for. Nicotinic acid was spiked by the same procedure as described for the calibration graph.

The basal value of nicotinic acid (endogenous nicotinic acid) in six healthy adults was not determinable (signal-to-noise ratio = 2—3). The signal-to-noise ratio of the peak height response of 0.1 μg/ml which was the lowest concentration in the calibration curve examined was approximately 10. In this experiment "not determinable" means less than 0.05 μg/ml, though this method is sensitive enough to detect less than 0.05 μg/ml.

Determination of metabolites

The assay method for two major metabolites of nicotinic acid, nicotinuric acid (glycine conjugate) and nicotinamide, in plasma was also investigated. They could be assayed simultaneously by using the same sample solution pre-

pared for the nicotinic acid assay by HPLC; the only change was in the mobile phase (Fig. 2). Standard solutions containing 0.5, 1, 2, 3, 4, and 5 μg/ml nicotinuric acid, and 1, 2, 4, 6, 8, and 10 μg/ml nicotinamide were prepared. The calibration graphs were constructed by the same method as that of nicotinic acid, and they were linear with correlation coefficients of 0.9992 and 0.9985 for nicotinuric acid and nicotinamide, respectively. As the procedure for sample preparation was developed focusing on nicotinic acid, it is not necessarily satisfactory for each metabolite. However, without an additional volume of plasma and newly prepared samples by the other methods, they can be assayed using the sample solution prepared for nicotinic acid assay under the same HPLC conditions except for the change in the mobile phase.

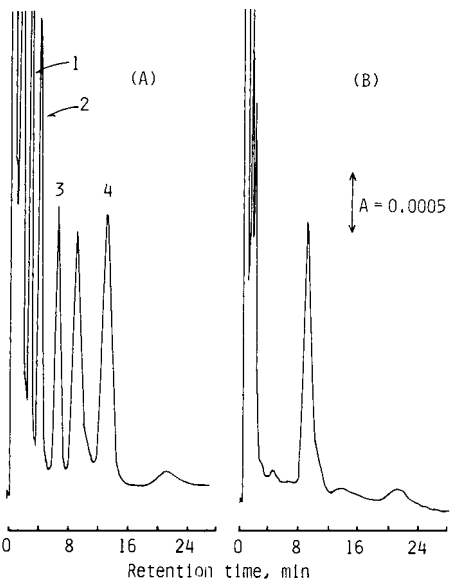

Fig. 2. High-performance liquid chromatograms of plasma sample spiked with 1 μg/ml nicotinic acid, 4 μg/ml nicotinuric acid, and 8 μg/ml nicotinamide (A), and of plasma blank (B). Mobile phase: NaH_2PO_4—H_3PO_4 (78 : 22). Peaks: 1 = nicotinic acid, 2 = internal standard, 3 = nicotinuric acid, 4 = nicotinamide.

Plasma concentration profiles

By using the newly developed assay method, the monitoring of plasma concentrations of nicotinic acid and its metabolites was performed. The plasma concentration profiles are shown in Fig. 3. After the rapid increase of nicotinic acid in large excess, it was rapidly eliminated from plasma. Nicotinuric acid, on the other hand, increased with the increase of the concentration of nicotinic acid and was gradually eliminated. The concentrations of nicotinamide were non-determinable (< 0.5 μg/ml) at all sampling times.

It is possible to determine the low plasma concentrations of nicotinic acid specifically and sensitively by the method described in this report. Our results suggest that the method is useful for monitoring nicotinic acid which is a significant index of its pharmacological effects and side-effects.

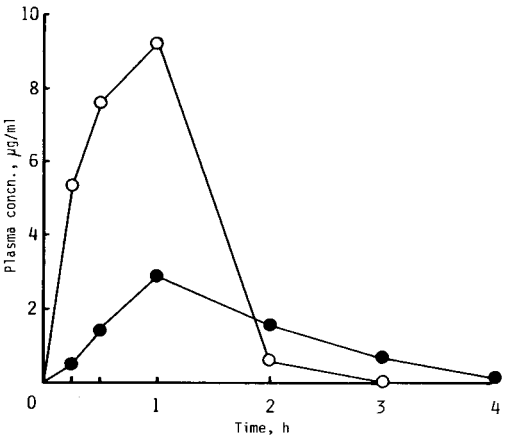

Fig. 3. Plasma concentration profiles of nicotinic acid (○) and nicotinuric acid (●) after oral administrations of 500 mg of nicotinic acid.

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Note

Rapid method for the determination of the phospholipid subclass distribution in human breast milk samples

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Phospholipids are distributed ubiquitously in living matter, including nutrients [1—4]. Their correct analysis is therefore of great interest to clinical, biochemical and nutritional researchers.

Separation of the subclasses sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) is commonly achieved by thin-layer chromatography (TLC) [5—7] or by liquid column chromatography (LC) [8—12]. These techniques allow good separation; however, quantitation of the subclasses remains a problem, especially in the case of the TLC, which is the method chosen when only small sample amounts are available [13]. Appropriate high-performance liquid chromatographic (HPLC) techniques are still in the process of being developed and, in addition, expensive laboratory equipment is needed.

In a recent publication from our laboratory a TLC method for the analysis of the phospholipid subclass distribution in milk was described. Good separation could be obtained by using solvent systems of different polarity, and quantitation was achieved by phosphorus assay [14, 15]. Although this method gives good results, it is somewhat inconvenient for the analysis of large series of samples because it is a time-consuming process. We have therefore developed a new method for the quantitation of the subclass distribution, which is based on reflectance measurement of properly sprayed TLC plates.

EXPERIMENTAL

Materials

Lipid standards were obtained from Sigma, St. Louis, MO, U.S.A. Nano-Sil 20 TLC plates (20 × 20 cm) came from Macherey-Nagel, Düren, G.F.R. All

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chemicals and organic solvents in use were of analytical grade and were purchased from E. Merck, Darmstadt, G.F.R. Human milk samples were frozen immediately after sampling and stored at −30° C.

Instrumentation

Reflectance measurement was performed on a dual-wavelength chromatogram scanner, Model CS-910 from Shimadzu, Kyoto, Japan. Spots were measured in the dual-wavelength mode -- sample beam at 600 nm and reference beam at 450 nm — when molybdatophosphoric acid was used as spraying reagent. Plates sprayed with hydroxylamine—ferric chloride or ammonium heptamolybdate—perchloric acid were measured in the same mode at appropriate wavelengths (605 and 700 nm, 600 and 700 nm, respectively). Slit size was set to 10 mm (height), 0.5 mm (width). The linearizer was adjusted as described in the manual of the CS-910. The scanner was connected to a Sigma 10 data processor (Perkin-Elmer, Überlingen, G.F.R.). Quantitation was achieved by normalisation of the peak areas.

Methods

Preparation of lipid extracts from human milk, TLC and determination of phospholipids by phosphorus assay were carried out as described previously [14, 15]. For quantitative TLC scanning, samples (20 μl of lipid extract, or standard, containing a total of 1--10 μg of phospholipids) were applied as spots on the TLC plates.

Triglyceride and fatty acid standards (1--50 μg per spot) were chromatographed on Nano-Sil 20 plates using hexane—diethyl ether—methanol—acetic acid (70:20:10:1, v/v) in a first run (8 cm) and hexane-diethyl ether—chloroform—acetic acid (70:20:10:1, v/v) in a second run (17 cm).

The fatty acid composition of the phospholipids was determined as described elsewhere [16].

Molybdatophosphoric acid was used in ethanolic solution (20%, w/v) [17], and plates were kept at 80° C for exactly 10 min after spraying with this reagent. Hydoxylamine—ferric chloride and ammonium heptamolybdate—perchloric acid reagents were prepared and applied according to the description of Krebs et al. [17]. They were used within 1 h after preparation.

RESULTS

Three lipid-staining reagents — hydroxylamine—ferric chloride (HF), ammonium heptamolybdate—perchloric acid (AM) and molybdatophosphoric acid (MP) — were studied as to their usage for quantitative reflectance measurements of TLC-separated phospholipids. Only on using MP did an intensive color develop when milk samples containing about a total of 1 μg of phospholipid were subjected to chromatography. Much larger amounts (20 μg of total phospholipids per spot) had to be applied to obtain a comparable color intensity when using the other reagents. This, however, led to overloaded TLC plates, due to the high triglyceride concentration in milk, and therefore the separation of phospholipid subclasses was significantly impaired.

A more detailed investigation of the observed color reactions was done by

using different fatty acid, triglyceride, and phospholipid standards. When 50 μg of lipid standard were applied, AM reacted only with lipids containing phosphorus, and MP only with unsaturated lipids, while HF reacted with all lipids under investigation. Therefore, because unsaturation of the fatty acids derived from the phospholipids of different biological materials might differ, AM and HF would seem to be more appropriate than MP for phospholipid quantitation. However, the better colour development makes MP the only one really suitable for this purpose. This caused us to investigate the colour reaction of MP with fatty acid standards of different degrees of unsaturation and chain length. These standards were applied as a spot and developed in the solvent systems for free fatty acids as described under Experimental. All unsaturated fatty acids had identical R_F values and, as mentioned above, only they reacted with MP. As a result, the degree of unsaturation and the chain length could be shown to have no influence on the color density.

For the analysis of the phospholipid subclass distribution in milk, samples were also applied as a spot onto the TLC sheets and separated as described elsewhere [14]. By analyzing the TLC sheets with the TLC scanner it could be demonstrated that signals are linear over the range 0.3—3 μg of a single phospholipid subclass or 1—10 μg of total phospholipids applied. This fairly wide range could only be obtained when the linearizer of the CS-910 was properly set. The lower limit for the phospholipid subclass quantitation was therefore 5 mg of total phospholipids per 100 ml of milk. Without linearization, linearity was only obtained for 0.5—1.5 μg of a single phospholipid subclass applied.

As SPH and lyso-phospholipids have only one fatty acid per molecule, data obtained for these subclasses have to be multiplied by a factor of 2 to compensate for the resulting differences in colour development.

Fig. 1 shows that the signal for PE compared to that for PC is high, although these two phospholipid subclasses occur in almost equivalent amounts in human milk [18]. This discrepancy can be explained as a factor of the different R_F values [19—21]. We could demonstrate by using reference mixtures of PC and PE having the same degree of unsaturation, that PE gives at least a 30% larger peak area than PC. The area for PI and PE obtained from milk samples were therefore corrected by multiplication with a response factor, which could be obtained by division of the PC area by the PE area (standards with the same degree of unsaturation). This factor has to be calculated for each TLC plate analyzed. Table I summarizes the relative response factors (RRF) for individual phospholipid subclasses as these have been used for the processing of raw data.

Fivefold determinations of the phospholipid subclass distribution in three milk samples gave variances ranging from 2.1% to 3.2% for SPH, PC and PE, and from 4.3% to 5.5% for PS and PI. These variances are much smaller than those observed when using the phosphorus assay [15]. Many analyses of a single milk sample over a six-month period showed a very good reproducibility with a variance not exceeding the values described above.

A reflectance chromatogram of the phospholipid subclasses derived from human milk sprayed with MP is shown in Fig. 1. It demonstrates that all subclasses are resolved well.

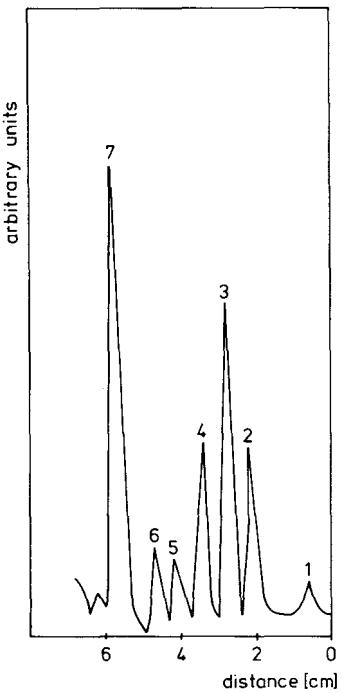

Fig. 1. Reflectance chromatogram of TLC-separated human milk phospholipids stained with MP. 1 = application spot, 2 = SPH, 3 = PC, 4 = PS, 5 = LPE, 6 = PI, 7 = PE. Details under Experimental.

TABLE I

RELATIVE RESPONSE (RRF) FOR REFLECTANCE MEASUREMENT OF TLC SEPARATED PHOSPHOLIPID SUBCLASSES

Plates were sprayed with MP. Details under Results.

Phospholipid subclass	RRF
Lyso-PC	2.000
SPH	2.000
PC	1.000
Lyso-PS	2.000
PS	1.000
Lyso-PE	2.000
PI	0.700*
PE	0.700*

*RRF values were obtained by dividing the PC area by the PE area from a standard mixture containing equivalent amounts of these phospholipids.

Table II shows the comparison of the phospholipid distribution in nine human milk samples determined by reflectance measurement and phosphorus assay. The analyzed milk samples were donated by several mothers at different stages of lactation.

TABLE II

MOLAR DISTRIBUTION OF PHOSPHOLIPID SUBCLASSES IN HUMAN BREAST MILK SAMPLES OBTAINED FROM DIFFERENT MOTHERS

Data, expressed as percentage total phospholipid, are the average of two separate analyses.

Sample	Method*	SPH	PC	PE
1	a	27.0	33.9	31.7
	b	25.5	32.9	33.5
2	a	31.6	17.2	27.8
	b	31.8	19.4	29.3
3	a	29.3	21.6	28.1
	b	31.3	24.7	27.5
4	a	30.0	32.3	34.0
	b	29.1	31.5	33.0
5	a	26.9	26.7	25.7
	b	28.9	22.4	27.5
6	a	25.0	20.5	35.7
	b	23.1	18.8	35.3
7	a	28.1	23.0	35.6
	b	29.0	23.6	33.7
8	a	25.7	23.1	32.3
	b	28.0	25.5	34.5
9	a	27.5	23.5	31.1
	b	22.7	23.0	35.9

*a = phosphorus assay; b = reflectance measurement.

DISCUSSION

The quantitative determination of the phospholipid subclass distribution in human breast milk based on reflectance measurement as described herein compares well with the determination by phosphorus assay [14]. In order to show the validity of the new method, we analyzed a total of nine different human breast milk samples, which were obtained from several mothers at different stages of lactation. Their total phospholipid content, subclass distribution and the fatty acid composition therein was therefore expected to show rather significant variations [16]. As seen from Table II, the data obtained by the two different methods are comparable, even though the variations of the subclass composition between the samples are considerable. This can only be explained by the fact that, despite the changes in the fatty acid composition of total phospholipids during lactation, the total unsaturated fatty acids therein remains relatively constant [16].

Due to the phosphorus threshold of the TLC plates used, data for SPH, PC and PE obtained by phosphorus assay showed considerable variations in multiple analyses of the same sample (variance: SPH $\approx$ 13%, PC $\approx$ 10%, PE $\approx$ 10%) [15].

The reproducibility of data from reflectance measurements was better. To obtain such reproducible values, however, some care had to be taken. Spotting, developing and spraying had to be performed without any delay between these

particular steps. Furthermore, chromatographic conditions, e.g. chromatographic tank saturation, had to be considered. The final phospholipid determination (phosphorus assay or reflectance measurement) could, however, be done later. The colour of the MP-sprayed plates was stable for three days when the plates were stored in the dark. During this period no changes of the subclass distribution could be observed upon repeated scanning.

Taking into account the results presented above, the described method should also be useful for the determination of the phospholipid subclass distributions of biological material other than human breast milk. Of course, appropriate response factors would have to be evaluated to process the raw data. For serial analysis, necessary in many biological studies, these additional experiments may take only a small part of the time that would be needed for performing a phosphorus assay for all samples under investigation.

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CHROMBIO. 1434

Note

Quantitation of the urinary methylhistidine isomers by a combination of thin-layer and fluorometric techniques

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In the course of research devoted to the separation of the hydroxyproline isomers by thin-layer chromatography after derivatization with the fluorophore 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), we found that one of the rare interfering substances was 3-methylhistidine (3-MeHis) [1]. We investigated the suitability of this technique for the evaluation of the two methylhistidine isomers, 1-methylhistidine and 3-methylhistidine, and found it as valuable as ion-exchange column chromatography and more versatile.

The interest for the determination of the methylhistidine isomers has been increasing recently. 3-MeHis is a derivative of histidine found in actin and myosin [2]. In this respect it is particularly abundant in muscle. 1-MeHis, on the other hand, is formed in a peptide of the neuromuscular junction, the function of which is still unkown. Tallan et al. [3] found 3-MeHis as a normal component of human urine and pointed out its relationship to the muscular condition. The two MeHis isomers are not reused in protein synthesis and seem to be excreted through the urine without modification or degradation, thus they are useful as urinary markers of the muscular turnover.

The usual analysis techniques are based on ion-exchange chromatography either with the amino acid analyzer [4,5] or with a colorimetric method using ninhydrin and *o*-phthalaldehyde [6—8]. The evaluation may also be performed by high-performance liquid chromatography [9] or gas chromatography [10].

The technique that we propose comprises a preliminary fast step of ion-exchange chromatography on Dowex 50-X2, permitting the separation of the basic amino acids which are then combined with the fluorophore 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The fluorescent NBD derivatives of His, 1-MeHis and 3-MeHis are separated by thin-layer chromatography on silica gel

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and quantitated by the use of a spectrofluorometer equipped with a thin-layer recording device.

MATERIAL AND METHODS

Chemicals (analytical grade) are purchased from Prolabo (Paris, France) or Merck (Darmstadt, G.F.R.); NBD-Cl is bought from Aldrich-Europe (Beerse, Belgium). The standard amino acids are obtained from Calbiochem (Los Angeles, CA, U.S.A.), except 3-hydroxyproline which was prepared in the laboratory [11]. Resin Dowex 50-X2 (50—100 mesh) is purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). The chromatographic plates are Merck silica gel plates without fluorescent indicator (ref. 5721).

Prior to analysis, the urine samples are stored in the freezer at −80°C. They are deproteinized by heating at 100°C for 2 min, then centrifuged at 1500 *g* for 10 min. It was verified on several proteinuric urines that the precipitate does not contain any MeHis. One milliliter of clear supernatant is mixed with 1 ml of 12 *M* hydrochloric acid and hydrolysed in sealed tubes for 18 h at 105°C. The hydrolysates are evaporated under a stream of nitrogen and the residue is taken up in a few drops of water and evaporated again. This operation is repeated twice. The residue is dissolved in 1 ml of 0.1 *M* sodium phosphate buffer, pH 6.4. This solution is centrifuged at 1500 *g* for 5 min and 100 μl of clear supernatant are layered on the top of the Dowex 50-X2 column.

Ion-exchange chromatography on Dowex 50-X2

A 3 × 0.7 cm column of Dowex 50-X2, 50—100 mesh (total capacity 1.2 mequiv.) is equilibrated in the triethylammonium form by passing 10 ml of a solution of triethylamine—ethanol—water (25: 25: 50, v/v) and washing with distilled water until the pH of the effluent is 6.6.

The sample, prepared as previously described, is deposited and the resin washed with 20 ml of distilled water. Most of the mineral salts and all the neutral and acidic amino acids are not bound and leave the column in the first 6 ml of effluent. The column is then eluted with 15 ml of 2 *M* ammonium hydroxide solution. The eluate is evaporated to dryness under a stream of nitrogen at room temperature and the residue dissolved in 0.1 ml of ethanol—water (50:50, v/v).

Derivatization and thin-layer chromatography

To 0.1 ml of the alcoholic solution are added 0.1 ml of a 3.0 *M* solution of triethylamine in ethanol and 0.1 ml of a 0.03 *M* solution of NBD-Cl in ethanol. The mixture is incubated at 65°C for 30 min. The NBD derivatives are stable for weeks if stored at 4°C in the dark. They are separated by thin-layer chromatography according to the following procedure. Samples of 5 μl of the derivatized solution are deposited in triplicate on a starting line which is 1.5 cm from the lower edge of a 20 cm × 20 cm silica gel plate. Standards of 1-MeHis and 3-MeHis ranging from 200 to 500 pmoles are spotted on both sides of the unknown sample. The spots are dried under a cold stream of air and predeveloped in methanol put in the bottom of a thin-layer chromatography glass jar. The predevelopment is stopped when the spots have travelled 5 mm. This step pro-

duces very thin spots all deposited on the same starting line at 2 cm from the lower edge.

The plate is dried in an oven at 65°C for 5 min and transferred to a second chromatography jar previously saturated with the solvent chloroform—methanol—ethyl acetate—acetone—triethylamine (70:15:10:10:5, v/v). The development takes 75 min and the solvent front is 16 cm from the bottom of the plate. The plate is dried at 65°C for 5 min and stored in the dark.

The fluorescent spots are recorded with a Farrand Model Mark I spectrofluorometer equipped with a thin-layer recording device. The excitation light is set at 340 nm with an additional violet filter absorbing light over 500 nm and a slot of 1 cm × 0.5 mm. The emitted light is measured at 525 nm with an additional yellow filter to absorb radiation under 450 nm.

The concentrations of the unknown samples are obtained from a standard curve drawn every day by plotting the values of the area of the peaks corresponding to the spots of known amounts of standards 1-MeHis and 3-MeHis run in parallel. In practice, the heights of the peaks may be used because they are highly correlated to the area under the chromatographic conditions described.

RESULTS AND DISCUSSION

This paper describes a new technique for the evaluation of the urinary excretion of 1-MeHis and 3-MeHis. Five unknown samples may be deposited on every plate, together with four standards containing known amounts of 1-MeHis and 3-MeHis. One technician can easily perform ten urine analyses in a day.

The chromatographic solvent furnishes an excellent separation of histidine and the methylhistidine isomers (Fig. 1). No interference was found by any of

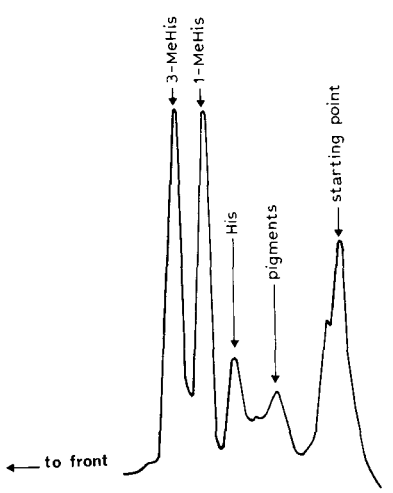

Fig. 1. Scanning of a thin-layer chromatogram showing the peak of NBD-3-MeHis and NBD-1-MeHis from a hydrolysate of urine. Mobile phase: chloroform—methanol—ethyl acetate—acetone—triethylamine (75: 15: 10: 10: 5, v/v). Development: 75 min at room temperature. Spectrofluorometer: Farrand Mark I, sensitivity 0.3, scan speed 120 mm/min.

the usual basic amino acids (Table I). The preliminary ion-exchange step permits the elimination of 3-hydroxyproline which has the same mobility as 3-MeHis on silica gel thin layers.

TABLE I

MOBILITY ON SILICA GEL PLATES OF DIFFERENT BASIC AMINO ACIDS AFTER DERIVATIZATION WITH NBD-Cl

Mobile phase: chloroform—methanol—ethyl acetate—acetone—triethylamine (75: 15: 10: 10: 5, v/v). Development: 75 min at room temperature.

Amino acid	R_F
Histidine	0.12
1-Methylhistidine	0.16
3-Methylhistidine	0.18
Arginine	0.04
Ornithine	0.01
Lysine	0.02
Hydroxylysine	0.01
γ-Aminobutyric acid	0.32

The lower limit of quantitation is 10 pmoles for 3-MeHis and 30 pmoles for 1-MeHis, which is more than sufficient for analysis in urine. The fluorescence intensity is highly dependent on the amino acid bound to NBD. If the fluorescence of 4-hydroxyproline is used as a reference [1] with an arbitrary fluorescence of 100, His gives a fluorescence equal to 23.1, 3-MeHis 54.8 and 1-MeHis 21.3.

The fluorescence of 1-MeHis and 3-MeHis is linear from 10 to 400 pmoles. The measurements are usually performed with the sensitivity of the apparatus set at 0.3, which permits a simple evaluation of the usual urinary concentrations corresponding to deposition ranging from 200 to 400 pmoles.

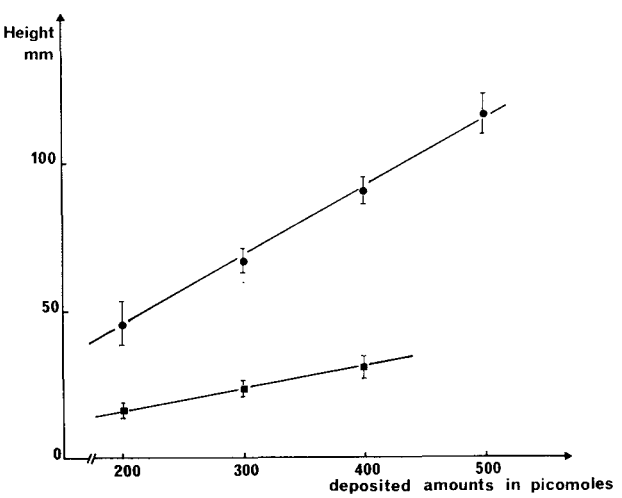

Fig. 2. Linearity of fluorescence with: (•) 3-MeHis, range 200—500 pmoles; (▪) 1-MeHis, range 200—400 pmoles. Bar: mean ± S.D. (ten determinations).

The reproducibility was tested by measuring the concentration of 3-MeHis on ten different samples of the same urine and was found to be 220.4 ± 4.6 μmoles/l (mean ± S.D.). The same urine was supplemented with 120 nmoles/ml of standards 3-MeHis and the results of five evaluations were 335.6 ± 4.4 μmoles/l, corresponding to a recovery of 97.3 ± 1.3%.

The results obtained for the urines of twenty normal adult subjects (11 males and 9 females) in the case of 3-MeHis correspond well with the reference values published by other authors using different methods (Table II). In the case of 1-MeHis, the value obtained in the urine of the same normal adult subjects was 71.4 ± 45.4 μmoles/day. Supplementation with 120 nmoles/ml of standard 1-MeHis gives a final recovery of 95.6 ± 3.7%. There are no statistically significant differences between male and female values.

TABLE II
RESULTS OBTAINED FOR 3-METHYLHISTIDINE AND 1-METHYLHISTIDINE IN URINE FROM TWENTY NORMAL ADULTS AND COMPARISON WITH THE REFERENCE VALUES PUBLISHED BY OTHER AUTHORS

Reference	3-MeHis (μmoles/day)	1-MeHis (μmoles/day)	Subjects
Yates et al. [4]	176 ± 45.1	—	20 { 17 females, 3 males
Ward and Cooksley [6]	151—154.6	—	3 { 2 females, 1 male
Bilmazes et al. [12]	167—252	—	4 males
Neuhaüser and Fürst [13]	299.4 ± 23.8	—	12 females
	545.4 ± 35.2	—	12 males
Bigwood et al. [14]	180—520	130—930	15 { 9 females, 6 males
Mussini et al. [15]	159.34 ± 19.72	795.42 ± 99.30	4 males (7—12 years old)
This paper	242.4 ± 65.4	71.4 ± 45.4	20 { 9 females, 11 males

Only two references give the normal range for this urinary metabolite [14, 15]. They both furnish a range higher than our data. This might be explained by an incomplete separation of the basic amino acids in ref. 14 published a long time ago and by the fact that the analyses of ref. 15 concerned the urines of four subjects whose ages ranged from 7 to 12 years. It is likely that the excretion of this metabolite increases during puberty.

This new method provides some advantages in comparison to the one used previously. It is faster, easily applicable to urine analysis in clinical chemistry and allows the evaluation of the second isomer 1-MeHis. In several pathological urines, we found an amount of 1-MeHis higher than that of 3-MeHis. We expect the evaluation of 1-MeHis to be of interest in the same pathological cases as 3-MeHis (for instance, in muscular diseases) and maybe to introduce some new semeiological applications.

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Note

Determination of γ-aminobutyric acid in rat brain using an isotachophoretic analyzer

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γ-Aminobutyric acid (GABA) has been known to be present in considerable amounts in the brains of animals. It has been implicated as a major inhibitory neurotransmitter.

The determination of this compound has been achieved by using an amino acid analyzer, automated high-performance liquid chromatography [1], ion-exchange liquid chromatography after reaction with *o*-phthalaldehyde [2], enzymatic methods [3—5], and thin-layer electrophoresis [6]. GABA has been used as a terminating electrolyte [7] for the determination of other amino acids on an isotachophoretic analyzer, but isotachophoresis has never been used for the determination of GABA in biological samples because a good terminator for detecting GABA had not been found. We devised a new simple method for detecting GABA in rat brain by using an isotachophoretic analyzer [8—12].

EXPERIMENTAL

Male Wistar strain rats weighing 200 g were killed by decapitation, then the brain was removed, blotted, weighed and used immediately for the estimation of GABA. The brain was homogenized with 4 volumes of 1.25% sulfosalicylic

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acid and centrifuged at 1400 *g* for 10 min. The supernatant was applied to a column containing 10 ml of Diaion SK-1 (H^+-form of sulfonated cation exchanger, 100 mesh, Mitsubishi Kasei Co., Tokyo, Japan), washed with deionized water and eluted with 2 *N* ammonia. The eluate was dried under reduced pressure and aliquots of the residue were analyzed on an isotachophoretic and amino acid analyzer (Hitachi Model 835 liquid chromatograph).

Apparatus

The capillary apparatus was a Shimadzu IP-1B isotachophoretic analyzer (Shimadzu, Kyoto, Japan). The determination of GABA in rat brain was carried out in a capillary tube (20 cm × 0.5 mm I.D.) maintained at a constant temperature of 20°C. The detector cell had an I.D. of 0.5 mm and a length of 0.05 mm. The migration current was 100 μA. The leading electrolyte was 0.01 *M* potassium acetate in 0.02% polyvinyl alcohol, titrated with 17 *N* acetate to pH 4.5. The terminating electrolyte was 0.01 *M* carnitine chloride. The chemicals used were of analytical grade.

RESULTS AND DISCUSSION

The fraction collected containing GABA described under Experimental contained all amino acids. Therefore, the analytical conditions for detecting GABA without interference from other amino acids were studied. At first, the following analytical system was used for detecting GABA as an anion. The leading electrolyte was 0.01 *M* hydrochloric acid and β-alanine (pH 3.1). The terminating electrolyte was 0.01 *M* basic amino acids (lysine, arginine and histidine). However, GABA and the terminating electrolyte were detected in the same zone due to the fact that they have almost the same potential gradient. Accordingly, we could not detect GABA as an anion under these conditions. We then tried to detect GABA as a cation. The leading electrolyte was the same as that described under Experimental. Adenine was used as the terminating electrolyte. Although GABA could be detected as a cation under these conditions, we did not obtain good results because the potential gradient difference between adenine and GABA was very small. Therefore, another terminating electrolyte, carnitine, was used. In this analytical system, GABA, the terminating electrolyte and other basic amino acids were well separated as shown in Fig. 1. The acidic and neutral amino acids could not be detected.

Fig. 1B shows an isotachophoretic run of the brain sample, and Fig. 1C that of a mixture of the brain sample and authentic GABA. The zones of GABA in the brain sample and authentic sample as shown in Fig. 1C just overlapped each other and were elongated.

The standard curves drawn by plotting zone length against concentration of authentic GABA under the analytical conditions described above are shown in Fig. 2. The slope of the curve for authentic GABA was linear from 0 to 200 nmole. The recovery curve of GABA obtained after treatment with the column of Diaion SK-1 as shown in Fig. 2 (B) was slightly less than that in Fig. 2 (A), but the curve was also linear.

The recovery of GABA after treatment of Diaion SK-1 was about 85—95%.

It was possible to detect 1 nmole of GABA in biological samples by using an

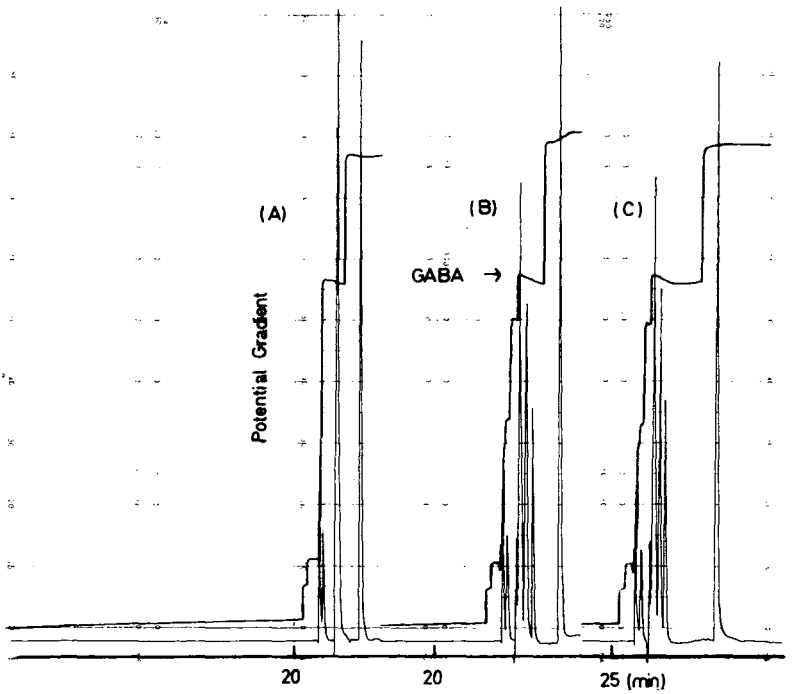

Fig. 1. Isotachophoretic runs of authentic GABA (A), brain sample (B) and a mixture of authentic GABA and brain sample (C). The leading electrolyte was 0.01 *M* potassium acetate and acetate, pH 4.5 (containing 0.02% polyvinyl alcohol). The terminating electrolyte was 0.01 *M* carnitine chloride. The migration current was 100 μA.

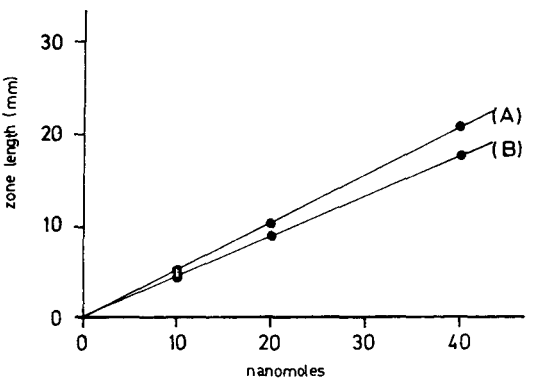

Fig. 2. Standard curves of authentic GABA (A) and GABA obtained after treatment of Diaion SK-1 (B). Analytical conditions as in Fig. 1.

isotachophoretic analyzer under the analytical conditions described in the Experimental section.

The comparison of the determination of GABA in rat brains using an isotachophoretic and an amino acid analyzer is shown in Table I. These two methods gave almost the same values. The results determined by using the isotacho-

TABLE I

COMPARISON OF GABA CONTENTS IN RAT BRAIN DETERMINED BY ISOTACHOPHORESIS AND AMINO ACID ANALYZER

Sample	GABA content (μmole/g wet weight)	
	Isotachophoretic analyzer	Amino acid analyzer
1	2.78	3.02
2	2.16	2.40
3	2.01	2.20
4	1.66	1.76
5	1.83	1.88

phoretic method described above for GABA levels in rat brain agreed well with earlier reports [1—5, 13]. This result shows that this method can be adequately utilized for the quantitative estimation of GABA in brain samples. The determination of GABA using the isotachophoretic analyzer presented here is simpler than with an amino acid analyzer, and should be very useful for determining GABA in rat brain.

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Note

Simple electrophoresis of glycosaminoglycuronans and the distinction of lung heparins from mucosal heparins

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So many electrophoretic systems have been devised and published for the analytical separation of glycosaminoglycans (GAGs) that it might seem pointless to suggest the use of yet another. It probably has to be accepted that no one simple continuous system will separate all samples of a single GAG class (e.g. dermatan sulphate) from all samples of every other class, but a system which will assuredly operate effectively in laboratories other than that of the devisor has definite advantages over a complex one which, for successful application, demands manipulative subtleties which may not be adequately described — perhaps because they are not even appreciated — in the published account.

The present system has been used only for the sulphated glycosaminoglycuronans; the migration of hyaluronate and of keratans has not been investigated. If we consider electrophoresis of GAGs on the most convenient medium, cellulose acetate membranes, two main kinds of simple continuous buffer system are in general use: one of low pH where molecular charge is the dominant factor determining separation, and another of more neutral pH containing a cation which binds with varying specificity to different classes of GAG. The first kind, originated by Mathews [1], separates heparin (Hep) from the less sulphated classes, but makes little distinction between the chondroitin sulphates (ChS), dermatan sulphate (DeS) and similarly sulphated heparans (HeS). The second kind has a wider application; in the form of barium acetate it was introduced by Wessler [2], and among the accessible metal cations barium remains the most useful for electrophoretic GAG separations. Differences in the solubility of the barium—GAG complexes have also been used to enhance certain separations [3]. Dietrich and Dietrich [4] in-

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troduced α,ω-diaminoalkane buffers, and the three- and four-carbon members of this series show a remarkable resemblance to barium in their separative, and presumably complexing, ability. These buffers yield more compact spots on cellulose acetate than the low pH systems, and will usually separate Hep, DeS and ChS from each other, but since the spread of R_F values is rather limited there is little discrimination within a class. The system described here gives separations which are essentially similar to those obtained with barium acetate or propane-1,3-diamine acetate, but with a greater spread of R_F values, and since the spots are at least as compact (depending of course on sample homogeneity) greater discrimination is possible.

Heparin is commonly obtained commercially either from porcine intestinal mucosa or from bovine lung tissue. The two types differ [5], and for pharmacological or other reasons it may be desirable to distinguish the one from the other [6]. In the electrophoretic system described here the two types differ in behaviour to a degree sufficient for this purpose, but the distinction may be made more positive by using the discontinuous system of Hopwood and Harrison [7].

EXPERIMENTAL

Bis(3-aminopropyl)amine (dipropylenetriamine, DPTA)* was obtained from Fluka and from Aldrich; Ch4S I, Ch6S I, DeS I and HeS I were standard samples (batch 1977) kindly donated by Professors Cifonelli and Mathews, University of Chicago. Ch6S II (ex shark) was obtained from Koch-Light, and DeS II (ex pig skin) from Seikagaku. HeS II and III were prepared in this laboratory from residues of commercial mucosal heparin production (galactosamine <1% total hexosamine, SO_3^-/CO_2^- ratios 1.0 and 1.65, respectively), as was DeS III. Ch4S II (some 6-isomer by infrared) and DeS IV (some ChS as impurity) were prepared in this laboratory from bovine lung heparin production residues. The sources of the heparin samples are given in the captions to the figures.

The brands of cellulose acetate used were: Shandon Celagram (identical to Elvi Microphor), Gelman Sepraphore III, Schleicher and Schüll CA-Elektrophoresefolien (very similar to the Sepraphore), and the wet-stored gels Whatman Cellogel and Cellogel RS. In the electrophoresis apparatus (Shandon Minimicroband, no longer available) the cellulose acetate strips, ca. 8 cm long, were suspended in air between filter paper wicks over gap of about 6.5 cm without intermediate support. There were no special features except that the buffer chambers were partitioned by antidiffusion barriers into electrode and wick compartments; the buffer in the latter therefore remains relatively unaffected by electrolytic changes.

The buffer used was 0.025 *M* in DPTA, 0.03 *M* in Mg^{2+} and ca. 0.12 *M* in $CH_3CO_2^-$, pH ca. 7.0. A concentrated stock solution (×10) became yellow on standing at room temperature, and then gave reduced resolution. For best results the buffer should be fresh. For the Shandon apparatus, to 150 ml water was added successively 10 ml 0.75 *M* magnesium acetate solution, 0.96 ml

*Alternative catalogue names: 3.3′-iminobispropylamine, 3.3′-diaminodipropylamine.

glacial acetic acid, 0.89 ml amine, and the whole made up to 250 ml.

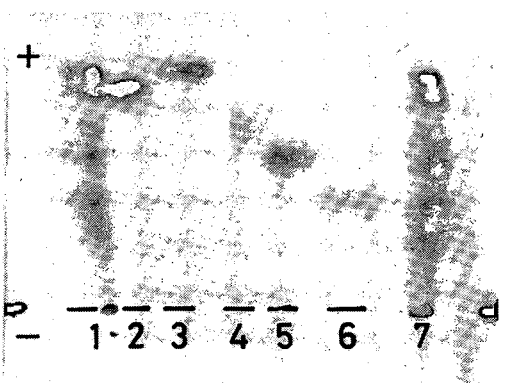
All operations were conducted at room temperature. Cellulose acetate strips of suitable width were equilibrated by floating on buffer for a minimum of 3 min (longer for wet-stored material), then laid on paper tissue or filter paper and loaded with about 0.2—0.4 μl GAG solution (2—5 mg/ml in water) in a zone of ca. 1 × 4 mm. Electrophoresis was performed with a current of about 1 mA/cm width; about 120 V was required. After about 1 h the fastest-moving GAG, Ch6S, had migrated about 30 mm, depending on temperature, and further electrophoresis was not usually advantageous. A number of runs may be made, reversing polarity each time to reduce changes in buffer composition, before the buffer needs to be renewed. Staining and destaining were carried out in the usual way, using Alcian Blue and 5% aqueous acetic acid.

For discrimination between lung and mucosal heparins the following electrophoretic system was used. The tank buffer was 1.0 *M* barium acetate, and the cellulose acetate was equilibrated before loading with 0.1 *M* barium acetate. After 30 min electrophoresis at about 1 mA/cm width the gel was removed and immersed for 2 min in 0.1 *M* barium acetate containing 30% ethanol, and, after blotting to remove excess buffer, electrophoresis was continued with the original tank buffer for a further 30 min. All other conditions were as described above.

RESULTS AND DISCUSSION

Fig. 1 displays the separation which may be obtained with a range of GAGs, and Fig. 2 indicates that DeS samples may show slight variations in mobility; substitution of DeS I for DeS III in Fig. 1 would destroy the separation between DeS and HeS I. One may speculate whether, in view of the close structural relationship between DeS and Ch4S, the faster migrating sample may have a higher ratio of glucuronate to iduronate.

Fig. 3 compares samples of ChS and also of the HeS—Hep sequence. The



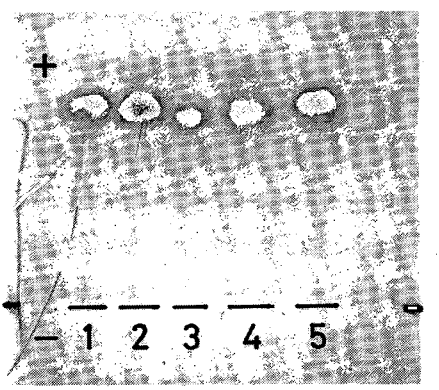


Fig. 1. DPTA + Mg^{2+} acetate buffer, as in text. Samples 1 and 7 = mixture of 2—6 with bovine lung heparin added, 2 = Ch4S I, 3 = Ch6S I, 4 = DeS III, 5 = HeS II, 6 = HeS III.

Fig. 2. Conditions as Fig. 1. Samples 1 and 5 = DeS I, 2 = DeS II, 3 = DeS III, 4 = DeS IV.

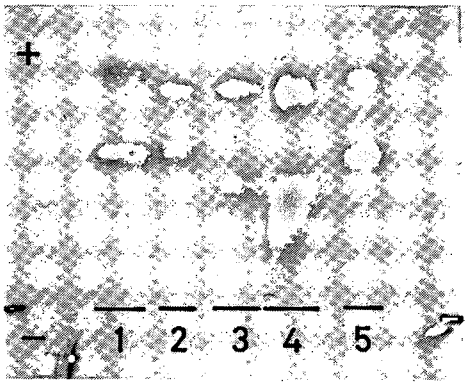


Fig. 3. Conditions as Fig. 1. Samples 1 and 5 = Ch6S I + DeS I, 2 = Ch4S I + HeS II, 3 = Ch4S II + HeS III, 4 = Ch6S II + porcine mucosal heparin.

clear-cut distinction between Ch4S I and Ch6S I (cf. Fig. 1) is not maintained for the other samples of this class. For heparan sulphates the migration rate is inversely related to the degree of sulphation, but (from results with other samples not included here) in a rather discontinuous manner. HeS I/II and HeS III may be representatives of two sub-classes of HeS, within each of which there is some variation in degree of sulphation — as is indeed the case with heparins.

The inherent heterogeneity within GAG classes, with consequent irregularities in electrophoretic behaviour, has recently been emphasised by Schuchman and Desnick [8]. Their conclusion however, that "only highly purified GAG preparations should be used as standards", appears to be wrong; it is surely desirable to prepare or otherwise find standards as close as possible in behaviour (implying also composition) to the corresponding constituents of the samples to be examined. Difficulties were encountered in trying to employ the electrophoretic procedure of Schuchman and Desnick [8], since EDTA salts available here did not yield the separations which they describe.

The composition of our electrophoresis buffer is not the outcome of an extensive research programme, but for those inclined to investigate it further a few comments may be useful. The amine acetate is quite difficult to use alone, for reasons involving conductivity and complexing strength. Quite a number of other metal acetates have been tried as likely co-ions; calcium behaves in almost identical manner to magnesium, but metals with stronger complexing ability were on the whole less satisfactory. Barium did not appear to improve the HeS and DeS separation.

Samples of commercial heparin subjected to electrophoresis in this system normally leave some material immobile at the point of loading, and the rest migrates as a diffuse zone (Figs. 1 and 3). The exact effect varies with temperature and obviously involves the formation of heparin—amine complexes with low solubility (cf. ref. 3), but with porcine mucosal heparin samples the mobile material migrates consistently faster than with bovine lung samples. However, although the DPTA buffer may be used to provide an indication of the tissue of origin of an unknown heparin — assuming it to be one of the two

usual kinds, porcine intestinal mucosal and bovine lung, and using samples of these for comparison — we have found no simple continuous system which is altogether satisfactory. Some trials with the discontinuous system of Cappelletti et al. [9] as modified by Hopwood and Harrison [7] were encouraging, provided that they were run with the gel strip suspended in air at ambient temperature as in our DPTA procedure; when we adapted our equipment to conform with the detailed Hopwood—Harrison procedure, confining the gel strip between melamine film (on a metal plate at 15°C) and parafilm under a flat 1000-g weight, we were not able to obtain satisfactory results. The procedure was further simplified by using only one intermediate buffer bath.

Fig. 4 was obtained at an ambient temperature of 27°C; at lower temperatures there is more non-mobile material with all samples, but the distinction is maintained. Samples 4 and 6 are typical of mucosal heparins, whereas sample 2 is the least sulphated of over 30 commercial heparin samples we have examined. All samples were essentially free of other GAGs. Discrimination between mucosal and lung heparins is also obtained with the procedure of Oreste and Torri [3], although we have found it difficult to obtain the distinctive and reproducible "fingerprints" described by these authors.

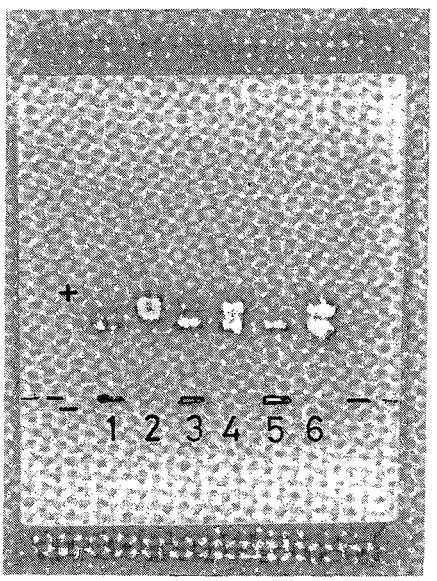


Fig. 4. Barium acetate buffers; conditions modified from Hopwood and Harrison [7] as described in text. Samples 1, 3 and 5 are bovine lung heparins; 1 = isolated in this laboratory, 3 = Upjohn lot 730EH, 5 = Choay lot FF783. Samples 2, 4 and 6 are porcine mucosal heparins; 2 = Terhormon lot 018, 4 = Bioiberica lot F4, 6 = Diosynth lot Hb 1659-I.

In the course of the trials of the Hopwood—Harrison electrophoresis procedure [7] it became clear from relative staining intensities that sample losses occur, particularly of the less sulphated GAGs, when the partly-run gels are soaked in the aqueous—ethanolic barium acetate solutions. Their procedure was of course designed primarily as an aid to the diagnosis of the mucopoly-

saccharidoses, and while for this purpose a reasonable consistency in the electrophoretic band patterns is essential, there is no requirement for an accurate representation of the proportions of the various GAGs present. Since our own system may not be effective in separating DeS from low-sulphated heparans such as are excreted in mucopolysaccharidoses, it may not be suitable for diagnosis except in conjunction either with chondroitinase B treatment or with galactosamine—glucosamine ratio determination (see ref. 10 for a simple procedure). Together with this latter determination, however, our system gives an unequivocal indication of the composition of a large majority of the miscellaneous GAG fractions we have had occasion to examine. Brief reference has been made to this electrophoresis method in a review article [11].

ACKNOWLEDGEMENT

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Note

Quantitative determination of *n*-dipropylacetamide in the plasma of epileptic patients by gas—liquid chromatography with nitrogen-selective detection

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n-Dipropylacetamide (DPM) is the primary amide of valproic acid (VPA), a widely used antiepileptic drug. DPM is commercially available for therapeutic use and its antiepileptic properties have been reported [1—5]. After ingestion, it is converted almost completely to VPA [6,7] so that its therapeutic dose in epilepsy is considered to be the same as that of VPA [6,7]. Similar doses of DPM or VPA during chronic therapy produce similar VPA plasma concentrations [6]; moreover, VPA plasma concentrations are used to adjust the therapy in patients taking DPM.

Gas chromatographic methods for the determination of plasma DPM concentrations have already been published [8,9] but they are very time-consuming [8] or have unsatisfactory sensitivity [9] for clinical use.

The plasma concentrations of DPM after normal therapeutic doses in epileptic patients were always found to be below 1 μg/ml, which is the detection limit of the analytical procedure used [6]. Even if the plasma concentrations of DPM are so low, their measurement may be important to investigate the metabolism and kinetics of this drug in patients during acute and chronic therapies.

The aim of this work is to indicate a more sensitive gas chromatographic method for the measurement of DPM in plasma of epileptic patients.

MATERIALS AND METHODS

Reagents and standards

n-Dipropylacetamide and *n*-tripropylacetamide (TPM, employed as an internal standard) were obtained from Sigma-Tau (Pomezia, Italy). All chemicals were of analytical grade.

Stock solutions of DPM and TPM were prepared in acetone to give a concentration of 1 mg/ml for each compound.

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Calibration samples were prepared at the time of the analysis by adding 0.05, 0.1, 0.25 and 0.5 μg of DPM in acetone to a series of test-tubes, which were then evaporated to dryness under vacuum at room temperature and thus redissolved into 0.5 ml of drug-free plasma.

Apparatus

A Carlo-Erba Fractovap 2150 gas chromatograph equipped with a nitrogen-selective detector and a Hewlett-Packard 3380 A recorder—integrator were used. The glass column, 1.5 m × 3 mm I.D., was packed with 10% diethyleneglycol-succinate—phosphate (DEGS-PS) on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The following flow-rates were used: hydrogen 36 ml/min; air 250 ml/min; carrier gas (nitrogen) 35 ml/min. The temperature of the column was 190° C; the temperature of the injector was 225° C. The sensitivity of the detector was checked daily.

Extraction procedure

Twenty-five microlitres of the diluted internal standard solution (10 μg/ml in acetone), 0.5 ml of 0.5 *M* H_3PO_4 and 8 ml of diethyl ether were added to 0.5 ml of plasma (calibration samples or patients' samples). The test-tubes were shaken mechanically for 10 min, then centrifuged for 10 min at 2000 *g*; 6.5 ml of the organic phase were transferred to a second test-tube and evaporated to dryness under vacuum at room temperature.

The residues were dissolved in 100 μl of *n*-hexane and 1—2 μl of this solution were injected into the gas chromatograph.

Calibration graphs were constructed of the peak-area ratio of DPM to TPM versus concentration of DPM. For each series of analyses a new calibration graph had to be prepared.

Recovery

Various amounts (0.05, 0.1, 0.25, 0.5 μg) of DPM were dissolved in 0.5 ml of drug-free plasma. The samples were extracted as described above but without adding TPM. The residues were dissolved in 100 μl of *n*-hexane containing 0.25 μg of TPM. A second series of standards was prepared simultaneously by extracting 0.5 ml of drug-free plasma and then adding both DPM and TPM to the dried extract at the concentrations above indicated. The recovery was calculated by comparing the peak-area ratios of the extracted standards to the ratios obtained from the standards to which DPM had been added after extraction. Thus this value was corrected by a factor representing the ratio between the diethyl ether volume added to the plasma and the diethyl ether volume subsequently removed and evaporated during the extraction procedure.

Due to the partial solubility of water in the diethyl ether, this value of recovery may be different from the actual value of absolute recovery; the difference should, however, be slight.

Linearity

The linearity was calculated by using the results obtained from the calibration graphs (from 0.1 to 1 μg/ml).

Interference from other antiepileptic drugs

To determine the potential usefulness of the procedure, we checked for possible interferences from other antiepileptic drugs (phenobarbital, mephobarbital, primidone, carbamazepine, phenytoin, ethosuximide and valproic acid) by chromatographing extracts from plasma of epileptic patients taking the above drugs and pure standards at normal therapeutic concentrations.

Plasma of patients

Samples of blood from patients receiving DPM orally thrice daily (at 8 a.m., 2 p.m., 8 p.m.) together with other antiepileptic drugs were taken in heparinized test-tubes just before the morning dose. The plasma was separated as soon as possible, immediately frozen and stored at −20° C until taken for analysis.

RESULTS AND DISCUSSION

Under our gas chromatographic conditions, only two of the antiepileptic drugs tested were seen on the chromatograms; valproic acid, which eluted in the front of the solvent, and ethosuximide, which had a retention time of 11.5 min. Since DPM and TPM had a retention time of about 2.6 min and 3.4 min, respectively, we can conclude that no antiepileptic drug tested (at normal

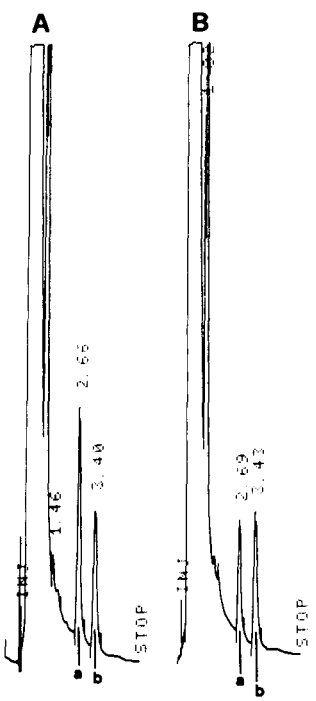

Fig. 1. Gas chromatographic response of two extracted plasma samples. (A) Extract from a calibration sample (0.5 μg of DPM per 1 ml of plasma). (B) Extract from plasma of a patient taking DPM (16 mg/kg per day), phenytoin and carbamazepine (DPM plasma level = 0.21 μg/ml); a = DPM; b = TPM.

therapeutic concentrations) interfered with the analysis. Moreover, no interference from endogenous plasma compounds was seen on the chromatograms of drug-free plasma samples.

Typical chromatograms of extracts from a plasma calibration sample and a plasma sample of a patient taking DPM, phenytoin and carbamazepine are shown in Fig. 1.

Calibration curves from extracted plasma showed a linear correlation between concentrations and respective readings: $Y = 1.79\ X + 0.009$, $r = 0.997$. To calculate these curves, a least-square linear regression method was used. The minimal amount of DPM detectable by the described procedure is about 20 ng/ml of plasma. The mean ± S.D. recovery from four analyses of plasma samples containing four different DPM concentrations (0.1, 0.25, 0.5, 1 μg/ml) was 90.2 ± 3.5% ($n = 16$).

In a series of 13 patients, with a DPM dosage of 10—40 mg/kg per day (mean ± S.D. = 27.1 ± 9.5) we found plasma concentrations of 0.03—3 μg/ml (mean ± S.D. = 0.668 ± 0.840) regardless of the co-medications. In the same samples, the concentrations of VPA, measured by gas—liquid chromatography [10], were 30—80 times higher than DPM concentrations.

The proposed method is, to the best of our knowledge, the most sensitive available to determine the concentrations of DPM in human plasma and it is suitable to study the pharmacokinetics and metabolism of DPM in human subjects.

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Note

Determination of apparent dihydralazine in plasma by gas—liquid chromatography and electron-capture detection

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Dihydralazine sulfate, the active ingredient of Nepresol®, is a potent vasodilator. Therapeutic doses are low, normally 25 mg. Measurement of the compound in biological fluids thus requires a method of high sensitivity. It has been shown that after administration of hydralazine, an analogue of dihydralazine, the major fraction of the compound measurable in plasma exists in the form of acid-labile hydrazones such as pyruvic acid hydrazone [1].

Previous methods were either not sensitive enough or have not been optimized to measure apparent dihydralazine [3—10]. The recently published specific method for free dihydralazine [11] is not sensitive enough for the measurement of therapeutic levels of dihydralazine in man.

This paper describes a method to assay apparent dihydralazine in plasma. The method is based on hydrolysis of the acid labile compounds followed by reaction with nitrous acid to form the azidotetrazolophthalazine (II, Fig. 1). This compound when isolated in crystalline form is a violent explosive and thus not thermally stable enough for gas chromatographic (GC) analysis. In a second step, the labile derivative is treated with sodium methylate to form the methoxytetrazolophthalazine (III, Fig. 1). This final derivative is stable and has excellent properties for its GC determination with electron-capture detection. 6-Trifluoromethyl-dihydralazine is used as an internal standard. After derivatisation, this compound forms two isomers. However, the ratio of the two peaks is constant (0.68) and thus any of the two peaks may be used for the quantitation.

Acid-labile hydrazones of dihydralazine in plasma of patients can be hydrolyzed to yield concentrations of apparent dihydralazine far in excess to the free dihydralazine as measurable without preceding hydrolytic treatment of plasma.

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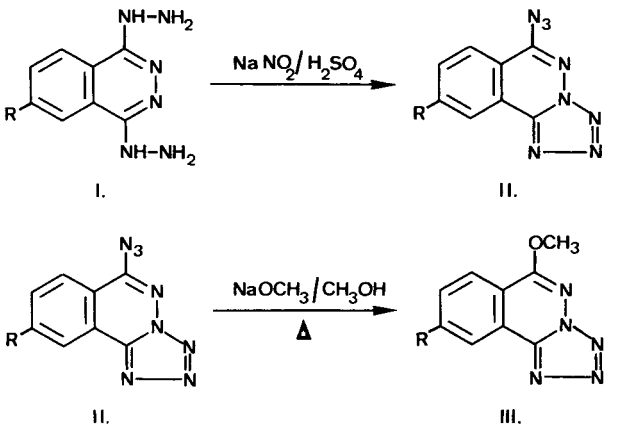

Fig. 1. Structures of dihydralazine (R = H) and 6-trifluoromethyl-dihydralazine (R = CF_3) (I), azidotetrazolophthalazine (II) and methoxytetrazolophthalazine (III).

MATERIALS AND METHODS

Reagents

All standard solutions (dihydralazine sulfate and 6-trifluoromethyl-dihydralazine hydrochloride) were prepared daily in 0.1 *N* hydrochloric acid. Toluene and methanol, laboratory grade, were distilled before use.

Sodium nitrite and sodium methylate were obtained from Fluka (Buchs, Switzerland). Buffer solutions were prepared as follows. pH 13.8 buffer: 0.225 mol potassium hydrogen phthalate and 1.575 mol sodium hydroxide, made up to 1 liter. pH 7.0 buffer: 0.041 mol disodium hydrogen phosphate and 0.028 mol potassium hydrogen phosphate, made up to 1 liter.

Procedure

A 20-ml extraction tube is wetted with 4 ml of 1.5 *N* sulfuric acid. After addition of 1 ml of plasma, 50 μl of internal standard solution (1 ng/μl) are added. The tube is stoppered and agitated in a water bath at 90°C for 25 min (100 rpm). After cooling to room temperature, 0.1 ml of 50% sodium nitrite solution is added, briefly mixed and left at room temperature for 15 min. Then 4 ml of buffer solution pH 13.8 are added to bring the pH to about 4.5. After addition of 5 ml of toluene, the mixture is shaken for 10 min on a horizontal mechanical shaker at 120 rpm. After brief centrifugation, the organic phase is removed and evaporated to dryness under a stream of nitrogen at 40°C. A solution of 8.6 μmol sodium methylate in 1 ml of toluene (with 5% methanol) is added to the dry residue and left at 50°C for 1 h; 3 ml of buffer solution pH 7 are then added and shaken for 10 min at 200 rpm. After brief centrifugation, the organic phase is removed and aliquots of 5 μl are injected into the gas chromatograph.

Gas chromatography

A Pye 204 gas chromatograph equipped with a ^{63}Ni electron capture detector was used. The column was a 1.5 m × 4 mm I.D. Pyrex glass column packed

with 3% OV-225 on Ultrapak Gas-Chrom Q, 230—270 mesh. The carrier gas flow-rate was 50 ml of nitrogen per min. The temperatures were: injector and column oven 250°C, detector 350°C. The retention times for the two derivatives were 6.6 min for the methoxytetrazolophthalazine and 4.0 min for the more abundant isomer of the trifluoromethyl-methoxytetrazolophthalazine. Chromatograms of a blank plasma sample and of a plasma sample spiked with 40 ng of dihydralazine are shown in Fig. 2.

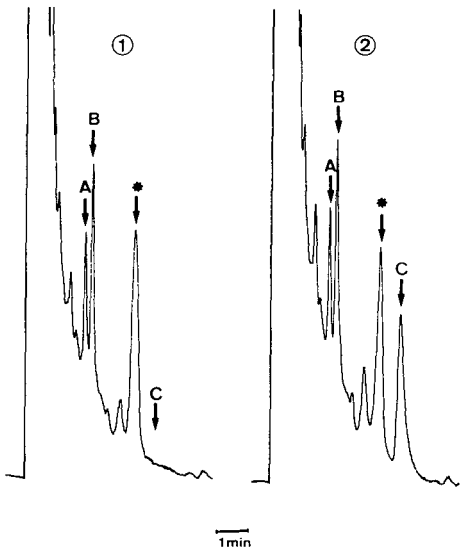

Fig. 2. Typical chromatograms of (1) extract of a blank plasma sample (1 ml) with 50 ng of internal standard, and (2) extract of a plasma sample spiked with 40 ng of dihydralazine sulfate and 50 ng of internal standard. Injected aliquot: 1/200th. The peak marked with an asterisk is an unknown constituent of plasma. (A, B) Isomers of the internal standard derivative (B is used for the quantitation of dihydralazine). (C) Derivative of dihydralazine.

Calibration graph

A calibration graph was prepared as follows. Blank plasma samples were spiked with solutions of dihydralazine in 0.1 *N* hydrochloric acid (0—330 ng/μl). The samples (n = 11) were then processed as described. The peak height of the dihydralazine derivative was divided by the peak height of the internal standard derivative and the ratio (H_x) plotted against initial dihydralazine concentrations.

Calculation of the linear regression resulted in a coefficient of correlation (r) of 0.9999 and a standard error of estimate (S_y) of 0.0086 H_x.

RESULTS AND DISCUSSION

Hydrolysis

Acid strength, time and temperature of incubation were optimized to obtain maximum yield of apparent dihydralazine using plasma pools from rats treated with dihydralazine (12 mg/kg per os). Sulfuric acid up to 6 *N*, incuba-

tion temperatures up to 100°C, and reaction times up to 120 min were tested. It was found that hydrolysis with 1.5 *N* acid at 90°C for 25 min produces optimal recoveries.

Stability

Dihydralazine is rapidly oxidized under alkaline conditions. Only about 20% of unchanged dihydralazine was found after 48 h storage in neutral aqueous solution. However, a solution of dihydralazine in 0.1 *N* hydrochloric acid stored under the same conditions was found to be stable.

Added to plasma, dihydralazine is very unstable, even when stored at −20°C. The addition of hydrochloric acid improves the stability only marginally. Without acid, about 80% of the dihydralazine is lost after 48 h of storage at −20°C, and with hydrochloric acid added (pH 1) about 60% is lost. In contrast to this observation, plasma samples from in vivo studies were kept up to 28 days at −20°C without detectable losses of apparent dihydralazine. The instability of dihydralazine seen in spiked biological samples refers to unchanged dihydralazine only. Dihydralazine proved quite stable in plasma samples obtained from animal studies, when the samples were measured by the GC technique for apparent dihydralazine.

This seeming discrepancy between findings with spiked biological or aqueous samples and with samples obtained from animals after dihydralazine administration, suggests that dihydralazine, like hydralazine [1, 2], forms metabolites which are stable upon storage but regenerate free dihydralazine upon hydrolysis in the analytical procedure.

Derivatisation

The nitrous acid reaction, according to Jack et al. [12], was found to be completed after 15 min at room temperature and the yields in water and plasma were about 80%. In blood the yields drop to about 50—60%.

The formation of the methoxy derivative is complete after 1 h at 50°C. The yields at concentrations up to 500 ng per sample are almost 100%. The final derivative is stable in toluene for several days at room temperature. The structures of the derivatives have been verified by gas chromatography—mass spectrometry (GC—MS).

The internal standard forms two isomers (6-trifluoro-4-methoxy- and 6-trifluoro-1-methoxy-tetrazolophthalazine) upon derivatization. Both isomers have been verified by GC—MS; however, on the basis of molecular ion and fragmentation data it is not possible to differentiate between the two isomers. The ratio between the two derivatives is very stable; it was found to be 0.68 ± 0.05 (mean ± S.D.) calculated from a series of 40 chromatograms. Thus, for convenience, the larger of the two peaks was chosen for the evaluation of the chromatograms.

Recovery, precision and limit of quantitation

Recovery of dihydralazine and precision were evaluated by analysing spiked samples. Fifteen samples were prepared with dihydralazine concentrations between 10 and 130 ng/sample. The differences between the found and the initial concentrations were between −0.6% and +12.2%.

Calculation of the linear regression between given and found concentrations resulted in a coefficient of correlation (r) of 0.9997 and a standard error of estimate (S_y) of 1.1927 ng.

The limit of quantitation is about 5 ng/ml plasma.

Application

One healthy, male volunteer received 12.5 mg (½ tablet) of Nepresol® in the morning 2 h before breakfast. Blood samples were collected immediately before and at specified times after administration of the oral dose. The blood samples were immediately centrifuged, the plasma removed and analysed as described above.

The peak plasma level was 87 ng/ml, attained 1 h after administration (Fig. 3). The plasma levels found are considerably higher than those found in an earlier study, where the acid-labile components were not completely hydrolysed [10]. However, the apparent biological half-life values are comparable (2—3 h).

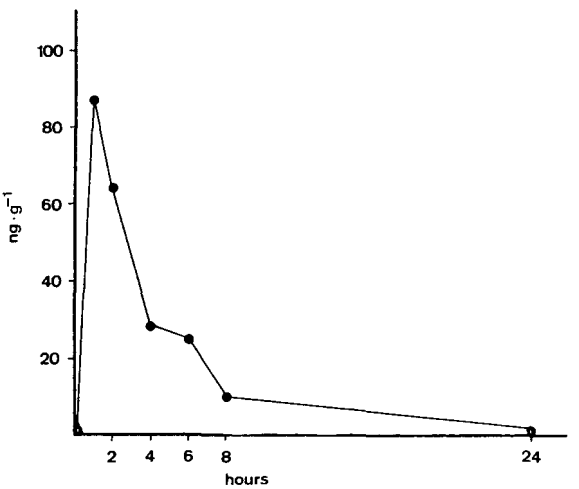

Fig. 3. Plasma levels of apparent dihydralazine after a single, oral dose of 12.5 mg of Nepresol in a healthy volunteer.

No measurable concentrations of free dihydralazine were found after analysis of the samples by the GC method with nitrogen-specific detection [11] (detection limit: ca. 20 ng/ml).

ACKNOWLEDGEMENTS

Thanks are due to Dr. K. Eichenberger, Ciba-Geigy, Basle, for the supply of internal standard and to Dr. W. Theobald, Ciba-Geigy, Basle, for providing the biological samples.

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CHROMBIO. 1442

Note

Gas—liquid chromatographic determination of etoperidone in plasma, serum, and urine

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(First received March 27th, 1981; revised manuscript received July 19th, 1982)

Etoperidone, 2-[3-(4-(*m*-chlorophenyl)-1-piperazinyl)-propyl]-4,5-diethyl-2,4-dihydro-3H-1,2,4-triazol-3-one monohydrochloride (Fig. 1a), is a psychotropic drug with antidepressant activity, and no cardiotoxic or cholinergic effects. The drug is potentially of particular utility in geriatric patients, since it is well tolerated, and appears to have a beneficial effect on certain of the psychosomatic parameters of ageing.

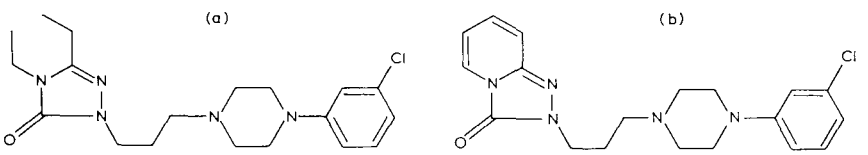

Fig. 1. Structures of etoperidone (a) and trazodone, the internal standard (b).

For metabolic and pharmacokinetic studies on this drug it was necessary to have available a method for its measurement in biological fluids. A similar compound, trazodone, has previously been determined by fluorimetry [1], gas—liquid chromatography (GLC) [2, 3], and high-performance liquid chromatography (HPLC) [4]. GLC with flame ionization detection has previously been used for the determination of etoperidone during animal studies with a sensitivity of 200 ng/ml [5]. In view of the low absorption of this compound in the near ultraviolet region, we felt that separation and detection by HPLC would not yield a method of adequate sensitivity; however, by using the extra selectivity which a nitrogen—phosphorus detector offers, a method has been developed with a sensitivity of 20 ng/ml in plasma or serum, and 5 ng/ml in urine.

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EXPERIMENTAL

Materials

Analytical-grade reagents were used throughout, and inorganic reagents were prepared using glass distilled water. Etoperidone as the hydrochloride was supplied by Sigma-Tau Research Labs. (Domezia, Rome, Italy). Trazodone as the hydrochloride (2-[3-(4-(*m*-chlorophenyl)-1-piperazinyl)-propyl]-*s*-triazole-[4,3-*a*]-pyridine-3-(2H)-one monohydrochloride) (internal standard, see Fig. 1b) was supplied by the same company.

Extraction procedure

Serum or plasma samples (1 ml) were transferred to 10-ml centrifuge tubes, spiked with internal standard solution (50 μl; 10 μg/ml of trazodone in methanol), and made alkaline with 5 *M* potassium hydroxide solution (200 μl). This mixture was extracted twice, each time with 5 ml of diethyl ether—petroleum ether (b.p. 40°—60°C) (1:1, v/v). Phases were separated by centrifuging for 5 min, the organic layers were combined in a clean tube, and reduced to low volume on a water bath. The remaining solvent was evaporated in a stream of nitrogen at room temperature, and the residue taken up in methanol (200 μl).

Urine samples (5 ml) were spiked with internal standard (50 μl; 10 μg/ml), and made alkaline with 5 *M* potassium hydroxide solution (500 μl). The sample was extracted twice with 5 ml of the ether mixture, separating the phases as above. The combined organic layers were extracted twice with 0.5 *M* hydrochloric acid (2 × 2.5 ml). These combined aqueous layers were made alkaline by the addition of 5 *M* potassium hydroxide solution (1 ml), and then extracted with the ether mixture (2 × 5 ml). The ether extract was evaporated and redissolved as described above.

Calibration procedures

Samples of blank serum were spiked with etoperidone at concentrations of 50—300 ng/ml, and carried through the extraction procedure described above. For urine, solutions of the drug in water were extracted as above. Water was used in place of urine as no significant difference was observed between the two, but water produced a much cleaner chromatogram. The standard curve for urine was prepared over the range 50—500 ng/ml, although the parent drug has only shown concentrations up to 100 ng/ml.

GLC conditions

Chromatography was carried out on a Sigma 3 gas chromatograph (Perkin-Elmer, Beaconsfield, Great Britain) fitted with a nitrogen—phosphorus detector. The output was taken via a CRS308 computing integrator (Infotronics, Stone, Great Britain) to a Perkin-Elmer Model 56 chart recorder. The column used was glass 1 m × 4 mm I.D., packed with 3% OV-17 on Chromosorb W HP 100—120 mesh, maintained at 290°C. The carrier gas was nitrogen at a flow-rate of 40 ml/min, and the injector and detector blocks were maintained at 350°C. Flow-rates of the combustion gases were optimised according to the manufacturer's instructions.

Linearity, sensitivity, and recovery

The detector gave a linear response from 2.5 to 600 ng of etoperidone injected on column. Under the operating conditions described, the method is capable of routinely detecting down to 20 ng/ml of etoperidone in plasma or serum and 5 ng/ml of etoperidone in urine. Total recovery is about 84% because of extraction and transfer losses.

RESULTS AND DISCUSSION

The calibration curve was constructed from six replicate measurements of concentration over the working range. The plot of peak area ratios against concentration was linear ($y = 0.002926x - 0.002$, correlation coefficient $r = 0.999$). The value of the intercept was not significantly different from zero.

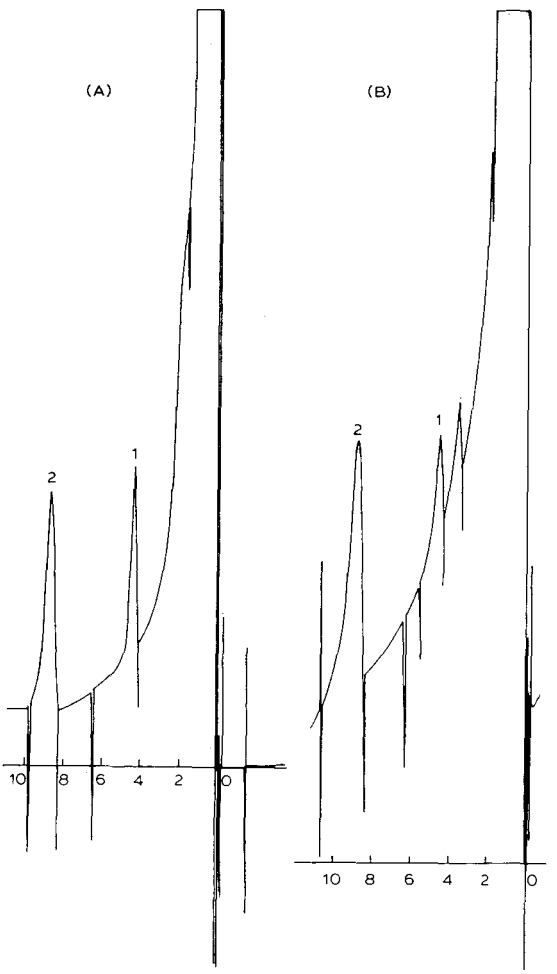

Fig. 2. Chromatograms from (A) a plasma sample containing 75 ng/ml of etoperidone, and (B) from the 0.5-h post-dose serum sample. Peaks: 1 = etoperidone, 2 = trazodone.

This method has been used for the analysis of samples collected after dosing a volunteer with a 50-mg capsule of etoperidone. The peak concentration in blood occurred between 0.5 and 1 h after dosing, and the concentration at 24 h was below the limit of detection (Table I). Insufficient samples were taken to examine the descending portion of the plasma concentration curve in detail, but it appears that the half-life is of the order of 1.5 h. Results from plasma and serum were similar for the lower concentrations of the drug, but the peak level values observed in plasma were noticeably lower than those from serum. No explanation is offered for this difference; however, the chromatogram resulting from serum has less interfering peaks than that from plasma (Fig. 2).

Results from urine were more complex, and are still under investigation. An amount of 8.2 μg of unchanged drug was found in urine collected between 0 and 6 h post-dose, and a further 0.7 μg up to 24 h. However, at least three metabolites were observed in the traces (Fig. 3). These compounds have not yet

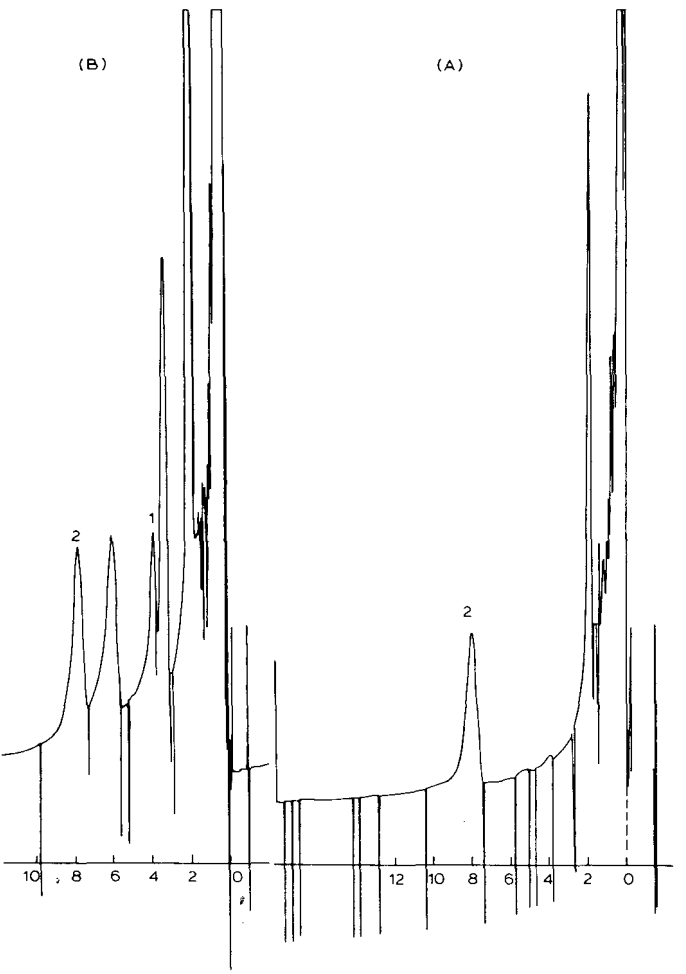

Fig. 3. Chromatograms from urine samples taken pre-dose (A), and 2 h post-dose (B). Peaks: 1 = etoperidone, 2 = trazodone.

TABLE I

CONCENTRATIONS OF UNCHANGED DRUG IN SERUM AND PLASMA, AFTER ADMINISTRATION OF A SINGLE ORAL DOSE OF 50 mg OF ETOPERIDONE

Time (h)	Concentration of etoperidone (ng/ml)			
	Serum		Plasma	
	Mean*	Coefficient of variation (%)	Mean*	Coefficient of variation (%)
0.5	305	0	199	2.5
1	321	3.1	221	6.6
1.5	129	7.3	103	3.4
2	88	4.0	75	0
3	50	2.0	58	1 sample
5.5	23	9.7	21	7.5
8	no sample		21	1 sample
24	none detected		none detected	

*Mean of two samples.

been identified, but all show larger peak areas than the etoperidone, and all are present for up to 36 h.

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Note

Sensitive high-performance liquid chromatographic analysis of moxalactam in biological fluids

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(First received April 13th, 1982; revised manuscript received June 30th, 1982)

Moxalactam (LY 127935; 6059 S) (Fig. 1) is a new parenteral semisynthetic β-lactam antibiotic in which an oxygen replaces the sulfur atom in the cephem

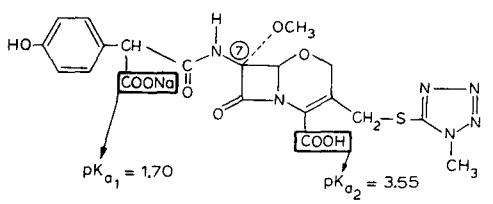

Fig. 1. Chemical structure of moxalactam.

ring. This antibiotic possesses two acidic functions: one on the cephem nucleus (as in cephalosporins), and the other on the side-chain. Moxalactam is a polar molecule, active in vitro against a variety of gram-positive and gram-negative bacteria including staphylococci, Enterobacteriaceae and *Pseudomonas* [1]. Plasma levels [2—4] and pharmacokinetics of moxalactam in normal volunteers [5—8] or in patients [9] have been determined by microbiological assay.

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Recent papers have described the use of high-performance liquid chromatographic (HPLC) methods for the quantitation of moxalactam in human urine [10] and to compare the pharmacokinetics of cefazolin and moxalactam after deproteination of the plasma [11]. Assay techniques used in pharmacokinetic studies of moxalactam must be capable of measuring plasma levels of at least 100 ng/ml. This report describes a sensitive (50 ng/ml) and selective ion-pair HPLC method for the quantitation of moxalactam present in biological fluids, and the application of this method in pharmacokinetic studies.

These results were presented in part at the 2nd Mediterranean Congress of Chemotherapy [12].

EXPERIMENTAL

Apparatus

A Waters Assoc. (Paris, France) HPLC system was used. This system includes a dual 6000A delivery system, a WISP 710 sample processor, and an M440 absorbance UV detector (280 nm fixed wavelength). An Omniscribe B 5000 recorder (Houston Instruments) (10 mV) was used. Data were analyzed by means of a computer program [13]. The column was a reversed-phase μBondapak C_{18} (10 μm; 30 cm × 3.9 mm I.D.) from Waters Assoc.

Drug standard, plasma and urine samples

Moxalactam (LY 127935) was supplied by E. Lilly Laboratories (Saint-Cloud, France). Five healthy adults (three women, two men) received a 1-g dose of moxalactam intravenously. The antibiotic was dissolved in sterile physiological saline (30 ml).

Blood samples (5 ml) were collected in glass tubes (Vacutainers, 10 ml) containing heparin, immediately before and at selected intervals following administration of the drug. The samples were immediately centrifuged at 1000 *g* for 10 min. The plasma fraction was carefully separated using a sera-clear and was then frozen at −70° C until taken for assay.

Urine samples were taken at selected intervals and they were also stored at −70° C until taken for assay, at which time they were directly injected into the chromatographic system after dilution with distilled water.

All standards were prepared by dilution of 1 g of moxalactam per 100 ml (primary stock solution) of distilled water to concentrations ranging from 2 μg/ml to 4 mg/ml. These standard solutions were used as the basis for repeatability tests and for the preparation of standard curves. Calibration curves were plotted from data obtained from standard solutions diluted with blank plasma (50 μl plus 950 μl blank plasma) to concentrations ranging from 0.1 μg/ml to 200 μg/ml.

Chemicals

Reagent grade ethyl acetate, acetonitrile (Uvasol grade), chloroform, hydrochloric acid, calcium chloride, potassium dihydrogen phosphate and disodium hydrogen phosphate (E. Merck, Darmstadt, G.F.R.) were used without further purification.

Procedure

In a 10-ml glass-stoppered centrifuge tube, 1.0 ml of plasma and 0.5 ml of 0.4 *M* hydrochloric acid were mixed with 7 ml of ethyl acetate and extracted by shaking for 5 min. After centrifugation, (5 min at 1000 *g*), a 6-ml portion of the organic phase was placed in a new 10-ml glass-stoppered centrifuge tube with 0.350 ml of phosphate buffer (pH 7). After the tube was shaken and centrifuged, the upper organic phase was discarded by aspiration, and the aqueous phase was washed with 5 ml of chloroform. Again the tube was shaken and centrifuged, and a 10—100 μl portion of the upper aqueous phase was chromatographed.

The mobile phase used was a solution of acetonitrile and phosphate buffer (pH 7) (170:822) containing per litre 25 mg of calcium chloride and 8 ml of tetra-*n*-butylammonium hydroxide (Pic A reagent, Waters). Prior to use, the eluate was passed through a Millipore filter (0.45 μm) and deaerated under vacuum.

The column flow-rate was 2 ml/min. The eluate was monitored at the UV wavelength of 280 nm and quantitation was based on the peak height.

RESULTS

A representative chromatogram from the plasma prepared with 5 μg/ml moxalactam and plasma samples obtained 8, 10, and 12 h after a 1-g intravenous injection are shown in Fig. 2. Neither control plasma nor urine samples gave any interfering peaks on the control chromatograms. Under the described chromatographic conditions, the retention time is 4.6 min. The relationship between plasma concentration of the drug and the peak height is linear: $Y = 1.02X + 0.68$, $r = 0.9995$, and $Y = 19.8X + 0.08$, $r = 0.9999$, in the calibration range 0.4--12.5 μg/ml and 12.5—200 μg/ml, respectively.

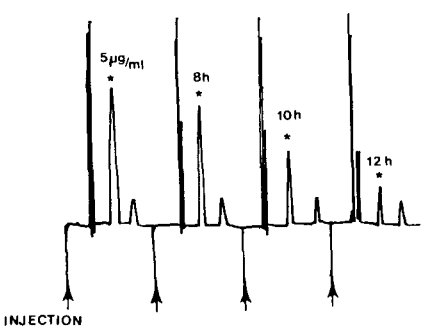

Fig. 2. Chromatograms of extracts of plasma spiked with moxalactam (5 μg/ml) and of plasma samples at various times after the intravenous administration of 1 g of moxalactam (a.u. = 0.05, injection volume = 25 μl).

The precision and accuracy of the plasma assays are summarized in Table I.

The mean plasma level after a 1-g intravenous injection of moxalactam (Fig. 3) was observed to decline in three phases. The average drug concentration at 12 h was 2.2 μg/ml and the biological half-life of the γ phase ($t_{1/2\gamma}$) was 2.74

TABLE I

WITHIN-DAY REPEATABILITY FOR PLASMA SAMPLES AT THREE CONCENTRATIONS

Concentration (μg/ml)	n	Injection volume (μl)	Sensitivity	Mean peak height (mm)	Standard deviation	Relative standard deviation (%)
40	10	10	0.05	172.6	3.9	2.3
5	6	40	0.05	75.3	2.7	3.6
0.5	9	40	0.01	39.5	5.1	12.9

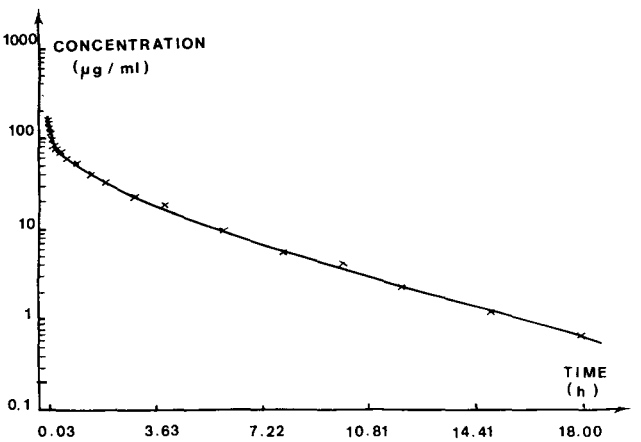

Fig. 3. Plasma concentration of moxalactam in a healthy volunteer following administration of 1 g intravenously. The concentration—time curve is fitted in a three compartment body model.

(± 0.28) h. For two subjects, plasma concentrations at 18 h were still at 0.53 μg/ml. The mean apparent volume of distribution was 0.26 l/kg. Approximately 57% of the administered dose was recovered in the urine within the first 12 h.

DISCUSSION

The extraction procedure is complex, due to the presence of the two acidic functions on the moxalactam molecule. The pK_a of the carboxylic acid groups was determined potentiometrically to be 3.55 for the group on the nucleus and 1.70 for the group on the chain (Fig. 1). To extract moxalactam by means of an organic phase, the pH has to be less than 1.7, as the pK_a for the acidic group on the side-chain is 1.7. Since the moxalactam molecule is polar, a polar organic solvent is required: ethyl acetate in this instance.

A reversed-phase system is used with a mobile phase at pH 7 to which counter-ion (tetra-*n*-butylammonium hydroxide) and calcium chloride were

added, since at pH 7 the two acidic functions of the moxalactam molecule are in ionised form.

The limit of sensitivity is at the 50 ng/ml level for plasma or urine samples (Fig. 4). This sensitivity level enables more accurate measurement of the plasma concentrations throughout pharmacokinetic studies. The small relative standard deviations amongst the averages obtained from repeatability tests affirm that this technique is accurate.

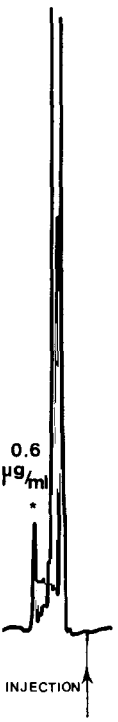

Fig. 4. Chromatogram of extract of plasma spiked with 0.625 µg/ml moxalactam (*) (a.u. = 0.02, injection volume = 50 µl).

Levels of moxalactam found to be present were lower than those of cefazolin, but generally higher than those of cephalosporins which have been studied in this laboratory (i.e. cefamandole, cefotaxime, cefoperazone, ceftazidime) [14, 15].

In summary, a method has been developed for the extraction and quantitation of moxalactam in plasma. This method enables determination of levels below 100 ng/ml of plasma. In terms of rapidity and selectivity, this method offers significant advantages over previously published microbiological procedures and has permitted the study of the pharmacokinetics of moxalactam.

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CHROMBIO. 1414

Note

Studies on the quantitation of chlorambucil in plasma by reversed-phase high-performance liquid chromatography

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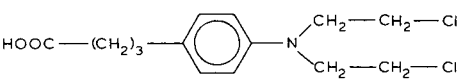
(First received April 14th, 1982; revised manuscript received June 28th, 1982)

Chlorambucil, 4-{*p*-[bis-(2-chloroethyl)amino]phenyl}butyric acid (Leukeran®), a bifunctional alkylating agent, is an antineoplastic drug widely used in the treatment of chronic lymphocytic leukemia, malignant lymphomas, ovarian carcinomas, and Hodgkins disease [1—4].



Limited pharmacokinetic studies on chlorambucil in humans are primarily due to the lack of a simple, sensitive, yet efficient, means of monitoring changes in concentration of the drug in biological samples. A number of UV spectrophotometric [5, 6] and chlorine titrimetric [5, 7] methods have been described for chlorambucil analysis. More recently, measurement by high-performance liquid chromatography [8—10] and gas chromatography—mass spectroscopy [11—13] has become possible. The high-performance liquid chromatographic (HPLC) method reported by Newell et al. [8] using a methanol gradient has a close retention time for chlorambucil and biological background. Hence, interference occurred at our hands. Leff and Bardsley [9] described an HPLC method which required derivatization of chlorambucil prior to analysis. In addition to being time-consuming, decomposition of chlorambucil may occur under this condition. For the method of Ehrsson et al. [10], there is no description of application to biological samples.

The assay procedure described herein involves a sensitive, rapid, isocratic HPLC analysis of chlorambucil.

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MATERIALS AND METHODS

Reagents

HPLC grade acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and a 0.2% solution of acetic acid (Fisher Scientific, Fair Lawn, NJ, U.S.A.) in water were the two solvents used. Both solvents were glass distilled and were filtered with Gelman 0.2-μm filters and degassed under vacuum prior to use. Chlorambucil was a gift from the Burroughs-Wellcome Co., Research Triangle Park, NC, U.S.A. All other chemicals and reagents were of the highest purity available and were obtained from commercial sources. Human blood plasma was provided by the University of Texas Medical Branch Blood Bank, Galveston, TX, U.S.A.

Instruments

All analyses were run on a Waters Assoc. Model 270 liquid chromatograph with microprocessor-controlled gradient system (Waters Assoc., Milford, MA, U.S.A.). This consisted of the 6000A and M45 solvent delivery systems, Model 720 system controller, Model 730 data module, and Model 710B WISP (Waters Intelligent Sample Processor). Separations were accomplished using Waters Assoc. Radial-Pak C_{18} cartridges containing 10-μm diameter particles. Absorption of the eluents was monitored with a Beckman Model 155 variable-wavelength detector (Altex, Berkeley, CA, U.S.A.).

Centrifugation of samples was achieved at 12,800 *g* with a desk-top, Model 5412 Eppendorf centrifuge (Brinkman Instruments, Westbury, NY, U.S.A.).

Standard curve of chlorambucil

Solutions of chlorambucil in acetonitrile were freshly prepared in concentrations of 0.5, 1, 2, 4, and 5 μg/ml and kept at −20°C. Solvent composition and sample injections were automatically carried out by the system controller and the automatic sample processor. The mobile phase consisted of an isocratic mixture of 65% acetonitrile and 35% dilute (0.2%) acetic acid. The flow-rate was set at 1 ml/min. Absorbance was monitored at 263 nm and recorded on the data module. The module chart speed was 1 cm/min, and quantitation of peak areas was carried out by the data module integrator. A standard curve was developed using the acetonitrile solutions of chlorambucil standards; 50 μl of each were injected.

Standard curve of chlorambucil in human plasma

Appropriate aliquots of the standard chlorambucil solutions were added to human plasma, obtained from the blood bank, to provide concentrations of 0.1, 0.5, 1, 5 and 10 μg/ml of the drug in plasma. Aliquots (100 μl) of chlorambucil-spiked plasma were taken from each tube, mixed with 4 volumes of acetonitrile (i.e. 400 μl of acetonitrile added to 100 μl of plasma), and vortexed. Macromolecular components precipitated by acetonitrile were separated from chlorambucil solution by centrifugation (2 min). To reduce further the concentration of unprecipitated plasma proteins and biological constituents that might interfere with the chromatographic separation of chlorambucil, the samples were rapidly frozen in a solution of dry-ice—acetone and again

centrifuged (2 min). Similar techniques were described by Chang et al. [14] for the analysis of melphalan, another nitrogen mustard used in the treatment of multiple myeloma. Aliquots (200 μl) of the upper, clear supernatants were drawn off, placed in vials and assayed immediately (75-μl injections). Extraction efficiency was 96 ± 8%. Various chlorambucil plasma concentrations were utilized to study the effects of freezing (in the above-mentioned dry-ice—acetone bath), acidification and volume of the extracting solvent on acetonitrile extraction efficiency of chlorambucil. In the above procedure plasma samples are acidified to pH 3.0 prior to extraction.

Clinical application of the analytical technique

Blood samples from patients undergoing chlorambucil chemotherapy were obtained under the supervision of David Gill, M.D., at the Department of Medicine, Division of Medical Oncology and Hematology of the University of Texas Medical Branch at Galveston. The patients were administered 18—40 mg chlorambucil tablets orally with water following an overnight fast. These patients were receiving chlorambucil for chronic lymphocytic leukemia. Blood samples (400 μl) were placed in heparinized micro-test tubes and centrifuged at 12,800 *g* for 2 min. The plasmas were subsequently prepared for analysis by the previously described procedure.

Time—course of chlorambucil in rats

Male Sprague—Dawley rats, six per group (250—300 g, Charles River, Wilmington, MA, U.S.A.) were treated orally with 20 mg/kg chlorambucil. Blood samples were taken from the supraorbital plexus into heparinized 500-μl microcentrifuge tubes and kept on ice. Blood samples were collected at 5, 15, and 30 min and at 1, 2, 4, 6, 12, and 28 h after treatment. Samples were processed for chlorambucil analysis as described before.

RESULTS AND DISCUSSION

Fig. 1 illustrates the HPLC resolution of chlorambucil in plasma. It shows chromatograms of normal blood plasma supplied by the blood bank (A) and of blank plasma spiked with 10 μg/ml chlorambucil (B). The retention time of chlorambucil by the previously described chromatographic conditions is 7.65 min. Detection of chlorambucil by this method is not accompanied by interference from plasma constituents.

Standard curve

The regression line for the standard curve of chlorambucil in acetonitrile is $Y = 0.00137X + 0.08869$, where Y is the concentration and X the absorbance; the correlation value is 0.9986. The standard curve of chlorambucil in plasma yields a regression line of $Y = 0.00279X + 0.01395$ with a correlation value of 0.9999. This procedure allows the quantification of chlorambucil in plasma with a high degree of accuracy. In separate determinations of the spiked standard chlorambucil sample (1 μg/ml) the range of difference in area of the chlorambucil peak is only ± 6% of the mean.

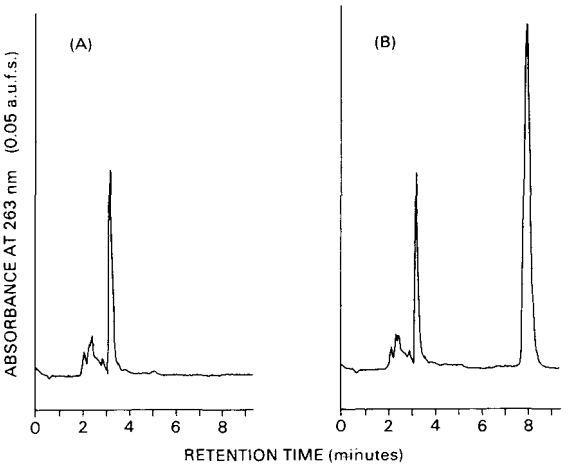


Fig. 1. HPLC analysis of chlorambucil in human plasma using an isocratic mobile phase of acetonitrile and 0.2% acetic acid buffer (65:35, v/v) with a flow-rate of 1 ml/min and UV detection at 263 nm. (A) Blank plasma; (B) plasma spiked with chlorambucil.

Effect of temperature

A major difference in extraction efficiency is observed when the extraction procedure is followed by freezing of the samples at −70°C versus not freezing (room temperature, 27°C). As seen in Fig. 2, failure to freeze the samples prior to centrifugation reduces the recovery of chlorambucil by approximately 60%. Low temperature provides efficient denaturation and precipitation of biological material. Hence, hydrophobic and other physical bonding forces between drug and biological macromolecules decrease and free drug concentration increases.

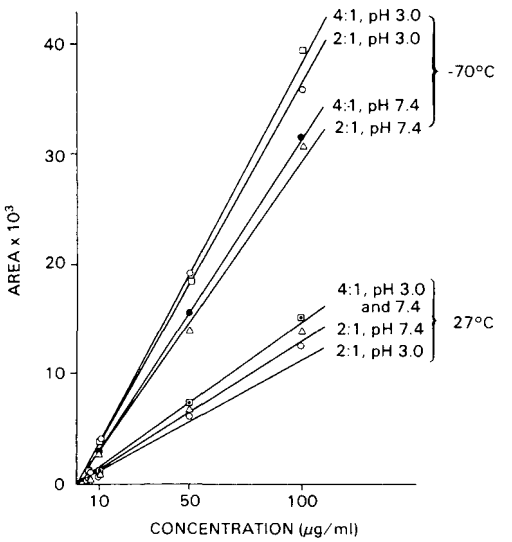


Fig. 2. Effect of pH, volume of extractant, and temperature on acetonitrile extraction efficiency of chlorambucil. (□) 4:1 acetonitrile/plasma ratio, pH 3.0; (○) 2:1 acetonitrile/plasma ratio, pH 3.0; (●) 4:1 acetonitrile/plasma ratio, pH 7.4; (△) 2:1 acetonitrile/plasma ratio, pH 7.4.

Effect of pH

Plasma samples spiked with various amounts of chlorambucil were acidified to pH 3.0 by adding 0.1 ml of 0.2 *N* hydrochloric acid to the sample prior to its extraction with acetonitrile. Acidification followed by extraction at 27°C had no effect on the extractability of chlorambucil from biological fluids as compared to unacidified plasma at this temperature. However, acidification followed by extraction and cooling of the mixture to −70°C enhanced the extraction efficiency of chlorambucil 15% over the unacidified samples (Fig. 2).

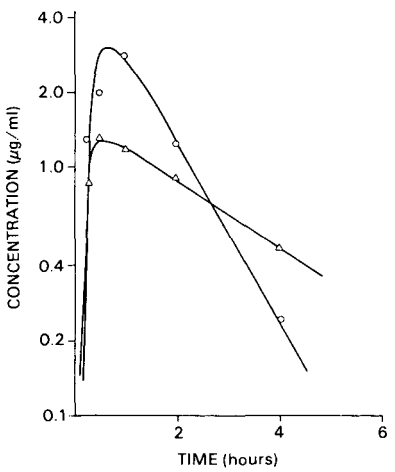
The nitrogen atom in the bis-2-haloethylamine moiety of chlorambucil is not strongly basic [15]. Ionization at pH 3 may occur, although it will be minimal, particularly at low temperatures. This minimal ionization is compensated by the unionized form of the carboxyl group of the butyric acid moiety of the compound, thus slightly enhancing its extractability in organic solvents.

Effect of solvent/plasma ratio

The effect of using various acetonitrile/plasma ratios for extraction of chlorambucil is also seen in Fig. 2. Using a 4:1 acetonitrile/plasma ratio resulted in an insignificant increase in the efficiency of chlorambucil extraction over the corresponding 2:1 ratio.

Application

The results of the analyses of chlorambucil concentration versus time in the plasma of two cancer patients receiving chlorambucil chemotherapy are shown in Fig. 3. Essentially, both patients showed a rapid attainment of peak drug concentration followed by a rapid decline in drug plasma levels. These data are in agreement with previous studies [9, 12]. A similar pattern is also observed in the analysis of rat data depicted in Fig. 4 (pooled results). The plasma levels of chlorambucil peak in less than 2 h, then decline rapidly with an approximate half-life of 1.79 h.



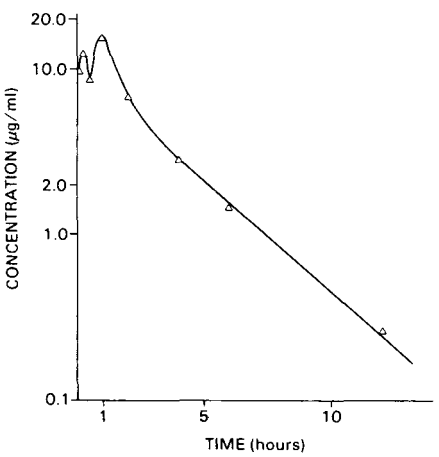


Fig. 3. Plasma levels of chlorambucil in two patients receiving chlorambucil chemotherapy.

Fig. 4. Concentration—time profile of chlorambucil in rats. Each point represents the mean of six animals. The standard error ranges from 0.69 to 2.61 μg/ml.

Elucidation of the pharmacokinetics of chlorambucil, hence its chemotherapeutic efficiency in patients, can be greatly enhanced by the use of a simple, yet efficient method of chlorambucil analysis. As such, significant advantages of the technique reported here can be readily seen. This technique allows quantification with a high degree of accuracy. Sample preparation is rapid and efficient. Clear resolution of the drug in biological samples is achieved with no background interference. Application of this technique toward patient studies is now in progress in our laboratory and should yield a better understanding of chlorambucil pharmacokinetics and the mechanisms of its chemotherapeutic and toxic actions in cancer patients.

ACKNOWLEDGEMENT

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Note

Determination of α-amanitin by high-performance liquid chromatography

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Amanita phalloides is one of the mushrooms most frequently responsible for fatal mushroom poisoning [1]. Various amatoxins (α-, β-, γ- and ε-amanitin, amanin, amanullin) (Fig. 1) have been identified, and of these α-amanitin is one of the most toxic and abundant [2—6].

Most published analyses of α-amanitin have involved separation by thin-layer chromatography [7—13] and radioimmunoassay [14, 15].

This paper reports a method using reversed-phase high-performance liquid chromatography (HPLC) with UV detection for the determination of α-amanitin.

MATERIALS AND METHODS

Chemicals and reagents

Standards were obtained from commercial sources; acetonitrile and methanol (HPLC grade) were obtained from Merck, Darmstadt, G.F.R. α-Amanitin was obtained from Boehringer, Mannheim, G.F.R. Water was demineralized, distilled in glass and filtered before use. Human blank serum was obtained

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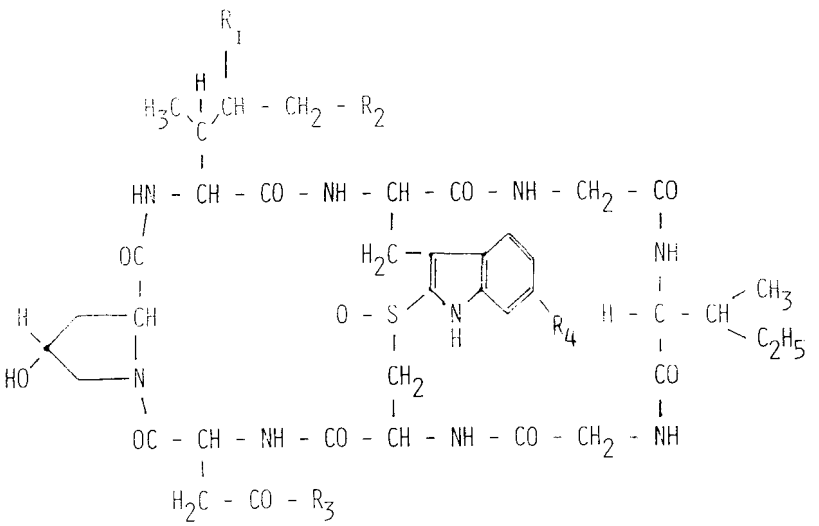


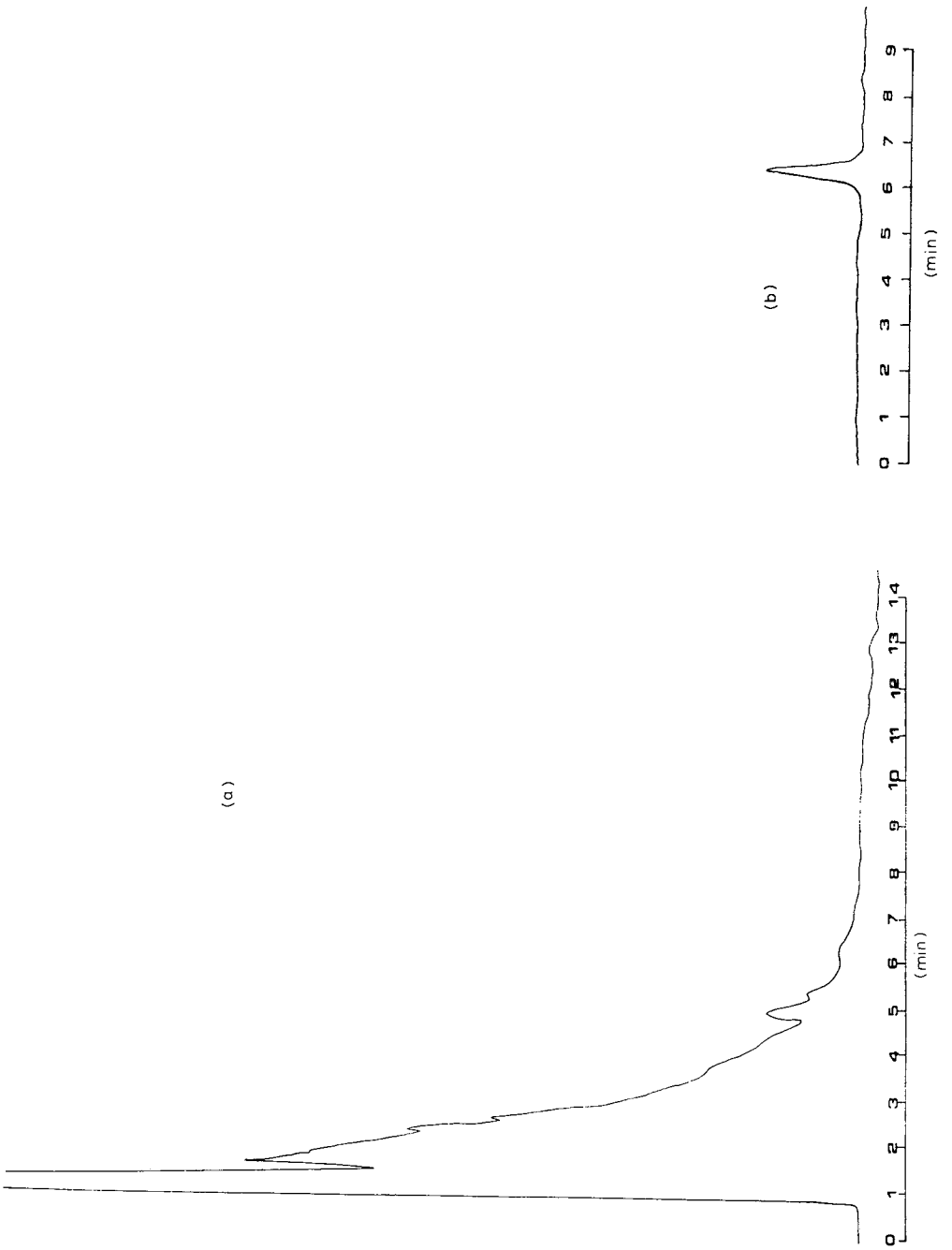
	R_1	R_2	R_3	R_4
α-Amanitin	-OH	-OH	$-NH_2$	-OH
β-Amanitin	-OH	-OH	-OH	-OH
γ-Amanitin	-OH	-H	$-NH_2$	-OH
ε-Amanitin	-OH	-H	-OH	-OH
Amanin	-OH	-OH	-OH	-H
Amanullin	-H	-H	$-NH_2$	-OH

Fig. 1. Chemical structures of amatoxins.

from A.V.I.S., Milan, Italy. The serum of poisoned patients was supplied by Trent Hospital, Trent, Italy.

Instrumentation

A Gilson (Villiers-Le Bel, France) high-performance liquid chromatograph was used equipped with a Holochrome (Gilson) dual-beam UV detector set at 302 nm, the wavelength of maximum absorption of α-amanitin. The column system consisted of a Spherisorb 5-μm ODS precolumn (Phase Separations, Queensferry, Great Britain) and a Spherisorb 5-μm (Phase Separations) analytical column (25 cm × 4.6 mm I.D.). It was eluted with acetonitrile—water (15 : 85) at a flow-rate of 1 ml/min. Injections were made with a Rheodyne 6-port valve fitted with a 20-μl sample loop. The output of the detector was displayed on a recorder (Model N 2, Gilson) having a full-scale range of 100 mV. Chart speed was 0.5 cm/min.


(a)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
(min)
(b)
0 1 2 3 4 5 6 7 8 9
(min)

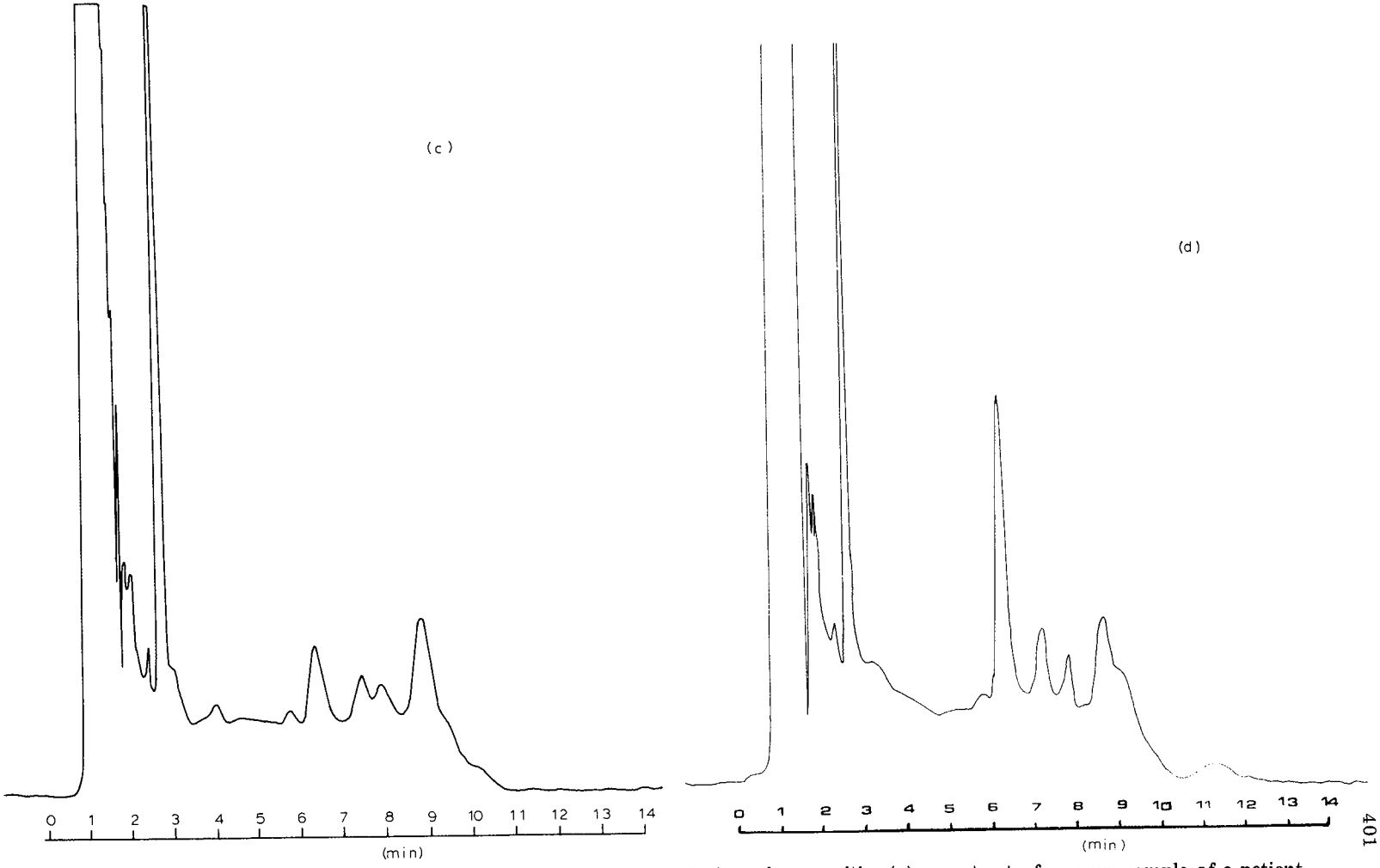


Fig. 2. HPLC profiles of: (a) an extract of blank serum; (b) a standard solution of α-amanitin; (c) an extract of a serum sample of a patient poisoned with *Amanita phalloides*; (d) an extract of a serum sample from a poisoned patient, spiked with α-amanitin.

Analytical procedure

At the beginning a calibration curve was obtained. Methanolic standard solutions corresponding to 20, 10, 5, 2.5 and 0.5 μg/ml α-amanitin were prepared. Each point is the mean of three determinations. Every injection was carried out filling the sample loop. A least-squares regression relationship between concentration and the peak height was calculated.

Serum of some patients poisoned by *Amanita phalloides* was extracted with methanol (1:2, v/v) modified from ref. 10). The sample was centrifuged twice at 2500 *g* for 15 min and the supernatant obtained was evaporated to a volume of 100 μl; 20 μl filling the sample loop were injected into the instrument. The same extraction method was carried out for the blank serum.

RESULTS AND DISCUSSION

The calibration curve of the standard solution gave good linearity. Least-squares linear regression analysis was used to determine the slope, y-intercept, and correlation coefficient. Using peak height: $y = 0.4710X - 0.0149$ (r= 0.9999).

The minimum detectable amount of α-amanitin by this method was about 10 ng (20 μl of a 0.5 μg/ml solution).

The chromatograms of samples from some patients poisoned by ingestion of *Amanita phalloides* show a peak at the same retention time of the standard, not present in the blank serum and becoming higher if the serum of the poisoned patient is spiked with α-amanitin.

A chromatogram obtained from blank plasma is shown in Fig. 2a. Fig. 2b shows a chromatogram of a standard solution of α-amanitin in methanol. Fig. 2c is a chromatogram of a sample from a patient who died from ingestion of *Amanita phalloides*. Fig. 2d shows a chromatogram of serum of a poisoned patient spiked with α-amanitin standard.

Samples from patients poisoned with *Amanita phalloides*, compared with authentic α-amanitin, show values approximately between 70 and 90 ng/ml of serum.

In conclusion the HPLC method described has the following advantages: (a) α-amanitin is separated from other serum components; (b) the chromatographic analysis can be completed within 15 min; (c) microquantities (ca. 50 ng/ml α-amanitin in human serum) can be determined.

The values we have found for α-amanitin in the serum of the poisoned patients relate to a limited number of cases. For this reason it will be necessary to obtain a larger number of samples to be able to effect a statistical evaluation. These studies should also take into consideration other biological fluids such as urine.

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Note

Simultaneous analysis of methyl methacrylate and methacrylic acid in blood by double isotope derivative dilution analysis

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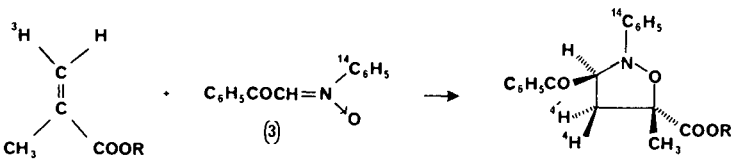
(First received April 27th, 1982; revised manuscript received June 30th, 1982)

Polymethylmethacrylate is widely used in orthopaedic surgery for the fixation of prostheses [1—4]. It has been used with particular success in total hip replacement, an operation that is being performed on an ever-increasing scale. Suggestions that the entry of methyl methacrylate into the blood circulation was responsible for the frequent and occasionally irreversible hypotensive episodes during operations for total hip replacement [3, 5—9] made it imperative that a method for the analysis of methyl methacrylate should be developed. We had shown that in blood in vitro, methyl methacrylate is hydrolysed enzymatically to methacrylic acid [10]. Since methacrylic acid has been reported in animal experiments to exert cardiovascular effects similar to those of methyl methacrylate [11] it was necessary to be able to determine levels of methacrylic acid as well as those of the methyl ester.

The rapid enzymatic hydrolysis of methyl methacrylate explains the wide discrepancies in published estimations of methyl methacrylate levels in blood samples from patients undergoing total hip replacement, when analysis was carried out by gas chromatography at unspecified times after withdrawal of the blood sample from the patient [12—16]. In order to circumvent this problem a procedure was devised whereby a mixture of methyl-(*E*)-[3-^{3}H]methacrylate (1) or the corresponding acid (2) was added to the blood sample immediately



(1) R = CH_3
(2) R = H

(4) R = CH_3
(5) R = H

eqn.1

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after withdrawal from the patient. The methacrylate, diluted by endogenous material, was re-extracted into halothane and treated with N-[U-^{14}C] phenyl-C-benzoylnitrone (3) to give the stable isoxazolidines (4) and (5) (eqn. 1). The reaction mixture was separated by high-performance liquid chromatography (HPLC) on a column of μBondapak C_{18} (Fig. 1), the individual derivatives (4) and (5) were collected and their $^{3}H/^{14}C$ ratios were determined by liquid scintillation counting. Concentrations of methyl methacrylate and methacrylic acid were calculated by comparing these isotope ratios with those in the adducts derived from reaction between undiluted methyl-(*E*)-[3-^{3}H] methacrylate (1) or (*E*)-[3-^{3}H] methacrylic acid (2) and the ^{14}C-labelled nitrone (3).

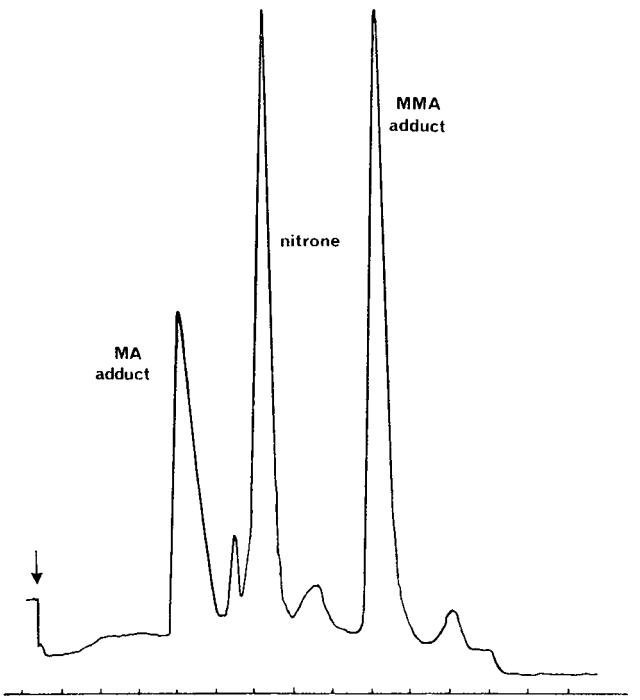

Fig. 1. High-performance liquid chromatogram of the product obtained by reacting methyl-methacrylate and methacrylic acid with N-phenyl-C-benzoylnitrone. For conditions, see under Experimental. MA adduct: derivative formed with methacrylic acid; MMA adduct: derivative formed with methyl methacrylate. The arrow indicates the point of injection. Detection of the adducts was made by monitoring their absorbance at 254 nm.

The required labelled methacrylic acid (2) was obtained using a procedure we have developed for the stereospecific synthesis of tritiated acrylates [17]. Conversion into the methyl ester was carried out using the method of Shaw et al. [18]. The ^{14}C-labelled nitrone was obtained by a modification of published procedures [19].

The results obtained by the application of these methods to the analysis of blood samples from patients undergoing total hip replacement have been described [20].

EXPERIMENTAL

Proton nuclear magnetic resonance (nmr) spectra were determined with a JEOL MH100 spectrometer and infrared spectra with a Hilger H900 Infrascan or Perkin-Elmer 357 Grating infrared spectrometer using KBr discs. HPLC was performed on a Waters Assoc. liquid chromatograph equipped with a Model 6000 solvent delivery system, fixed-wavelength (254 nm) UV detector and septum injector. Radio-activity was measured using a Packard Tri-Carb 2002 liquid scintillation counter, using solutions in Aquasol (New England Nuclear, Boston, MA, U.S.A.) or NEN 260 (Nuclear Enterprises Ltd., Edinburgh, Great Britain). All radiochemicals were purchased from The Radiochemical Centre, Amersham, Great Britain.

N-Phenyl-C-benzoylnitrone [as (3)]

The unlabelled material was prepared according to the published procedure [19].

N-[U-^{14}C]Phenyl-C-benzoylnitrone (3)

[U-^{14}C]Aniline. [U-^{14}C]Anilinium hydrogen sulphate (0.17 mg, 0.89 μmol, 50 μCi) and anilinium hydrogen sulphate (2.1 mg, 11 μmol) were dissolved in dilute ammonia (0.02 *M*, 5 cm^3). The solution was extracted with halothane (2 × 3 cm^3). The solvent was removed on a rotary evaporator at ambient temperature and the residue was dissolved in ethanol (1 cm^3).

[U-^{14}C]Nitrosobenzene. The ethanolic solution of [U-^{14}C]aniline was cooled to 6°C and treated with *m*-chloroperbenzoic acid (4.4 mg, 22 μmol). The mixture was stirred for 10 min, then transferred to a cooled (0°C) column (250 × 6 mm I.D.) containing basic alumina (10 g). The nitrosobenzene was eluted with ethanol (2 cm^3) and to the solution was added a solution of inactive nitrosobenzene (1 mg, 10 μmol) in ice-cooled ethanol (0.14 cm^3).

N-[U-^{14}C]Phenyl-C-benzoylnitrone (3). The ethanolic solution of [U-^{14}C]-nitrosobenzene was cooled to −5°C and added to a solution of N-phenacylpyridinium bromide (5 mg, 18 μmol) in water (0.5 cm^3) at 0°C. The mixture was cooled to −5°C and treated with aliquots (10 μl) of sodium hydroxide solution (0.125 *M*) every 2 min (total: 120 μl). The solution was purified by HPLC on a column of phenyl Porasil B (37–75 μm) (102 cm × 6 mm I.D.) in methanol–water (1:1). The fractions containing the labelled nitrone were combined and the solution (32 cm^3) was treated with inactive nitrone (20 mg, 86 μmol) in methanol (10 cm^3). The solution was extracted with halothane (3 × 10 cm^3), then the combined extracts were concentrated to 8 cm^3 under reduced pressure and dried ($MgSO_4$) at 0°C. The solution was filtered and evaporated, and the residue was dried over P_2O_5 under reduced pressure. The residue was dissolved in dry methanol (3 cm^3) and stored in nine equal aliquots in sealed ampoules at liquid nitrogen temperature until required. The purity of one aliquot was confirmed by HPLC as described above. Each aliquot contained 2.08 mg (estimated by UV) of the [^{14}C]nitrone (3), specific activity 57 μCi mmol^{-1}.

5-Methyl-2-phenyl-3-benzoylisoxazolidine-5-carboxylic acid (5)

Methacrylic acid [as (2)] (0.86 g, 10 mmol) was added to a solution of

N-phenyl-C-benzoylnitrone [as (3)] (2.23 g, 10 mmol) in ethanol (25 cm^3). The mixture was heated at 50°C for 90 min. The solution was cooled and the crystalline product was recrystallised (ethanol) to give the derivative [as (5)] (2.88 g, 93%) m.p. 131—132°C. ν_{max} 1702 (COOH) and 1680 (CO) cm^{-1}. τ ($C^2H_3O^2H/C^2H_3COC^2H_3$) 4.55 (dd, 1H, *J* 6 Hz, 8 Hz, H-3), 6.72 (dd, 1H, *J* 8 Hz*, 10 Hz*, H-4), 7.44 (dd, 1H, *J* 6 Hz, 10 Hz, H-4'), 1.96- 3.4 (m, 10H, C_6H_5N, C_6H_5CO), 8.39 (s, 3H, CH_3C). Found: C, 69.85; H, 5.5: N, 4.5. $C_{18}H_{17}NO_4$ requires: C, 69.45; H, 5.5; N, 4.5%.

(E)-[3-^{3}H] Methacrylic acid (2)

To a slowly stirred solution of (*E*)-3-bromo-2-methylpropenoic acid (0.27 g) in ^3H_2O (1 g, 5 Ci g^{-1}) at 5°C was added, in aliquots over a period of 48 h, sodium amalgam (2.5%, 5.5 g). Following the final addition of sodium amalgam, the mixture was stirred for a further 25 h at 0—5°C. The aqueous solution was decanted, the mercury was washed with water (3 × 1 cm^3) and the combined aqueous solutions were acidified (congo red) with dilute hydrochloric acid. The solution was extracted with ether (3 × 3 cm^3); the ethereal solution was dried ($MgSO_4$), filtered and evaporated. The residue was dissolved in ethanol and the yield was determined by UV (24 mg, 20%, specific activity approximately 1.5 Ci g^{-1}).

Methyl [3-$^3H_1$] methacrylate (methyl-(E)-[3-^{3}H]-2-methylpropenoate) (1)

[3-^{3}H] Methacrylic acid (0.17 g, 975 μCi g^{-1}) in hexamethylphosphoramide (5 cm^3) was treated with sodium hydroxide solution (25%, 0.35 cm^3). The mixture was stirred for 90 min, treated with iodomethane (1.14 g), stirred for 2 h at 25°C, treated with dilute hydrochloric acid (5%, 10 cm^3) and extracted with diethyl ether (2 × 7.5 cm^3). Hydroquinone (1 mg) was added to the combined ethereal extracts which were washed with water (2 × 2.5 cm^3), boiled under reflux with mercury (1 cm^3) and anhydrous magnesium sulphate for 1 h and allowed to stand for 12 h. The ethereal solution was decanted, filtered and evaporated to give methyl-(*E*)-[3-^{3}H] methacrylate (0.1 g, 50%), pure by gas—liquid chromatography (15% SE-30 on Chromosorb W, 70°C).

Simultaneous analysis of methyl methacrylate and methacrylic acid in blood

Blood samples (2 cm^3) were added to centrifuge tubes containing known amounts of tritiated methyl methacrylate and methacrylic acid. The mixture was homogenised using a vortex mixer, treated with citrate buffer (pH 3.0, 1 *M*, pH 3.0) re-homogenised and extracted with halothane (2 cm^3) on the vortex mixer. The mixture was centrifuged (4000 *g* for 10 min), and the serum and erythrocytes were aspirated off from the halothane solution. To the halothane solution was added a solution of N-[U-^{14}C] phenyl-C-benzoylnitrone (3) (20 μg) in ethanol (40 μl) and the mixture was heated at 50°C for 1 h. The solvent was removed by a stream of nitrogen at room temperature; the residue was dissolved in ethanol (100 μl) and injected on to a column of μBondapak C_{18} (10 μm) (Waters Assoc., Milford, MA, U.S.A.) (300 mm × 4 mm I.D.). The

*Presumed coupling constants. The signal due to H-4 was partly obscured by the signal due to solvent C^2H_3OH.

derivatives were separated (Fig. 1) by elution with acetonitrile—water (43.5: 56.5). The derivatives were collected in 2 cm^3 of eluent and added to 12 cm^3 of liquid scintillation fluid for radioactivity determination. Amounts (m) of unlabelled material present in the blood sample were calculated from the expression

$$m = \frac{m'(r_u - r_d)}{r_d}$$

where m' = amount of labelled methacrylate added, r_u = $^3H/^{14}C$ ratio in the isoxazolidine derived from undiluted [^{3}H]methacrylate and [^{14}C]nitrone, and r_d = $^3H/^{14}C$ ratio in the isoxazolidine formed between the diluted methacrylate from the blood sample and ^{14}C-labelled nitrone.

The sensitivity of this method is limited by the specific activities of the labelled compounds used in the analysis. In this study, the methacrylate used had a specific activity of $1.5 \cdot 10^5$ μCi $mmol^{-1}$ and the N-phenyl-C-benzoylnitrone had a specific activity of 57 μCi $mmol^{-1}$. The latter therefore limited the sensitivity of the method to 0.1 μg of methacrylate. With 100% recovery, this would give a total ^{14}C activity in the final sample of 126 disintegrations per minute (dpm), but since the recoveries obtained were less than this, final counts were of the order of 50 dpm (40 counts per min) which, being approximately twice the background count rate, were taken as the lowest acceptable level. Since 2-cm^3 blood samples were used in the clinical studies, the specific activity of the tritiated methacrylate used put an effective limit of sensitivity on the procedure of 0.05 μg (per cm^3 of blood) for both methyl methacrylate and methacrylic acid. The size of the aliquots of tritiated methacrylate also limited the sensitivity to about the same value. Aliquots of approximately 0.75 $\mu g/cm^3$ of blood were used. If this were diluted with 0.05 μg of endogenous methacrylate, the change in specific activity of the labelled methacrylate would be 7%, which was considered to be the level of accuracy of the final counting procedures (± 5%, approximately).

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Note

High-performance liquid chromatographic analysis of plasma levels of nalbuphine in cardiac surgical patients

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Nalbuphine, chemically derived from the opiate analgesic oxymorphone, is one of a new class of analgesics described as an agonist-antagonist. The administration of nalbuphine to patients with cardiac disease for anesthesia or analgesia is characterized by hemodynamic stability and lack of respiratory depression, even after large doses (> 1 mg/kg). However, studies of drug disposition and correlation of plasma levels with its effects have not been carried out for nalbuphine because a rapid, sensitive method for analysis has not been available.

Previously described methods for determination of plasma levels of nalbuphine are cumbersome and time-consuming [1], and thus have greatly limited understanding of the uptake, distribution and elimination of this drug. Knowledge of these factors will help to develop rational patterns of administration of the drug to surgical patients.

High-performance liquid chromatography (HPLC) using electrochemical detection has been used for the rapid analysis of nanogram quantities of morphine, oxymorphone, and other opioid analgesics.

EXPERIMENTAL

Materials

The nalbuphine and naloxone were obtained from Endo Laboratories Division of E.I. Dupont de Nemours, Wilmington, DE, U.S.A. The nalbuphine is a 10 mg/ml concentration with 0.1% sodium chloride, 0.94% sodium citrate, 1.26% citric acid anhydrous, 0.1% sodium metabisulfite and 0.2% of a 9:1 mixture of methyl- and propylparaben. Naloxone is available as a 0.4 mg/ml pre-

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paration in 8.6 ml of sodium chloride and 2.0 mg of 9:1 ratio of methylparaben and propylparaben.

Patients

The blood concentration of nalbuphine was determined in fourteen patients undergoing coronary artery bypass grafting (CABG) with mean age of 58 years (range 41—74) and seven patients having mitral valve repair or replacement (MVR) with mean age of 48.5 years (range 22—64), all of whom had given informed consent. The protocol was approved by the Human Investigation Committee of the University of Virginia School of Medicine. All patients were premedicated with nalbuphine 0.1 mg/kg and scopolamine 0.005 mg/kg intramuscularly 1.5 h prior to the study. After insertion of intravenous and intraarterial catheters, arterial blood was drawn for the control level of nalbuphine. The patients undergoing CABG then received nalbuphine 0.5 mg/kg body weight as a bolus through the central venous catheter. Blood was drawn after 5 min for determination of nalbuphine concentration. Additional increments of 0.5 mg/kg were given every 7 min to a total of 3 mg/kg and blood samples were taken 5 min after each increment. Blood samples were also taken at 2 min after skin incision and sternotomy, at the time of aortic cannulation for cardiopulmonary bypass, 30 and 60 min on cardiopulmonary bypass, immediately and hourly for 3 h postoperatively.

The patients having MVR received only 2 mg/kg total dose in increments of 1 mg/kg with determination of plasma levels 5 min after each dose. Otherwise, the sampling schedule was the same as for patients having CABG. No additional nalbuphine was given after the 2 mg/kg (MVR) or 3 mg/kg (CABG) dose. The amount of priming solution for the bypass circuit and the degree of hypothermia during bypass were noted.

Sample preparation

Plasma samples were prepared by centrifugation at 1040 *g* and frozen at −15°C until the time of analysis. Naloxone, 250 ng, was added to 1 ml of plasma as an internal standard. The sample was deproteinated with 0.25 *N* perchloric acid, vortexed, allowed to stand for 3—5 min, centrifuged and the supernatant collected. The supernatant was adjusted to pH 8 with 1 *N* sodium hydroxide and then extracted with 10 ml of ethyl acetate—2-propanol (9:1). After centrifugation the organic top layer was collected and evaporated to dryness with a gentle air stream. The sample residue was then redissolved in 1 ml of methanol and injected into the chromatograph using a 200-μl injection loop.

High-performance liquid column chromatography

An HPLC Bioanalytical Systems (West Lafayette, IN, U.S.A.), 25-cm Biophase ODS 5-μm chromatographic column and a Bioanalytical Systems LC-4A electrochemical detector in oxidation mode with a detector cell potential of 0.75 V were used. This voltage was chosen from the cyclic voltammograph obtained for nalbuphine (Fig. 1) on a Bioanalytical Systems Model CV-1B cyclic voltammeter. The mobile phase was 55% monobasic potassium phosphate (0.01 *M*) and 45% HPLC grade methanol which had been degassed and filtered through a 0.22-μm filter before use. The flow-rate was 0.8 ml/min resulting in

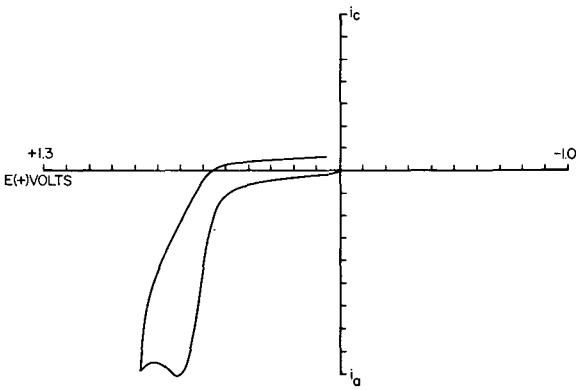

Fig. 1. Cyclic voltammograph of nalbuphine.

retention times of 400 sec for naloxone and 507 sec for nalbuphine. The plasma concentration of nalbuphine was determined from the ratio of the response of nalbuphine and naloxone in the samples when known amounts of naloxone had been added as the internal standard. A control plasma sample of nalbuphine containing 500 ng was analyzed daily in addition to the internal standard.

RESULTS

The analysis of nalbuphine was readily carried out using an electrochemical detector with HPLC. Plasma concentrations as low as 1 ng could be detected. The response of the detector was observed to be linear throughout the 1—400 ng range measured. Recovery from aqueous and plasma control samples was consistently 94% or greater. The daily control samples containing 500 ng of nalbuphine yielded concentrations of 496 ± 52.5 ng (mean ± S.D.) over a two-month period. The mean response factor ratio of nalbuphine to naloxone was 0.82 ± 0.02, an inter-day variation of 2.4% over 19 days. Within a given day the response factor varied 2.7%.

Fig. 2 is a chromatogram of human plasma containing internal standard naloxone. Fig. 3 is a typical chromatogram of nalbuphine extracted from human plasma. A small unidentified peak was seen after nalbuphine which may represent one of the metabolites. Since pure metabolites were not available, the source of this peak was not investigated. Plasma levels after premedication alone (control) were 14.6 ± 1.37 ng/ml in CABG and 14.6 ± 4.2 ng/ml in patients for MVR. There was an initial rapid increase in plasma nalbuphine as the 2 mg/kg (MVR) or 3 mg/kg (CABG) total doses were reached. Thereafter, a rapid decline occurred. Plasma levels in patients having MVR were consistently less than in patients undergoing CABG at all times. A small additional decline was associated with cardiopulmonary bypass. Blood levels decreased approximately 26% in both groups, with an approximately 50% increase in blood volume due to the use of the extracorporeal circuit in which the priming volume was 3042 ± 141 ml (CABG) and 2471 ± 245 ml (MVR) (mean ± S.E.M.). Systemic hypothermia to a mean of 25.3°C in both groups was accom-

Fig. 2. Chromatogram of human plasma with naloxone internal standard.

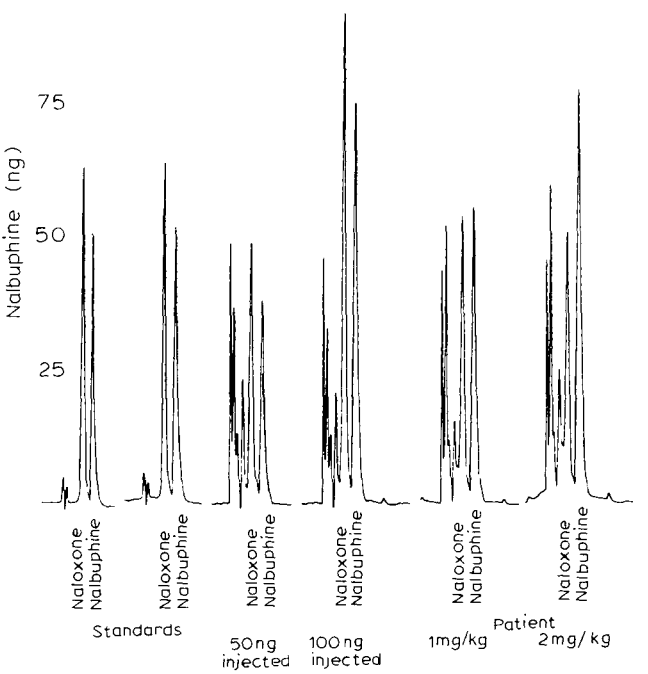

Fig. 3. Nalbuphine chromatogram in human plasma with naloxone as internal standard. Retention times: naloxone, 400 sec; nalbuphine, 507 sec.

panied by a further 76% decrease in plasma levels. Plasma levels of 45.5 ± 4.6 (MVR) and 72.6 ± 8.9 ng/ml (CABG), which exceeded or were comparable to known analgesic blood levels [2], were still present 3 h postoperatively (approximately 9 h from initial dose).

The $t_{½\beta}$ was determined from a log concentration plot against time for three samples drawn at hourly intervals postoperatively (Figs. 4 and 5) and was 3.0 h (MVR) and 3.5 h (CABG).

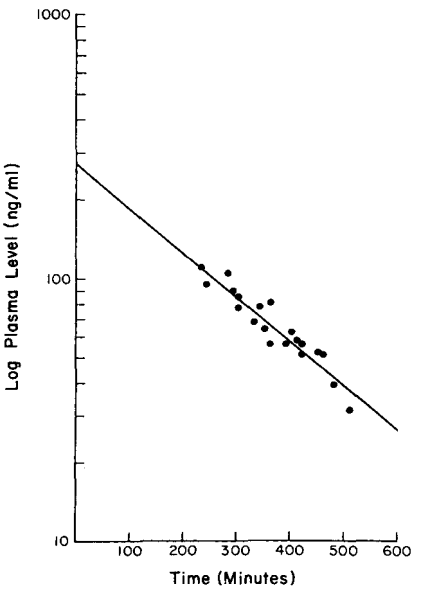

Fig. 4. Determination of $t_{½\beta}$ of nalbuphine in MVR.

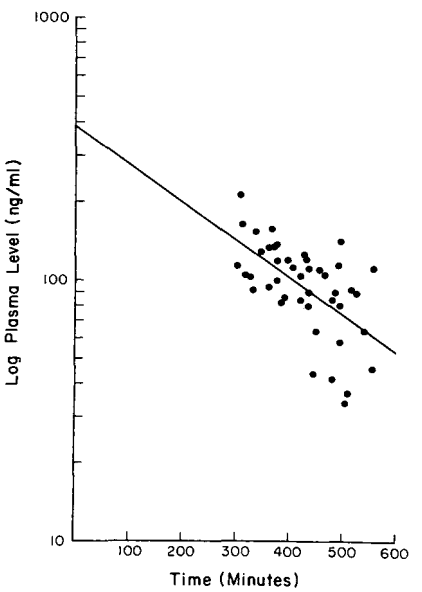

Fig. 5. Determination of $t_{½\beta}$ of nalbuphine in CABG.

DISCUSSION

Nalbuphine is metabolized in the liver to two metabolites, 14-hydroxy-7,8-dihydronormorphine and 14-hydroxy-7,8-dihydro-N-cyclobutyl-methylnormorphine (Fig. 6). It is excreted in the urine as unchanged nalbuphine, its conjugates, and the two metabolites [3] accounting for 71% of a dose [3]. The remaining drug is probably eliminated in the feces as a result of biliary excretion [3]. The assay described here was used for detection of nalbuphine and not for its metabolites. Our control samples were taken about 90 min following a premedication dose of 0.1 mg/kg which accounts for the lower plasma level than reported by previous investigators at 60 min after the dose [3]. Redistribution and metabolism of administered nalbuphine appears similar to that of other narcotics administered to cardiac patients [4]. Administration of heparin before bypass appeared to have no measurable effect on the nalbuphine plasma concentration. The nalbuphine level decreased with cardiopulmonary bypass due to dilution as has been reported with fentanyl [4]. Despite the use of

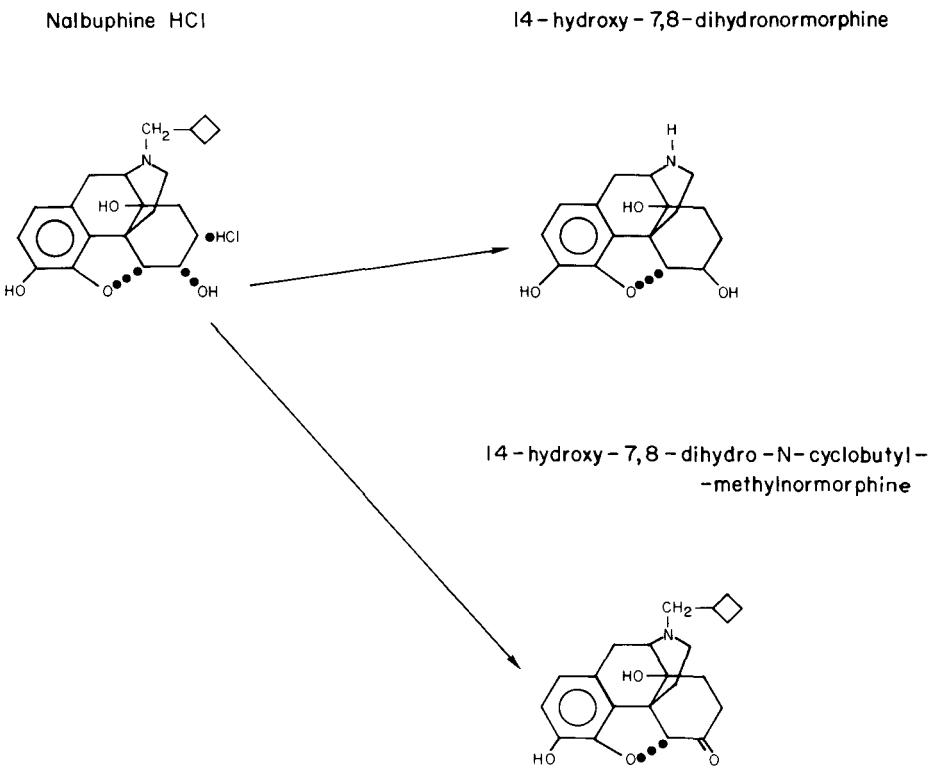

Fig. 6. Nalbuphine and its metabolites.

moderate systemic hypothermia, redistribution, metabolism, or excretion occurs during bypass. Because of the water-induced diuresis which usually occurs during cardiopulmonary bypass, the more rapid decline of plasma nalbuphine may be the result of greater urinary excretion of unchanged drug rather than metabolism. Since urinary levels of nalbuphine were not measured we can only speculate that this occurred. The presence of lower plasma levels in MVR at all sampling intervals was surprising and may be the result of a smaller total dose (2 mg/kg), greater urinary output due to diuretic administration, increased surgical bleeding, or the younger average age of patients [5]. Plasma concentrations, of more than 20 ng/ml, which are known to be analgesic [2], were present at 9 h after the initial dose. This is considerably longer than the 5 h reported by Weinstein et al. in dogs receiving 1 mg/kg doses [1]. It suggests that doses of nalbuphine in humans be spaced at longer intervals than the 3—6 h currently recommended.

The alpha or distribution half-life of nalbuphine has not been previously reported, but the beta or metabolism half-life of 3—3.5 h determined in this study is consistent with the duration of analgesia reported in humans by Tammisto and Tigerstedt [6]. The alpha or distribution phase of the plasma concentration curve could not be analyzed since single bolus dosing was not used and plasma sampling was not continued immediately after dosing.

The assay previously described for nalbuphine is a gas—liquid chromato-

graphic method using an electron-capture detector [1]. This method is time-consuming, requires dry conditions (relative humidity less than 50%) to derive the labile heptafluorobutyrate derivative of nalbuphine, and results in a recovery of only 70%. This paper describes a method which permits rapid assay of nalbuphine unaffected by moisture, and documents its applicability in clinical pharmacology. HPLC has been similarly used for the analysis of agonist narcotic drugs such as morphine [7] and oxymorphone [8]. It has also been used for analysis of the narcotic antagonists naloxone and naltrexone [8]. In the method used in the present study for the analysis of nalbuphine (a drug with mixed agonist and antagonist properties), naloxone was used as the internal standard. Common to all of these drugs is a phenolic hydroxy group. A dihydroxyphenolic residue common to the catecholamines epinephrine, norepinephrine, and dopamine allows them to be analyzed by this method and suggests that any drug containing this structure can be quantitatively analyzed by HPLC with an electrochemical detector.

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Note

Simple and sensitive high-performance liquid chromatographic procedure with electrochemical detection for the determination of plasma concentrations of trimeprazine following single oral doses

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The quantitation of phenothiazine drugs in the plasma of patients under treatment with these agents is difficult, due to the low levels encountered for such reasons as the low therapeutic doses used, a significant first-pass metabolism on oral administration, extensive metabolism to numerous metabolites, and a large volume of distribution partly resulting from extensive binding to multiple sites. This challenge of attaining adequate sensitivity (ng ml^{-1}) is compounded by the instability and adsorptive loss of phenothiazines in all stages of handling for analysis [1]. However, despite these problems there has in recent years been development of specific and sensitive analytical methods, which in some cases have been demonstrated as capable of quantitating the subnanogram plasma levels encountered 24 h and later after low single oral doses of phenothiazine drugs. These latter methods are generally either the more sensitive chemical methods, as for example those based on gas—liquid chromatography—mass spectrometry (GLC—MS) [1,2], and/or biological methods, especially those based on an immune response [1,3].

Trimeprazine is one of the more difficult phenothiazines to quantitate in plasma, being a potent antihistamine and antipruritic, the recommended maximum daily dosage not exceeding 15 mg as the tartrate salt. Indeed, although methods such as GLC with nitrogen—phosphorus dectection have been reported as being suitable for quantitating toxic levels of this drug [4], these authors are unaware of any suitable, sensitive and specific published procedure for quantitating trimeprazine in plasma after administration of the usual oral therapeutic doses. Recently a radioimmunoassay method, capable of quantitating 0.32 ng ml^{-1} of trimeprazine in a 200-μl plasma sample, was developed in these laboratories [5]. Since such methods are doubted for their specifity, a

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specific and sensitive chemical method was required to be developed in order to verify the biological procedure. Such a method, based on high-performance liquid chromatography with electrochemical detection (HPLC—ElCD), which is also simple and easy to adopt, was recently developed in these laboratories for chlorpromazine [6]. This paper reports a similar ultrasensitive (subnanogram) HPLC—ElCD method for trimeprazine, which for the first time allows determination of plasma concentrations after oral administration of therapeutic doses to patients. In fact, plasma concentration—time profiles in healthy volunteers up to 24 h after single 5-mg oral doses of trimeprazine tartrate were demonstrated.

EXPERIMENTAL

Materials

Trimeprazine tartrate (Panectyl®) was obtained commercially from Poulenc, Montreal, Canada. Prochlorperazine mesylate was a gift from Poulenc. HPLC solvents were distilled in glass prior to use; all other chemicals used were of the highest commercial grade available. Double-distilled deionized water was used to make stock solutions of trimeprazine and prochlorperazine. Appropriate dilutions of standard solutions made in distilled deionized water were placed in pooled fresh plasma obtained prior to analysis from blood collected from healthy volunteers.

Instruments

A Waters Model M-45 liquid chromatographic pump (Waters Scientific Co., Mississauga, Canada) fitted with a Model 7125 Rheodyne valve-loop (500-μl loop) injector, (Technical Marketing Associates, Calgary, Canada) was used. A Dupont Zorbax CN, particle size 5 μm, 250 × 4.6 mm column (Fisher Scientific and Co., Edmonton, Canada) was connected to a Bioanalytical Systems electrochemical detector (Technical Marketing Assoc., Calgary, Canada) operated in the oxidation mode at +0.9 V utilizing a 10-nA feed to a Model 56 linear recorder (Perkin-Elmer, Montreal, Canada). The mobile phase consisted of a 10% 0.1 *M* ammonium acetate buffer in acetonitrile. The mobile phase was degassed by Millipore filtration (Millipore Corp., Bedford, NA, U.S.A.) prior to use. The chromatograph was operated at ambient temperature with a flow-rate of 4 ml/min.

Preparation of standard curve

A stock solution of trimeprazine tartrate (100 μg ml^{-1} as the free base) was made monthly in distilled deionized water and stored at 4°C. Prochlorperazine mesylate stock solution (1000 μg ml^{-1} as the free base) was made monthly in distilled deionized water and stored at 4°C.

Appropriate volumes of a standard trimeprazine solution (100 ng ml^{-1}) diluted with distilled deionized water were added to fresh blank plasma (2.0 ml) and to this was added prochlorperazine (1.0 ml from a 100 ng ml^{-1} solution). The standard samples were then extracted in an identical fashion as unknown samples.

Extraction of samples

The extraction procedure was similar to that described earlier for the extraction of chlorpromazine [6]. To a 10.0-ml PTFE-lined screw-capped centrifuge tube (13 × 100 mm) were added 1.0 ml of sample plasma, 1.0 ml of fresh control plasma, 1.0 ml of prochlorperazine solution (concentration 100 ng ml^{-1}) and 0.5 ml of saturated sodium carbonate solution. The tube was mixed by Vortex for 5 sec and 5.0 ml of extraction solvent (3% isopropanol in pentane) were added. The tube was tightly capped and mixed for 15 min on a SMI multitube shaker (Canlab, Edmonton, Canada) followed by cnetrifugation for 10 min at 1725 *g* at ambient temperature. The upper organic layer was transferred to a clean test tube and the aqueous phase extracted as above with a further 5.0 ml of 3% isopropanol in pentane. The combined organic extracts were evaporated to dryness in a dry bath at 65°C. The residue was reconstituted in 200 μl of acetonitrile and 100 μl were injected into the chromatograph.

Plasma level study

Two healthy male volunteers weighing 61 kg and 85 kg were fasted overnight and then administered 5 mg of trimeprazine with 200 ml of water. Blood samples were collected in heparinized tubes (Venoject®, Becton Dickinson, through Canlab, Edmonton, Canada) avoiding contact of the blood with the rubber stopper. Samples (8.0 ml) were drawn at 0, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 24.0 h following drug administration. The blood samples were centrifuged and the plasma stored at −20°C until the time of analysis.

Quantitation and recovery study

Standard curves for trimeprazine were constructed by chromatographing spiked plasma samples and plotting peak height ratios versus concentration of the drug. Standard curves were established at the same time as analysis of samples from volunteers.

For the determination of recovery of trimeprazine and prochlorperazine at least five replicates at levels of 5.0 ng ml^{-1} and 1.0 ng ml^{-1} were carried out for trimeprazine and seven replicates at 50 ng ml^{-1} were carried out for prochlorperazine. The absolute peak heights obtained for the extracted recovery samples were compared with peak heights obtained for fresh standards of trimeprazine and prochlorperazine made in mobile phase.

RESULTS AND DISCUSSION

As shown in Fig. 1, trimeprazine gave a sharp symmetrical peak with a retention time of 2.74 min, while prochlorperazine gave a peak at 5.56 min. The peak for trimeprazine shown in Fig. 1b represents a spiked sample of plasma containing 0.5 ng ml^{-1}. Also shown in Fig. 1 is a typical chromatogram obtained from a plasma sample taken from a healthy volunteer (61 kg) 6 h after the administration of an oral dose of trimeprazine (5-mg tablet). The lower limit of sensitivity of the assay as described in the experimental section was 0.25 ng ml^{-1} with a detection limit of 0.125 ng ml^{-1}.

Table I gives an estimate of the accuracy of the described procedure. The

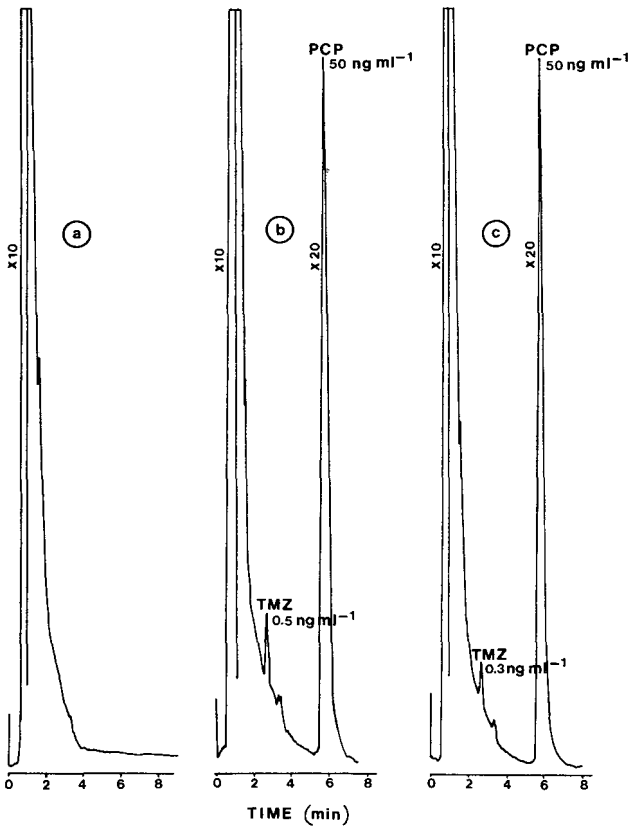

Fig. 1. Chromatograms of trimeprazine (TMZ) and prochlorperazine (PCP) obtained using electrochemical detection. (a) Blank plasma; (b) plasma (2.0 ml) spiked with 0.5 ng ml^{-1} trimeprazine; (c) plasma (2.0 ml) obtained from a healthy volunteer (61 kg) 6 h after an oral dose of trimeprazine tartrate. Chromatographic conditions and sample handling were as described in the Experimental section.

TABLE I

HPLC ESTIMATION OF TRIMEPRAZINE ADDED TO PLASMA

$y = mx + b$, where $m = 0.0507$, $b = 0.0027$. Mean = 3.42, $r^2 = 0.9995$.

Amount added (ng)	n	Mean peak height ratio	S.D.	C.V. (%)
0.25	6	0.0130	0.007	5.40
0.50	6	0.0262	0.0012	4.40
1.0	6	0.0544	0.0019	3.51
2.5	6	0.1277	0.0051	3.98
5.0	7	0.2573	0.0047	1.83
10.0	7	0.5072	0.0070	1.38

standard curve determined from these data had a slope value of 0.0507 and y-intecept of 0.0027. The correlation coefficient for the linear regression line drawn through these points was 0.9995 with a mean coefficient of variation of 3.42%. These results are in line with the accuracy obtained in a similar procedure for the HPLC—ElCD analysis of chlorpromazine reported earlier from these laboratories [6].

The mean percentage recoveries of trimeprazine at 5 ng ml^{-1} and 1 ng ml^{-1} were found to be equivalent with an overall value of 81.6 ± 1.3% (Table II).

TABLE II

RECOVERY OF TRIMEPRAZINE AND PROCHLORPERAZINE FROM PLASMA

Drug	Amount added (ng/ml) plasma)	n	Mean amount recovered (ng)	Percentage recovery mean ± S.D.)
Trimperazine	5.0	7	4.08	81.61±1.15
	1.0	5	0.82	81.64±1.98
Prochlorperazine	50.0	7	43.16	86.31±1.33

When this method of analysis was applied to the determination of the pharmacokinetics of trimeprazine after oral administration of low doses (5 mg) to healthy volunteers, profiles such as that shown in Fig. 2 were obtained. The peak plasma concentration of trimeprazine in this volunteer (85 kg) was 1.75 ng ml^{-1}, while the corresponding peak value for the other volunteer was 0.75 ng ml^{-1}. As can be seen in Fig. 2, the method described permits the determination of plasma trimeprazine concentrations up to 24 h after a single 5-mg oral dose.

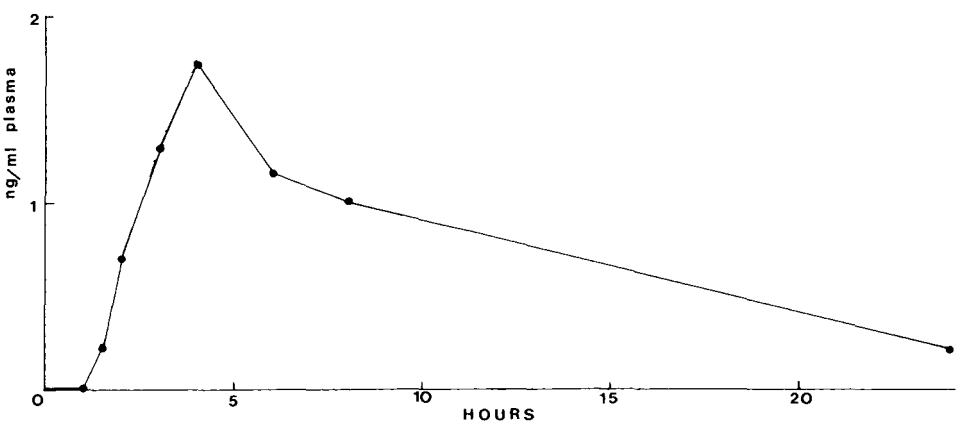

Fig. 2. Plasma concentration—time profile for a healthy volunteer (85 kg) after receiving an oral dose of trimeprazine tartrate (5-mg Panectyl tablet).

The described HPLC—ElCD method allows for the first time the determination of plasma concentrations of trimeprazine following a single therapeutic oral dose of this phenothiazine. In addition, plasma levels can be quantitated as late as 24 h post administration with sufficient sensitivity and specificity to

permit pharmacokinetic analysis of this drug. It is felt that this method of analysis will be of use in the study of pharmacokinetic and bioavailability parameters of this phenothiazine in healthy volunteers either at the steady-state level or even after low (5 mg) single oral doses. Studies of this type are currently being investigated in these laboratories.

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Note

Analysis of piperacillin using high-performance liquid chromatography

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Piperacillin sodium, a new semisynthetic penicillin derivative, has been shown to be effective in the treatment of many serious infections associated with gram-positive and gram-negative organisms including anerobes [1,2]. The ability to study the disposition and elimination of this antibiotic is dependent on accurate, sensitive, and specific methods to measure it in biological fluids. It is also desirable to monitor levels of piperacillin during therapy of certain serious infections caused by organisms in which the inhibitory concentration is relatively high. In some instances piperacillin concentration must be determined in the presence of other antimicrobial agents. Published studies [3—5] have employed microbiological assays to measure piperacillin in biological fluids. However, these assays are relatively cumbersome and potentially subject to interference from concurrently administered antibiotics.

The purpose of this paper is to describe a rapid high-performance liquid chromatographic (HPLC) assay which requires 200 μl of sample and is not subject to interference from other commonly used antibiotics such as penicillin-G, ampicillin, chloramphenicol, gentamicin, and kanamycin.

MATERIALS AND METHODS

Chromatography

Assays were performed using a Perkin-Elmer Series 2 liquid chromatograph equipped with an LC75 variable-wavelength detector and interfaced to a Sigma-10B data system (Perkin-Elmer Corporation, Norwalk, CT, U.S.A.). All analyses were performed on a 10-μm Waters μBondapak C_{18} 30 × 3.9 cm column (Waters Assoc., Milford, MA, U.S.A.) maintained at 50°C. A guard column packed with 10 μm size C-18 pellicular material (Supelco, Bellefonte, PA,

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U.S.A.) was installed between the injector port and the analytical column. The flow-rate was 2.0 ml/min and the effluent was monitored at 230 nm.

Reagents

Piperacillin sodium salt was supplied by Lederle Labs. (Pearl River, New York, NY, U.S.A.). 5-Ethyl-5-p-tolylbarbituric acid (ETBA) was purchased from Applied Science Labs. (State College, PA, U.S.A.). Methanol and acetonitrile were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were reagent grade.

The mobile phase was 22% acetonitrile in 0.1 *M* sodium acetate buffer, adjusted to a pH of 4.6 with a few drops of glacial acetic acid. This solution was freshly prepared and degassed under vacuum just prior to use.

A stock solution of 1.0 g/l piperacillin was prepared by dissolving an appropriate amount of piperacillin salt in methanol. The stock standard was then diluted with drug-free serum, urine, or cerebrospinal fluid (CSF) to provide assay standards over a concentration range of 1.0—200 mg/l. The internal standard (20 mg/l ETBA in methanol) was also used as the protein-precipitating agent. This solution was stored at −10°C and kept ice cold during sample processing.

Procedure

Two hundred microliters of sample (serum, CSF, or diluted urine) were placed in a 10 × 75 mm disposable glass tube. Depending on the time of urine collection in relation to the time of the dose, urine dilution ranged from 1:100 at peak time (0.1 h post dose) to 1:10 (3—4 h post dose). Two hundred microliters of ice-cold methanol containing the internal standard were added to the tube, which was then vortexed for 30 sec. The samples were placed on ice for 5 min, then centrifuged for 15 min at 1600 *g* in a refrigerated centrifuge. The clear supernatant was transferred to a clean 10 × 75 mm test tube and kept on ice until analyzed. Thirty microliters of supernatant were injected onto the column.

RESULTS

Fig. 1 shows typical chromatograms of (A) drug-free serum containing the internal standard; (B) drug-free serum reconstituted with 20 mg/l piperacillin and the internal standard; and (C) a patient sample in which the determined concentration of piperacillin was 43.8 mg/l. The retention times for piperacillin and internal standard were 3.7 and 6.5 min, respectively. The concentration of piperacillin was calculated from the integrated area under the peaks and was linearly related to the internal standard area over the concentration range 1.0—200 mg/l. Standard curves were identical with serum, urine, or CSF. The mean recovery of piperacillin from serum samples was 85%.

Within-run precision was evaluated by processing aliquots of a prepared piperacillin serum pool and day-to-day precision was evaluated by assaying samples on consecutive days (Table I). Stability studies were also conducted using drug-free sera reconstituted with the drug. Aliquots of these samples were frozen at −70°C and analyzed over a 3.5-month period. As shown in Table II,

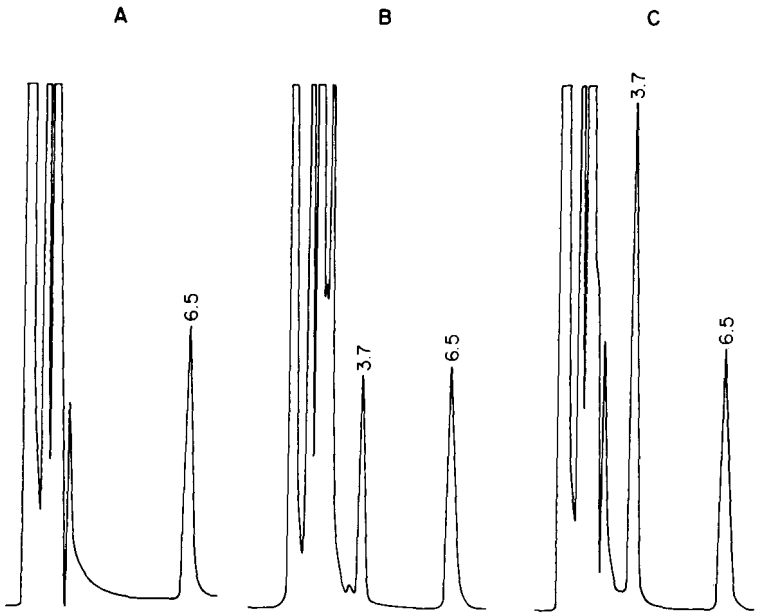

Fig. 1. Chromatograms obtained from (A) drug-free serum containing the internal standard, (B) drug-free serum reconstituted with 20 mg/l piperacillin, and (C) patient's serum in which the determined concentration of piperacillin was 43.8 mg/l. The retention times for piperacillin and the internal standard were 3.7 and 6.5 min, respectively. The early eluting peaks are unidentified extractants which do not interfere with the assay.

TABLE I

PRECISION OF SERUM PIPERACILLIN ANALYSIS

	Within-run	Day-to-day
Amount added (mg/l)	20.0	50.0
Amount found		
Mean (mg/l)	20.7	50.0
S.D. (mg/l)	1.3	3.7
C.V. (%)	6.1	7.4
n	14	32

TABLE II

STABILITY OF PIPERACILLIN

The samples were stored frozen at −70°C.

Day	Value (mg/l)	Day		Value (mg/l)
1	20.0	48		20.7
8	22.3	50		24.3
9	19.1	73		25.7
10	18.6	106		25.0
31	20.8		Mean:	21.8
			S.D.:	2.6

there was no appreciable change in drug concentration. The accuracy of the method was further validated by blindly assaying five serum samples containing 0.0—133.0 mg/l piperacillin which were supplied by Lederle Labs. The results of these analyses are shown in Table III.

TABLE III

ANALYSIS OF PIPERACILLIN BLIND-CHECK SAMPLES

$r = 0.99$.

	Assay concentration (mg/l)	Actual concentration (mg/l)
Sample 1	142.2	133.0
Sample 2	13.2	13.0
Sample 3	86.8	83.0
Sample 4	60.0	60.0
Sample 5	N.D.	N.D.

DISCUSSION

Measurement of antibiotic concentrations has traditionally depended on bioassays using a sensitive strain of bacteria. However, bioassays are increasingly being replaced by non-biological assays which offer distinct advantages. HPLC has proved to be one of the more practical and popular assay tools for this purpose. Antibacterial bioassays generally require a period of incubation by which the assay time can range from 4 to 12 h or more. Sample preparation and chromatography, however, typically require no more than 10 to 20 min per sample. Bioassays tend to be relatively nonspecific and subject to random variation inherent in biological systems. In contrast, HPLC conditions can be standardized to provide a high degree of reproducibility and specificity. This is particularly advantageous when the compound in question must be measured in the presence of concurrently administered antibiotics.

The method described here offers several advantages over traditional bioassays. The required sample size is small, a particularly important consideration when working with infants and children. The specificity, sensitivity, and accuracy of the method make it useful for pharmacokinetic studies. In addition, short assay time and simplicity make the method attractive for the routine monitoring of piperacillin levels during therapy. This method may be readily instituted in any laboratory with HPLC capability.

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Note

Assay of trimethoprim in plasma and urine by high-performance liquid chromatography using electrochemical detection

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(Received June 11th, 1982)

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine] (TMP) is a synthetic antibacterial, which interferes with folic acid metabolism by inhibiting dihydrofolate reductase (EC 1.5.1.3) [1]. The drug is administered either alone or in combination with a sulphonamide.

Various analytical methods for quantitation of TMP in body fluids have been used, including microbiological assay [2], spectrofluorimetry [3], autoradiography [4], differential pulse polarography [5], thin-layer chromatography with densitometry [6], and gas—liquid chromatography [7].

Several high-performance liquid chromatographic (HPLC) methods for determination of the drug in biological fluids have appeared [8—15]. Both normal-phase [8,11,15], reversed-phase [9,10,13,14], and ion-pair systems [12] have been employed. Clean-up procedures vary from simple precipitation of proteins [9,10] to extraction with chloroform [8,11], methylene chloride [12] or ethyl acetate [13—15].

The aim of this investigation was to develop a sensitive method, which enables handling of small volumes of plasma samples containing TMP at the 0.1 ppm level. Only small plasma volumes could be provided, as the assay was used in pharmacokinetic studies of TMP in newborn pigs. Existing methods for determination of TMP were not found suitable for measurements at the 0.1 ppm level, which was close to the detection limit using UV detection.

However, the electrochemical detector working in the oxidative mode has proved to be of value in trace analysis of, for example, phenols and aromatic amines [16]. Therefore, it seemed to be a promising alternative to use electrochemical detection (ElCD).

Suitable conditions (e.g. simple extraction procedure, small retention volumes) for use in pharmacokinetic studies involving a large number of samples were elucidated.

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EXPERIMENTAL

Apparatus

HPLC was performed with a Waters Model 6000 solvent delivery system connected to a Waters Model 440 UV detector (280 nm) and an electrochemical detector consisting of a Model 656 electrochemical detector and a Model 641 VA detector (Metrohm, Switzerland). Working electrode: glassy carbon. Reference electrode: Ag/AgCl. Auxiliary electrode: glassy carbon. The detectors were connected in series with the electrochemical detector downstream. The injection port was a Rheodyne Model 7125 loop injector provided with a 100-μl loop. An Omniscribe Model 5111-5 recorder (Houston Instruments, Houston, TX, U.S.A.) was employed. A stainless-steel HPLC column (Knauer, 120 × 4.6 mm I.D.) connected to a 40-mm precolumn of similar type was used. Both columns were packed with Nucleosil C_{18} (5 μm) particles (Macherey-Nagel, Düren, G.F.R.). The mobile phase was 0.07 *M* KH_2PO_4 (pH 4.75)—methanol (3:1). The procedure was carried out at a flow-rate of 1.5 ml/min (200 bars).

Chemicals

TMP was a gift from Syntex, Grindsted, Denmark. Sulphamethoxazole (SMZ) was donated by DAK-laboratories, Copenhagen, Denmark. All other chemicals were of analytical grade.

Standard solutions of TMP and SMZ were made from stock solutions of the compounds in 10% methanol by diluting with mobile phase. For recovery studies TMP solutions were made by diluting the stock solution with 0.1 *M* sodium hydroxide.

Internal standard

During the study SMZ was used as internal standard because chromatographic behaviour of this compound in combination with TMP is well described [9,10,13,14]. SMZ was dissolved in mobile phase and added to the samples after extraction, and thus functioned as a volume marker. Besides, the internal standard could check stability of the electrochemical detector within-day.

Procedure

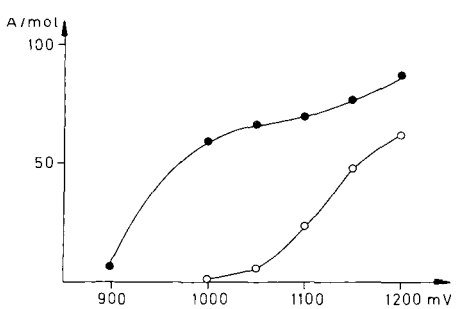
A 250-μl plasma sample together with 50 μl of 0.1 *M* sodium hydroxide and 1500 μl of ethyl acetate was pipetted into a 3-ml polypropylene tube. The tube was stoppered, mixed for 15 sec on a vortex mixer, and centrifuged for 1 min at 18,000 *g*. Then 1000 μl of the organic phase were transferred to another polypropylene tube and evaporated under a stream of nitrogen at 40°C. The residue was redissolved in 250 μl of mobile phase containing internal standard. After mixing for 30 sec on a vortex mixer and centrifugation for 1 min at 18,000 *g*, 10—100 μl were injected into the HPLC system. Urine samples were treated in the same way after diluting ten times with distilled water.

Concentrations of TMP in plasma or urine were measured by comparing peak height ratios of TMP/internal standard with peak height ratios of extracts from

blank plasma/urine samples with known amounts of TMP added. For recovery studies extracted blank plasma/urine samples with known amounts of TMP added were compared with TMP standard solutions.

RESULTS AND DISCUSSION

The response characteristics of the described ElCD system are shown for TMP and internal standard in Fig. 1. It is seen that TMP has not reached its plateau at 1200 mV. Comparison of the sensitivity of ElCD and UV detection of TMP is shown in Fig. 2.



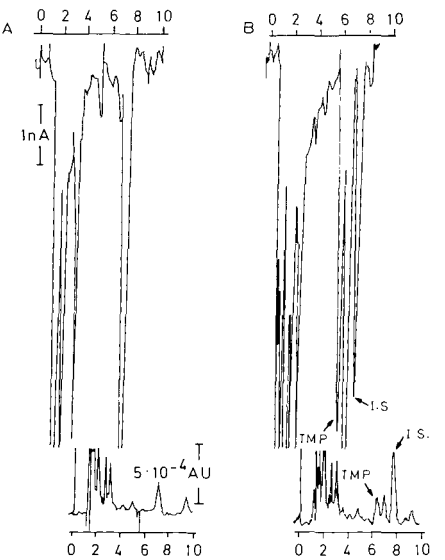


Fig. 1. Applied potential vs. output current. Output current is determined by injecting the same sample [250 ng of TMP (○), 70 ng of sulphamethoxazole (●)] at varying potentials.

Fig. 2. Electrochemical detection at 1200 mV (top) and UV detection at 280 nm (bottom) of (A) blank plasma extract, (B) plasma extract from a 67-day-old pig 7 h after intravenous injection of 5 mg/kg TMP. Concentrations of TMP and internal standard (I.S.) are 0.25 ppm and 0.07 ppm, respectively.

At 1200 mV a linear relationship was found between the concentration of TMP and the ratio of the peak heights of TMP/internal standard in the concentration range studied (1—250 ng on-column sample weight).

The detection limits, i.e. the minimum plasma concentration detectable, defined as twice the baseline noise for UV detection and twice a non-specific long-term noise in the case of ElCD, was found to be 0.01 ppm (ca. 2 pmol injected) using ElCD (1200 mV), and 0.1 ppm using UV detection (280 nm), when extractions were performed as described above.

Recoveries from the extraction procedure are presented in Table I.

When the required pressure for working the system at 1.5 ml/min exceeded 233 bars, the top filter of the precolumn was renewed (after several hundred injections) or, if necessary, the precolumn was repacked.

TABLE I

RECOVERY OF TMP ADDED TO PIG PLASMA AND URINE

The data represent mean ± S.D. for five determinations of samples at each concentration level.

	TMP concentration (ppm)	Recovery (%)
Plasma	0.1	98.1 ± 3.7
	0.5	89.6 ± 2.0
	1.0	93.6 ± 2.2
	5.0	87.9 ± 2.1
Urine	25	83.7 ± 0.4

Employment of the electrochemical detector in HPLC analysis of TMP in plasma has the advantage over UV detection that the sensitivity is enhanced. Working at high potential will often impair the analysis, because many compounds are detected and thus capable of interfering the assay. When analysing TMP, interfering peaks can be avoided by a single extraction with ethyl acetate. The choice of 1200 mV was a compromise between sensitivity and selectivity. Working the glassy carbon electrode above 1200 mV is, furthermore, not recommended by the producer. Besides, the long-term baseline noise was increased by applying a higher potential.

In conclusion, we have found that ElCD at 1200 mV is suitable for determination of TMP in blood plasma and urine, and the sensitivity by using this type of detection is superior to 280-nm UV detection. A minor drawback is a non-specific long-term baseline noise, which is present at high oxidation potentials, while electrode passivation is an unimportant problem which can be overcome by routine repolishing. Actually, the chromatographic system was run for several weeks (30—40 injections daily) before repolishing of the glassy carbon electrode was necessary. The fact that the applied potential was placed on the ascending part of the curve did not cause serious problems. Injection of the same standard several times during the day gave rise to variation in the order of 2—3%.

Though the method was developed with the purpose of studying the pharmacokinetics of TMP in pigs during the first weeks after birth [17], it was also found suitable for analysis of human plasma.

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Note

Fluorimetrische Bestimmung von Amilorid in Humanplasma mittels Dünnschichtchromatographie

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(Eingegangen am 19. April 1982)

Obwohl das kaliumretinierende Diuretikum Amilorid (N-Amidino-3,5-diamino-6-chloropyrazin-carboxamid) schon lange therapeutisch eingesetzt wird, liegen nur wenige pharmakokinetische Untersuchungen vor. Noch 1979 wurde von der Herstellerfirma in dem Informationsprospekt zu Moduretik® angegeben, dass bei üblicher Dosierung der Serumspiegel von Amilorid zu niedrig sei, um fluorimetrisch erfasst werden zu können, Quantitative Bestimmungen von Amilorid in Humanplasma wurden daher mit ^{14}C-markiertem Amilorid durchgeführt [1—4]. Für pharmakokinetische Untersuchungen am Tier wurde ausserdem von Baer et al. [5] eine photometrische Bestimmungsmethode beschrieben. Der Wirkstoff wird dabei aus alkalisiertem Plasma mit Ethylacetat extrahiert und vor der spektralphotometrischen Bestimmung in eine saure, wässrige Lösung rückextrahiert. Die verwendeten Dosen von 4 mg/kg lagen jedoch wesentlich höher als die therapeutische Gabe beim Menschen (Einzeldosis 5—20 mg Amilorid—HCl). Metaboliten von Amilorid wurden bisher nicht gefunden [3, 4, 6].

Wegen der zunehmenden Bedeutung, die kaliumretinierende Diuretika insbesondere in Kombination mit Thiaziden zur Hypertoniebehandlung in den letzten Jahren gewonnen haben, erschien es uns sinnvoll, die Kinetik von Amilorid genauer zu untersuchen und dazu eine Bestimmungsmethode zu entwickeln, die empfindlich genug ist, um Amilorid-Plasmaspiegel beim Menschen ohne Verwendung von radioaktiv markiertem Material zu bestimmen. Bei dem im folgenden näher beschriebenen Verfahren wird der Wirkstoff aus alkalisiertem Plasma mit einem *n*-Butanol—Diisopropylether-Gemisch extrahiert und der Amiloridgehalt nach chromatographischer Trennung durch direkte Messung der Nativfluoreszenz auf der Dünnschichtplatte bestimmt.

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EXPERIMENTELLER TEIL

Geräte

Chromatogramm-Spektralphotometer KM3 mit Quecksilberdampf-Mitteldruck-Lampe ST41 der Firma Carl Zeiss; Perkin-Elmer-Recorder Modell 56; Perkin-Elmer-Integrator Modell M3B; Linomat III der Firma Camag mit 100-µl Hamilton-Spritze; Tecam-Heizblock mit Stickstoffbegasung.

Chemikalien

Amilorid—HCl-Dihydrat stellte die Firma MSD (München, B.R.D.) zur Verfügung. Die Lösungsmittel (p.a.-Qualität) sowie die Dünnschichtchromatographie (DC)-Platten (Kieselgel 60 HPTLC, ohne Fluoreszenzindikator) wurden von der Firma Merck (Darmstadt, B.R.D.) bezogen. Die Glasgeräte wurden mit Silikonimprägnierer der Firma Roth behandelt.

Extraktion

In einem silikonisierten Sovirel®-Glas werden 1.0 ml Plasma mit 0.2 ml 1 *N* Natronlauge und 5.0 ml eines Gemisches aus *n*-Butanol und Diisopropylether (1:1, v/v) versetzt. Nach 60-min Schütteln wird 10 min scharf zentrifugiert. 2.0 ml der Oberphase werden bei 80°C unter Stickstoffbegasung bis zur Trockene eingeengt; die Glaswände werden dabei mit 0.2 ml Methanol—Diisopropylether-Gemisch (3:2, v/v) abgespült.

Chromatographie

Der Rückstand wird in 50 µl dieses Lösungsmittelgemisches aufgenommen und 20 µl werden bandförmig (6 mm) auf die DC-Platte aufgetragen. Bei der Bestimmung von Plasmaproben werden pro Platte drei Plasmastandards aufgetragen, die ebenso aufgearbeitet werden. Zunächst wird in Ethylacetat chromatographiert (Laufstrecke 8 cm, hR_F = 0). Anschliessend wird die Platte in 2-Propanol—Diisopropylether—Ammoniak 25% (70:30:10, v/v/v; nach Kammersättigung) entwickelt (Laufstrecke 8 cm, hR_F = 34). Nach dem Trocknen wird in Paraffin—Cyclohexan (1:2, v/v) getaucht und erneut getrocknet.

Messbedingungen

Zur Fluoreszenzanregung wird die Platte mit Licht der Wellenlänge 365 nm bestrahlt (Spalt 0.5 × 8 mm). Die Emission durch den M436-Monochromatfilter wird gemessen (Fig. 1). Die Auswertung erfolgt über das Flachenintegral der Fluoreszenzortskurve.

Präzision und Richtigkeit

Zur Untersuchung der Linearität des Verfahrens wurden je drei Plasmastandards mit folgenden Amilorid-Konzentrationen aufgearbeitet: 0.5 ng/ml, 1.0 ng/ml, 2.0 ng/ml, 5.0 ng/ml, 10.0 ng/ml und 50.0 ng/ml. Die Standardabweichung wurde mit jeweils acht Plasmastandards gleicher Konzentration bestimmt. Die Wiederfindungsrate wurde durch den Vergleich einer Lösung der Amilorid-Base in Methanol—Diisopropylether (3:2, v/v) mit aufgearbeiteten Plasmastandards verschiedener Konzentrationen berechnet.

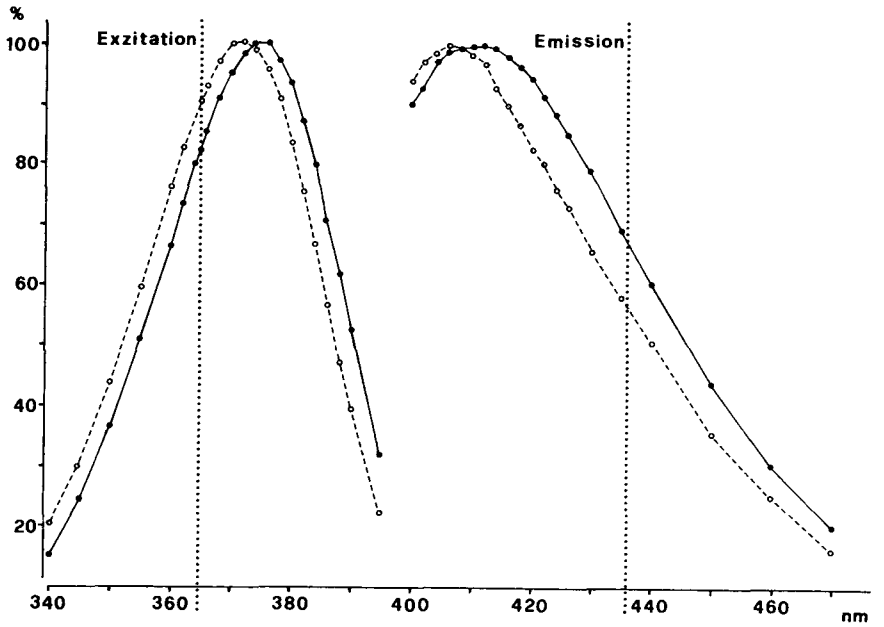

Fig. 1. Exzitations- und Emissionsspektren von Amilorid auf einer DC-Platte nach Chromatographie. Abszisse: Wellenlänge in nm; Ordinate: relative Fluoreszenzintensität in %. ○ — — — ○, vor dem Tauchvorgang; ●——●, nach dem Tauchen in Paraffin—Cyclohexan.

Anwendung der Methode

Zwei Personen wurden vor dem Frühstück zwei Tabletten zu je 5 mg Amilorid—HCl appliziert. Venenblut wurde nach 0, 1 bzw. 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 48 h entnommen. Das erhaltene Citratplasma wurde bis zur Bestimmung bei —18°C eingefroren.

ERGEBNISSE

Die Flächen unter den Fluoreszenzortskurven sind den vorhandenen Substanz-Konzentrationen im Bereich von 0—50 ng/ml direkt proportional. Der Korrelationskoeffizient beträgt 0.999, die Gerade verläuft annähernd durch den Nullpunkt. Die Ergebnisse der Untersuchung der Standardabweichung sind in Tabelle I angegeben. Die Grenze der quantitativen Erfassung in Plasma liegt bei 0.2 ng/

TABELLE I

RELATIVE STANDARDABWEICHUNG (n=8) IN ABHÄNGIGKEIT VON DER AMILORIDKONZENTRATION

Amilorid-Base (ng/ml)	Standardabweichung (SD_{n-1}) (%)
2	3.3
5	3.0
10	2.5
20	2.4

ml. Die Wiederfindungsrate beträgt ca. 62%.

Die gemessenen Plasmaspiegel eines Probanden und eines Patienten mit Niereninsuffizienz sind in Fig. 2 dargestellt. Die angegebenen Werte sind Mittelwerte aus mindestens zwei getrennten Bestimmungen [7].

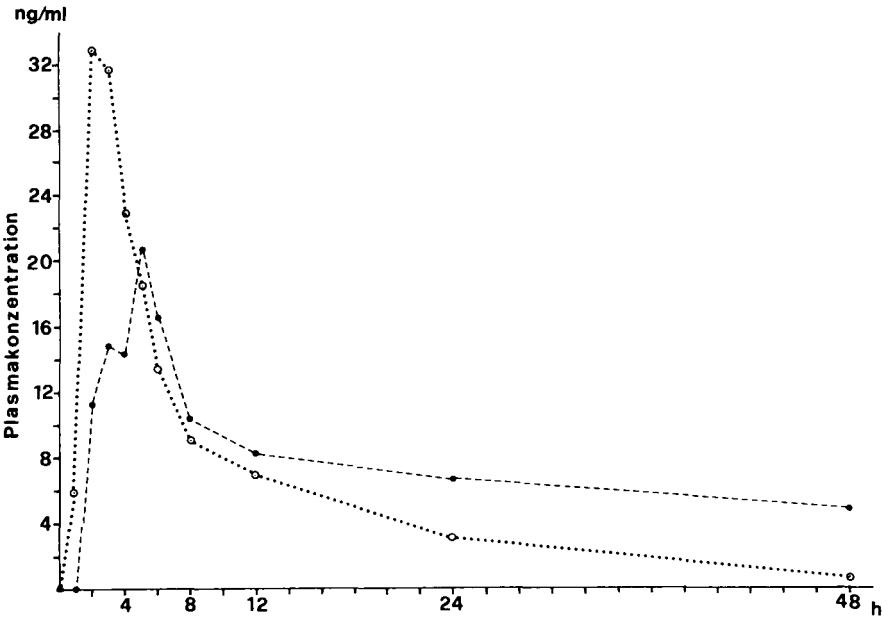

Fig. 2. Plasmaspiegelkurve von Amilorid nach Applikation von 10 mg Amilorid–HCl an (o·····o) einen Probanden und (• — — — •) einen Patienten mit Niereninsuffizienz.

DISKUSSION

Das vorliegende Verfahren erlaubt es, nicht radioaktiv markiertes Amilorid in Humanplasma nach einmaliger, therapeutischer Dosierung schnell und einfach zu bestimmen. Die Linearität der Bestimmungsmethode erstreckt sich über einen Bereich von mehr als zwei Zehnerpotenzen. Mitextrahierte Plasmabestandteile, die die eigentliche Chromatographie und die anschliessende Auswertung stören, werden durch Vorentwickeln der DC-Platte mit reinem Ethylacetat abgetrennt. Insgesamt ermöglicht das Verfahren eine selektive Bestimmung des Wirkstoffes (Fig. 3).

Das Tauchen der DC-Platte in Cyclohexan–Paraffin erhöht die Fluoreszenzintensität um ca. 80% bei gleichbleibendem Grundrauschen. Auf diese Weise wird der Messfehler erheblich reduziert und die Empfindlichkeit der Methode verbessert. Ausserdem bleibt die Grösse des Mess-signals über längere Zeit erhalten. Als Nebeneffekt des Tauchvorganges werden das Exzitations- und das Emissionsmaximum geringfügig in den längerwelligen Bereich verschoben (Fig. 1). Um eine optimale Reproduzierbarkeit beim Arbeiten mit sehr kleinen Mengen Amilorid zu erhalten, sollte unbedingt darauf geachtet werden, dass die Glasgeräte silikonisiert sind, andernfalls kann die Standardabweichung erheblich zunehmen. Durch das Arbeiten mit Dünnschichtplatten

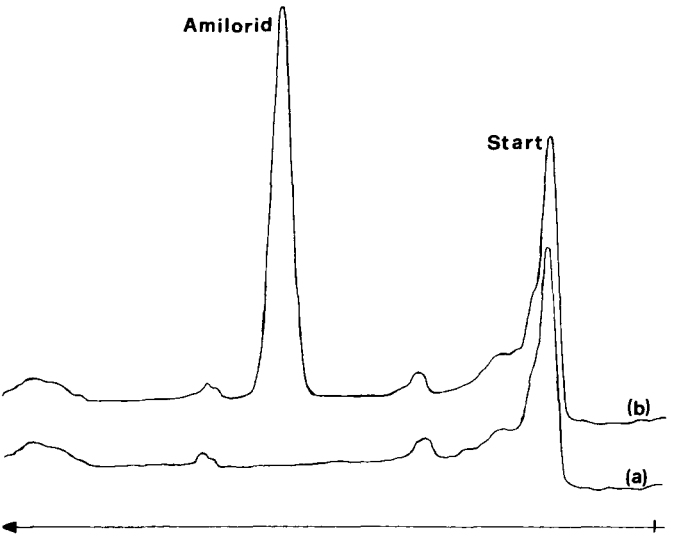

Fig. 3. Fluoreszenzortskurve eines entwickelten Chromatogramms. (a) Leerplasma, (b) Plasma mit 10 ng/ml Amilorid-Base.

als chromatographischem System ist die Methode auch gut für halbquantitative Untersuchungen ohne genaue densitometrische Auswertung geeignet.

DANK

Der Dr.-Robert-Pfleger-Stiftung danken wir für eine Sachbeihilfe.

LITERATUR

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CHROMBIO. 1410

Letter to the Editor

Analysis of barbiturates in blood by high-performance liquid chromatography

Sir,

In their recent paper, Gill et al. [1] have described a procedure for the analysis of barbiturates in blood by high-performance liquid chromatography. They have used an ODS-silica column for optimal separation and a mobile phase of pH 8.5 for enhanced detection sensitivity. However, the suppliers of ODS-silica columns recommend 7.5, the highest pH of the mobile phase to be used with these columns. In some applications mobile phase of pH 8 has been used after it has been saturated with silica by use of a precolumn to avoid any damage to the analytical column. In our experience mobile phase of pH greater than 8 causes a void in the column and the efficiency of the column, which is quite expensive, is lost within a few days.

I am sure that the readers of this paper would like to share with the authors their experiences with the life and performance of their ODS-silica column with the use of a mobile phase of pH 8.5.

St. Joseph's Hospital,
Hamilton, Ontario (Canada)

R.N. GUPTA

1 R. Gill, A.A.T. Lopes and A.C. Moffat, J. Chromatogr., 226 (1981) 117.

(Received May 5th, 1982)

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CHROMBIO. 1411

Letter to the Editor

Analysis of barbiturates in blood by high-performance liquid chromatography

Sir,

In a recent letter [1] Dr. Gupta has made some valuable comments concerning the use of alkaline eluents in high-performance liquid chromatography (HPLC) with particular reference to our recent paper [2] describing the analysis of barbiturates in small volumes of blood. In this procedure, using an ODS-silica column, we advocated the use of a pH 8.5 eluent to enhance detection sensitivity and optimise separation. Dr. Gupta relates their experience of a rapid loss in column efficiency with eluents of pH >8.

The dissolution of silica in aqueous solutions at high pH is well known but our experience has shown that ODS-silica columns have reasonable lifetimes (weeks rather than days) when used with the pH 8.5 eluent providing that sensible precautions are taken. We include a short column packed with silica (40 μm) between pump and injector to ensure that the mobile phase contains dissolved silica before entering the analytical column [3] and maintain a continuous flow such that the packing material is never in contact with static eluent. At the end of each working day the column is thoroughly washed with methanol—water (50:50, v/v) before stopping the flow.

Voids at the top of ODS-silica columns have occasionally been encountered after periods of extensive use but we do not consider this to be an overwhelming problem and certainly not outweighing the usefulness of the assay. Such voids are generally easy to repair by topping up with packing material hence restoring the performance of the column and extending its life. In addition, we pack our own HPLC columns rather than buying expensive pre-packed columns and finding this rapid and straightforward would urge others to do the same. By so doing costs are considerably reduced and column deterioration is not seen as such a serious disaster.

We thank Dr. Gupta for highlighting this problem area.

Home Office Central Research Establishment,
Aldermaston
(Great Britain)

R. GILL
A.C. MOFFAT

0378-4347/82/0000—0000/$02.75

1 R.N. Gupta, J. Chromatogr., 233 (1982) 437.
2 R. Gill, A.A.T. Lopes and A.C. Moffat, J. Chromatogr., 226 (1981) 117.
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(Received June 8th, 1982)

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Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1466

Book Review

Thin layer chromatography: Quantitative environmental and clinical applications, edited by J.C. Touchstone and D. Rogers, Wiley, New York, Chichester, Brisbane, Toronto, 1980, XIX + 561 pp., price £21.50, ISBN 0-471-07958-8.

This book contains 36 papers which were presented at the symposium "Clinical and Environmental Applications of Quantitative Thin-Layer Chromatography" held in Philadelphia on January 15—17, 1979. Judging by the contents it is possible to conclude that the editors selected the authors of some of the communications on purpose so that the volume, contrary to other symposium proceedings, represents a concise whole. The contents of the book can be classified into three parts: 1. History and techniques of thin-layer chromatography (TLC); 2. Toxicological and environmental analysis; and 3. Biomedical applications.

In the first part, which consists of seven papers, fundamental chapters about TLC techniques are presented in the review form. Recent advances in the techniques are respected. Most of the papers are on a high level; the chapter about quantitative densitometry is, unfortunately, less perfect though it should have been given more attention at least because of the book's title. The chapter about history includes some new facts and discloses some as yet neglected papers from the "prehistory" of TLC. It is rather inappropriate to decrease the contribution of Egon Stahl in this field. The general impression of this part is that of a very personal approach.

The second part, which is by far the largest and is worthy of high esteem, summarizes papers devoted to toxicological and environmental analysis. Attention is paid mainly to aflatoxins and other mycotoxins. From the series of excellent reviews and original papers, I would at least like to mention the chapter by S. Nesheim on aflatoxins which also presents a wealth of references (197 quotations). Of the papers several chapters about nitrosamines, polycyclic hydrocarbons and others can be mentioned.

In the area of biomedical applications the attention is focused on lipid analysis, some catecholamine metabolites, prostaglandins and cholesterol. Several papers deal with drug monitoring (e.g., the introduction paper by Fenimore and Davis).

As I have mentioned at the beginning, this book is written on a very high level in spite of the fact that the volume represents symposium proceedings.

This is underlined by the definite structure of the book and the volume can certainly bring valuable information to most workers involved in environmental and biomedical TLC. This, however, does not mean that the book is not lacking in some areas that are important for both mastering TLC and further development of the method. Thus, for example, though it is said in the introduction that it is necessary to combine theory and practice there is not a single chapter devoted to theory. Similarly, in the chapter about quantitative chromatography nothing is said about the theoretical background. Another interesting chapter that would probably be welcomed is a comparison of TLC and high-performance liquid chromatography, a topic frequently discussed, today. Perhaps, these problems will be the subject of another symposium and of another volume in this series. The editors can be congratulated for their editing of this monograph which is meticulously arranged and printed. The repetition of two lines in the Preface and the Overview (p. XIV) is one of the few mistakes that one can find in the book.

Prague (Czechoslovakia) KAREL MACEK

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CHROMBIO. 1459

Book Review

Biological/biomedical applications of liquid chromatography III, edited by G.L. Hawk, Marcel Dekker, New York, Basel, 1981, XV + 420 pp., price Sfr. 148.00, ISBN 0-8247-1297-8.

The third volume of the above monograph represents a collection of 22 papers which were presented at the 3rd Liquid Chromatography Symposium held in Boston, MA, U.S.A., October 11 and 12, 1979. The arrangement of this volume is analogous to the arrangement of the first two volumes [for review see J. Chromatogr., 181 (1980) 516 and 183 (1980) 260]. The papers present predominantly original results from the area of biomedical research and clinical chemistry, and several papers are devoted to drug monitoring. The difference of this volume to the previous ones reflects the fact that the symposium naturally mirrored the trends in biomedical applications in 1979. Therefore, most papers (about one third) were devoted to amino acids, peptides, proteins and enzymes. Other papers are orientated to the analysis of catecholamines and their metabolites, nucleic acids and their components, carbohydrates and prostaglandins. From the area of drug analysis reports are included on the monitoring of disopyramide, anticonvulsants and a gastric secretory agent ICI 125,211. From the methodical point of view, attention has been paid to radial compression separation systems and to pre- and post-column derivatization.

It is obvious that the editor paid considerable attention to preparing the manuscripts for publication. It is not clear, however, why he has not arranged individual papers according to the categories of compounds separated. Quotation of references is not uniform in this volume. It is worth mentioning that, for example, in the paper of Waterfield and Scrace there are a number of errors in the references. For example, the authors quoted three papers that should have been published in the present volume, but it is impossible to find any one of these. The book is completed by a Glossary (which has been transferred from the preceding volume) and a Subject Index.

It can be concluded that the book will be useful mainly for people involved in basic biomedical research. It is expected that it will be welcomed especially by those who took part in this meeting. With regard to the wide scatter of themes involved it is questionable whether the book will be bought by the readers interested in one or two papers in their area of interest.

Related articles published in *Journal of Chromatography*, Vols. 248 and 249

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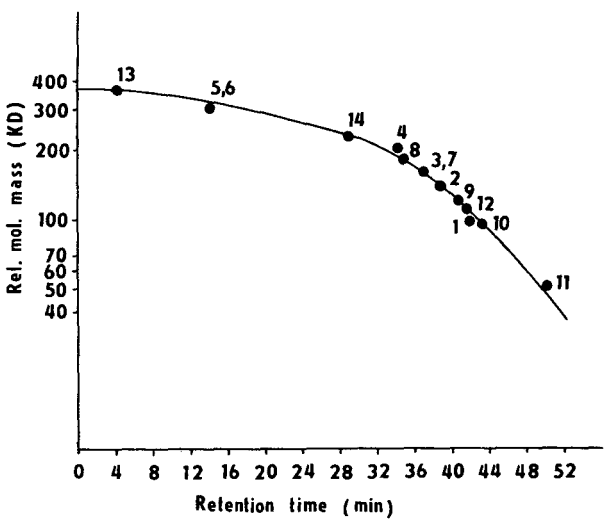
Errata

J. Chromatogr., 230 (1982) 409—414
Page 412, Table I: Relative molecular mass of No. 6, $[\alpha_1(III)]_3$ should be 300,000 and retention time (min) should be 14.0.
Page 413, Fig. 4 should read:



J. Chromatogr., 232 (1982) 197—202
Page 198, 10th line: "(70:20:20:20, v/v)" should read "(70:20:20:10, v/v)"

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. A free reprint can be obtained by application to the publisher.)

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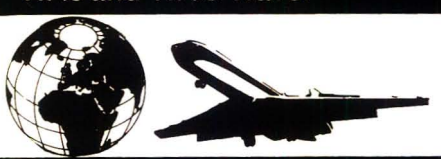

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