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BIOMEDICAL APPLICATIONS

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Contents (continued)

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edited by A. ZLATKIS, *Houston, TX, USA and* C.F. POOLE, *Detroit, MI, USA*

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CHROMBIO. 999

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

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(First received March 19th, 1981; revised manuscript received June 23rd, 1981)

SUMMARY

A fluorescence high-performance liquid chromatographic method is described for the determination of 17-oxosteroids in biological fluids. 17-Oxosteroids in urine samples are extracted with dichloromethane after enzymatic hydrolysis (β-glucuronidase—sulfatase), and dehydroepiandrosterone sulfate in serum samples is solvolysed with sulfuric acid in ethyl acetate. 17-Oxosteroids are labeled with dansyl hydrazine in trichloroacetic acid—benzene solution, and then chromatographed on the microparticulate silica gel column using dichloromethane—ethanol—water (400 : 1 : 2) as the mobile phase. The eluate is monitored by a fluorophotometer at 365 nm (excitation) and 505 nm (emission). Linearities of the fluorescence intensities (peak heights) with the amounts of various 17-oxosteroids were obtained between 60 and 1000 pg. The assay proved satisfactory with respect to sensitivity, precision and accuracy. The results obtained by a radioimmunoassay and this method were in good agreement (r = 0.964, n = 81) for serum dehydroepiandrosterone sulfate. This method is also useful for the simultaneous determination of individual 17-oxosteroids in serum and urine.

INTRODUCTION

The measurement of the so-called total 17-oxosteroids in serum and urine samples has been widely used in routine clinical analysis. However, in certain clinical conditions, such as adrenogenital syndrome, carcinoma of the adrenal cortex and gonadal disorders, it is desirable to obtain information about the urinary excretion of individual 17-oxosteroids. Many methods have been reported for the determination of individual 17-oxosteroids in biological fluids, including radioimmunoassay [1—3], gas chromatography [4, 5], and gas chromatography—mass spectrometry [6]. Recently, a high-performance liquid chromatographic (HPLC) method [7] was also applied. However, the sensitivity of

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HPLC methods was too low due to the use of a refractive index or UV detector. Especially 17-oxosteroids have no strong UV-absorbing groups in their structures. 2,4-Dinitrophenylhydrazine was used as a labeling reagent in order to increase the detection limit [8, 9]. Fluorescent labeling techniques [10] have been used for several years. In a previous paper [11] we used dansyl hydrazine as an fluorescent labeling reagent for Δ^4-3-oxosteroids, such as cortisol and 11-desoxycortisol.

In this paper, we describe a highly sensitive fluorescence HPLC method for the determination of 17-oxosteroids in biological samples.

EXPERIMENTAL

Materials

Dehydroepiandrosterone (DHEA) and androsterone (AN) were obtained from Tokyo Kasei Co. (Tokyo, Japan), and etiocholanolone (ETIO), androsta-3,5-diene-17-one, androstanedione, Δ^4-androstene-3,17-dione, 11-oxo-androsterone (11-oxo-AN), 11-oxo-etiocholanolone (11-oxo-ETIO), Δ^5-androstene-3β,16α-diol-17-one (16-OH-DHEA), 11β-hydroxyandrosterone (11-OH-AN), and 16α-hydroxyandrosterone (16-OH-AN) were purchased from Sigma (St. Louis, MO, U.S.A.). Potassium salt of dehydroepiandrosterone sulfate (DHEA-S) was prepared using DHEA and sulfamic acid according to the description of Joseph et al. [12]. (Anal. calc. for $C_{19}H_{27}O_5SK \cdot 1/2\ H_2O$: C, 53.57; H, 6.99. Found: C, 53.07; H, 6.88.) Dansyl hydrazine was of reagent grade (II) from Sigma. Sodium pyruvate, trichloroacetic acid, dichloromethane, ethyl acetate and other chemicals were obtained commercially. Helicase (β-D-glucuronidase–arylsulfatase) was purchased from Boehringer Mannheim—Yamanouchi Co. (Tokyo, Japan).

Apparatus

An Hitachi Model 634 high-speed liquid chromatograph, an Hitachi Model 204 spectrofluorophotometer equipped with a xenon lamp, and a Jasco FP-110 fluorescence spectrophotometer equipped with a mercury lamp were used.

Reagent solutions

Dansyl hydrazine solution (0.2%, w/v): a 0.2% (w/v) solution of dansyl hydrazine was prepared by dissolving 20 mg of dansyl hydrazine in 10 ml of ethanol, and stored at 4°C until used.

Sodium pyruvate suspension (0.5%, w/v): a 0.5% (w/v) suspension was prepared by suspending 50 mg of fine powder of sodium pyruvate in 10 ml of benzene; it should be vigorously mixed before use.

Trichloroacetic acid—benzene solution (0.5%, w/v).

Stock solution of DHEA-S: an aliquot of DHEA-S was dissolved in ethanol to make a stock solution (10 μg/ml), and stored at 4°C until used.

DHEA-S standard solution: aliquots of 0.75 ml, 1.0 ml and 1.5 ml of the DHEA-S stock solution were taken, evaporated under a stream of nitrogen gas, and each was then dissolved in redistilled water and made up to 100 ml before use.

Synthesis of dansyl hydrazone of DHEA. Dansyl hydrazine (159 mg) was

added to a solution of DHEA (150 mg) in 0.25% trichloroacetic acid—benzene solution and the mixture was heated at 60°C for 45 min on a water-bath. Sodium pyruvate (300 mg) was added to the reaction mixture and heated for 15 min on a water-bath. After cooling to room temperature, the solvent was removed by evaporating to dryness under reduced pressure. The residue was dissolved in dichloromethane and washed respectively with 0.25 *N* sodium hydroxide solution and water. After drying with sodium sulfate, evaporation of the solvent afforded a yellow solid, which was recrystallized from ethanol—diethyl ether to give colorless needle crystals (yield: 152 mg), m.p. 231—233°C. Anal. calc. for $C_{31}H_{41}N_3O_3S \cdot 1/2\ H_2O$: C, 68.38; H, 7.72; N, 7.72. Found: C, 68.30; H, 7.76; N, 7.54. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 2800 (Ar—$N(CH_3)_2$), 1614 (—N=C—), 1590, 1579, 1500 (aromatic). NMR (C^2HCl_3) δ ppm: 2.85 (s, 6H, N—Me_2), 0.60 (s, 3H, C(19)—Me), 1.00 (s, 3H, C(18)—Me), 3.35 (d, 1H, C(3)—OH), 3.50 (m, 1H, C(3)—H), 5.30 (d, 1H, C(6)—H), 7.20 (t, 1H, arom.), 7.29 (s, 1H, —SO_2NH—N=), 7.52 (t, 1H, arom.), 8.25—8.64 (4H, arom.). Mass spectrometry *m/z*: 535 (M^+).

Chromatographic conditions

Stainless-steel columns 250 mm × 4 mm I.D. and 250 mm × 4.6 mm I.D. were packed with Hitachi gel No. 3042 (silica gel, particle size 5 μm) and Zorbax SIL (particle size 5—6 μm), respectively. The eluent was the organic layer separated from the mixture dichloromethane—ethanol—water (400:1:2, v/v) after shaking for 30 min. Flow-rate, column pressure, and column temperature were 1 ml/min, 35 kg/cm^2, and 35°C, respectively. The effluent was monitored at 505 nm emission against 350 nm or 365 nm excitation with an Hitachi Model 204 spectrofluorophotometer or a Jasco FP-110 fluorimeter.

Procedure

Serum sample. To 50—100 μl of serum in a centrifuge tube is added water to a volume of 1.5 ml. After the addition of 5.0 ml of dichloromethane, the contents of the tube are mixed with a Vortex-type mixer for 1 min and centrifuged at 1000 *g* for 2 min. Unconjugated steroids are extracted into dichloromethane. One milliliter of the supernatant aqueous layer is taken exactly, transferred to another tube and then mixed with a mixture of 10 ml of ethyl acetate and 0.1 ml of concentrated sulfuric acid for 1 min. After discarding the aqueous layer, the ethyl acetate layer is incubated for 3 h at 40°C. The ethyl acetate layer is washed successively with 1 ml of 1 *N* sodium hydroxide solution and 2 ml of water. After drying with anhydrous sodium sulfate, 7.0 ml of the ethyl acetate layer are transferred to another tube and evaporated to dryness under a stream of nitrogen gas. The resultant residue is assayed by the procedure described below.

Urine sample. To 1.0 ml urine in a centrifuge tube are added 500 μl of 2 *M* acetate buffer (pH 5.2) and 40 μl of Helicase. The contents of the tube are then mixed and incubated overnight at 37°C. After incubation, 6 ml of dichloromethane are added and mixed with a Vortex-type mixer for 1 min. The aqueous layer is discarded and 4 ml of the dichloromethane layer are transferred to another tube and evaporated to dryness under a stream of nitrogen gas. The resultant residue is assayed by the following procedure.

Labelling reaction. To the evaporated residue in a test tube are added 0.2 ml of 0.5% trichloroacetic acid—benzene solution and 0.1 ml of dansyl hydrazine solution. After incubation for 20 min at 60°C, 0.2 ml of sodium pyruvate suspension is added and warmed for 10 min at 60°C. The solvent is evaporated to dryness under a stream of nitrogen gas. To the residue in a test tube are added 3.0 ml of dichloromethane and 1.0 ml of 0.25 *N* sodium hydroxide solution; the tube contents are mixed with a Vortex-type mixer for 1 min, followed by washing with 2 ml of water. The aqueous layer is discarded and the dichloromethane layer is dried by addition of anhydrous sodium sulfate. In the assay of urine sample, an aliquot of this extract solution is injected into the chromatograph. Two milliliters of dichloromethane extract obtained from each serum sample are transferred to another tube and evaporated to dryness under a stream of nitrogen gas. The resultant residue is redissolved by the addition of 100 μl of chloroform, of which an aliquot is injected into the chromatograph.

RESULTS

Fluorescence spectrum

Dansyl hydrazone of DHEA was prepared by the reaction of DHEA with dansyl hydrazine in trichloroacetic acid—benzene solution. As shown in Fig. 1, dansyl hydrazone of DHEA has an excitation maximum at 350 nm and an emission maximum at 505 nm. It was stable in chloroform for at least 1 week at 4°C without any change of fluorescence. The fluorescence of the solution obtained from the assay procedure was also stable for 3 days at 4°C.

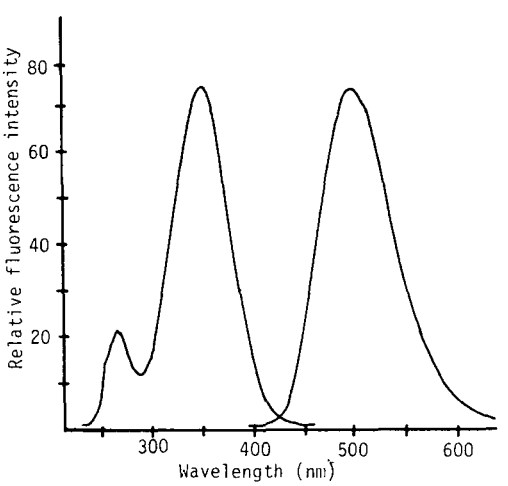

Fig. 1. Excitation and emission spectra of the dansyl hydrazone of dehydroepiandrosterone in chloroform. Excitation maximum = 350 nm; emission maximum = 505 nm.

Optimal conditions of dansylation

Though a 0.02% dansyl hydrazine solution was used in the previous paper, 17-oxosteroids could not be quantitatively labeled under the same conditions. Therefore, various factors were examined with DHEA. A 0.2% dansyl hydra-

zine solution was used in this method because the excess reagent could be removed by extraction after the reaction with pyruvate. Fig. 2 shows the effects of reaction time and the concentration of trichloroacetic acid solution on the fluorescence intensity (assessed by peak height in the chromatogram). The reaction time required to reach a maximum and constant peak height decreased with increased trichloroacetic acid concentration in benzene up to 1.0%. Using 0.2% dansyl hydrazine solution, the peak height reached a maximum at 20 min with 0.5% trichloroacetic acid solution. Though the peak height showed a slight decrease after 20 min, 0.5% trichloroacetic acid solution was used and a reaction time of 20 min was decided upon. The effect of temperature on the reaction was examined. It was decided to employ a reaction temperature of 60°C because the maximum peak height was obtained by 60°C, and the unknown minor peaks appeared at 80°C.

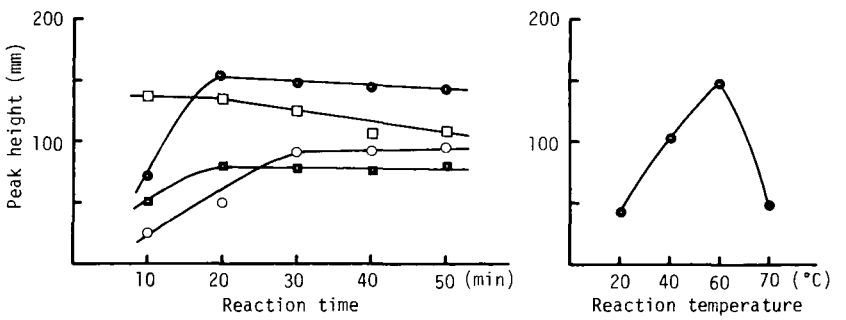

Fig. 2. Effects of trichloroacetic acid concentration, reaction time and reaction temperature on fluorescence intensity (peak height). The concentrations of trichloroacetic acid—benzene solution were: 2.0% (▪), 1.0% (□), 0.5% (•) and 0.25% (○).

Selection of eluent

Many solvent systems were examined in order to obtain the complete separation of 17-oxosteroids. The organic layer of dichloromethane—ethanol—water (400:1:2) was found to be suitable when used with an Hitachi gel No. 3042 column and a Zorbax SIL column. The chromatograms presented in Fig. 3A and B show a good separation of standard 17-oxosteroids including androsta-3,5-diene-17-one, androstanedione, androstadienedione, Δ^4-androstene-3,17-dione, AN, DHEA, ETIO, 11-oxo-AN, 11-oxo-ETIO, 16-OH-DHEA, 11-OH-AN, and 16-OH-AN.

Working curves and sensitivities

Typical working curves are shown in Fig. 4. When the Jasco FP-110 fluorimeter was used as detector, standard working curves of AN, DHEA, and ETIO showed linearities in the range of 60 pg to 1 ng, corresponding to 0.2—3.4 pmol. When 0.1 ml of serum sample was used for the assay, the detection limit of DHEA sulfate was about 0.6 μg/dl from this working curve.

Solvolysis conditions of sulfates

Most 17-oxosteroids are excreted as sulfate or glucuronide conjugates. To

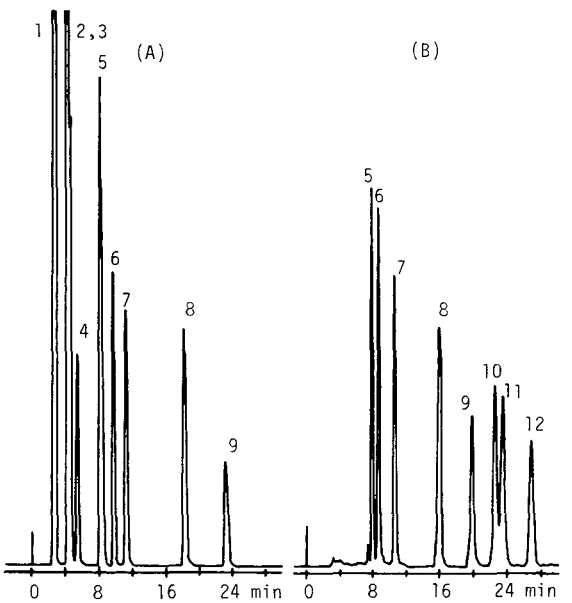

Fig. 3. Chromatograms of dansyl hydrazone derivatives of 17-oxosteroid standard mixture. Peaks: 1 = androsta-3,5-diene-17-one, 2 = androstanedione, 3 = androstadienedione, 4 = androst-4-ene-3,17-dione, 5 = androsterone, 6 = dehydroepiandrosterone, 7 = etiocholanolone, 8 = 11-oxoandrosterone, 9 = 11-oxoetiocholanolone, 10 = 16α-hydroxydehydroepiandrosterone, 11 = 11β-hydroxyandrosterone, 12 = 16α-hydroxyandrosterone. (A) Hitachi gel No. 3042 (250 × 4 mm I.D.) column; mobile phase, dichloromethane—ethanol—water (400:1:2), 1 ml/min; Hitachi 204 fluorescence detector (excitation 350 nm; emission 505 nm). (B) Zorbax SIL (250 × 4.6 mm I.D.) column; JASCO FP-110 fluorescence detector (excitation 365 nm; emission 505 nm); other conditions as in (A).

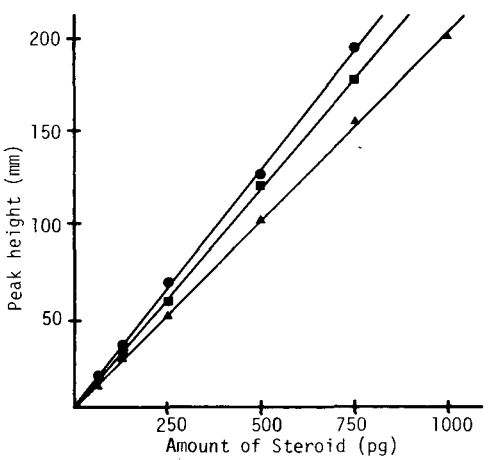

Fig. 4. Standard curves for 17-oxosteroids: androsterone (•), dehydroepiandrosterone (▪), etiocholanolone (▲).

assay DHEA sulfate in serum or urine, various hydrolysis procedures were examined. In the case of acid hydrolysis, the duration of hydrolysis is very critical; less than 10 min caused incomplete hydrolysis and more than 10 min lead to a gradual destruction of steroids and an increased number of unknown peaks. The amount of free DHEA obtained from the sulfate conjugate was only 50.2% of the calculated amount by acid hydrolysis and unknown peaks other than that of the dansyl hydrazone of DHEA appeared in the chromatogram. Therefore, hydrolysis of DHEA sulfate was done by solvolysis with ethyl acetate—sulfuric acid as reported by Burstein and Lieberman [13]. DHEA sulfate was almost quantitatively hydrolyzed to yield 94.5% DHEA after incubation for 3 h at 40°C. This value agreed closely with the yield reported by Kulpmann and Breuer [14].

Enzymatic hydrolysis

Urine samples containing both sulfate and glucuronide conjugates were hydrolyzed with Helicase according to the literature [5, 15].

Recovery and reproducibility

The recovery test was carried out by determining pooled serum samples spiked with known amounts of DHEA sulfate. As illustrated in Table I, the recoveries of added DHEA sulfate varied from 97% to 106.5% with the coefficient of variation (C.V.) ranging from 1.1 to 5.8%. Interassay variations were also measured with three serum samples containing 43.4, 62.5, and 97.9 μg/dl DHEA sulfate, respectively. The respective C.V. values were 2.1, 0.7 and 2.6% (five replicate determinations).

TABLE I

RECOVERIES OF DEHYDROEPIANDROSTERONE SULFATE FROM SERUM

Sample	Added (μg/dl)	Found (μg/dl)	Recovery (%)	*n*	C.V. (%)
Serum A	0	55.1	—	10	1.10
	75	130.5	100.5	10	3.62
	150	214.9	106.5	10	5.75
Serum B	0	109.0	—	5	2.75
	200	303.0	97.0	5	4.48

Typical chromatograms of serum and urine samples

Fig. 5A shows a chromatogram of a normal serum sample containing AN and DHEA. Fig. 5B—D are typical chromatograms of urine samples obtained from patients with ovarian cystoma, adrenogenital syndrome, and hypertension, respectively. 11- and 16-oxygenated 17-oxosteroids were measurable simultaneously by increasing the sensitivity of the detector.

Comparisons with radioimmunoassay and a colorimetric method

To assess the reliability of the HPLC method for determination of DHEA sulfate in serum, DHEA sulfate levels in serum samples from 81 patients were

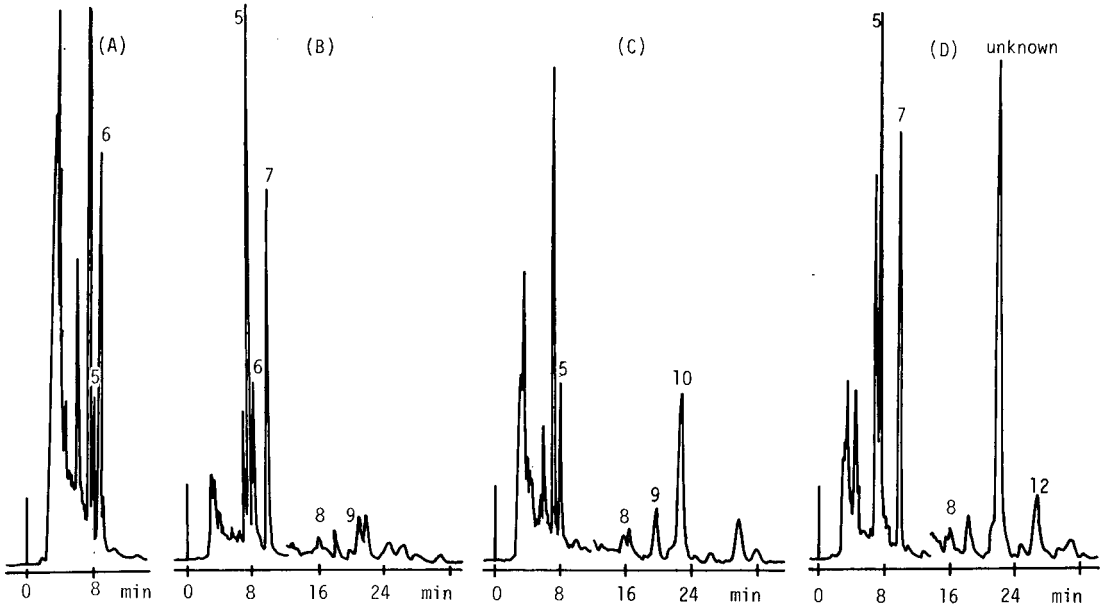

Fig. 5. Typical chromatograms of human serum and urine samples. (A) Normal human serum (male; age, 22): 5 = AN sulfate (32.8 μg/dl), 6 = DHEA sulfate (106.0 μg/dl). (B) Ovarian cystoma patient's urine (female, 21): 5 = AN (1.94 mg/day), 6 = DHEA (0.5 mg/day), 7 = ETIO (1.78 mg/day), 8 = 11-oxo-AN (38 μg/day), 9 = 11-oxo-ETIO (28 μg/day). (C) Adrenogenital syndrome patient's urine (male, 2): 5 = AN (22 μg/day), 8 = 11-oxo-AN (1.6 μg/day), 9 = 11-oxo-ETIO (5.24 μg/day), 10 = 16α-OH-DHEA (30 μg/day). (D) Hypertension patient's urine (male, 70): 5 = AN (4.32 mg/day), 7 = ETIO (4.64 mg/day), 8 = 11-oxo-AN (0.26 mg/day).

determined by both HPLC and radioimmunoassay (RIA). The RIA method used here is a direct assay of DHEA sulfate without hydrolysis after removing free steroids by dichloromethane extraction [16]. As shown in Fig. 6, the

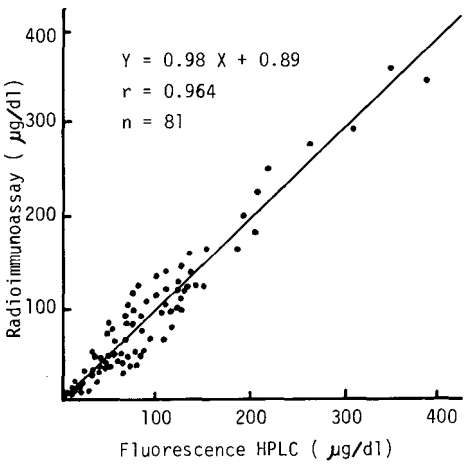

Fig. 6. Correlation between fluorescence HPLC and RIA values of dehydroepiandrosterone sulfate in serum.

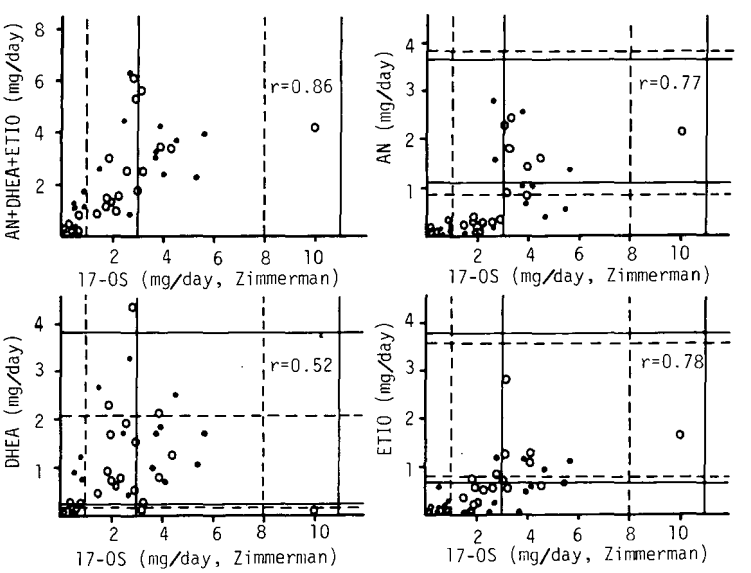

Fig. 7. Comparisons of results obtained with fluorescence HPLC and Zimmerman method for the determination of 17-oxosteroids in urine. (•) male, (○) female; normal value of male (straight line); normal value of female (broken line).

correlation coefficient was 0.964 and the regression line $Y = 0.98\ X + 0.89$, where X equals the values determined by the HPLC method.

Furthermore, the individual 17-oxosteroid levels in urine samples were determined by the HPLC method and the results were compared with the total values of 17-oxosteroids obtained by an ordinary colorimetric method used in routine assay. The results are shown in Fig. 7A—D.

DISCUSSION

Several reports on the use of HPLC to determine steroids in biological fluids have been published [7, 17], but the sensitivity of HPLC was too low due to the use of a UV detector. Especially, 17-oxosteroids have no strong UV-absorbing group in their molecules so that their detection limits were more than 1 μg using a refractive index detector. In order to increase the sensitivity in the assay of 17-oxosteroids, 2,4-dinitrophenylhydrazine has been used by several workers [8, 9, 18]. The 2,4-dinitrophenylhydrazone derivatives of 17-oxosteroids were easily separated by thin-layer chromatography or HPLC, and could be detected in quantities as low as 1 ng. Though these methods have been applied to the assay of 17-oxosteroids and their conjugates in urine samples, they could not be applied to the assay of 17-oxosteroids in serum samples. In this report a fluorophotometric HPLC method for the determination of 17-oxosteroids in serum and urine samples has been developed. Dansyl hydrazine was used as a fluorescent pre-labeling reagent. In the previous paper [11] cortisol in serum or urine was determined sensitively by fluorescence HPLC using dansyl hydrazine as an pre-labeling reagent. Though 17-oxosteroids react with dansyl hydrazine in the presence of acid at room temperature as described in the previous paper [11], the yield of hydrazone derivatives was low and the peak of excess hydrazine interfered with the separation of some 17-oxosteroids and other unknown peaks appeared in chromatogram. Therefore, the reaction conditions of labeling were examined and the optimal conditions were selected as described in Procedure.

The chromatographic conditions were also selected to give a complete separation between dansyl hydrazones of 17-oxosteroids and the fluorescent coexisting substances in serum or urine samples in the shortest possible analysis time. As shown in Fig. 3, good separation of dansyl hydrazone derivatives of 17-oxosteroids can be achieved with an Hitachi gel No. 3042 column or a Zorbax SIL column using the organic layer of dichloromethane—ethanol—water (400 : 1 : 2, v/v) as mobile phase. The detection limit of the 17-oxosteroids was about 60 pg from the working curves, as shown in Fig. 4. Then, using 0.1 ml of serum or 1.0 ml of urine as sample in routine assay, the detection limits are 0.5 or 0.7 μg/dl, respectively. The detection limit depends on the efficiency of the fluorescence detector and the final injection volume. When the residue in the assay tube is dissolved in 100 μl of solvent at the final step, the detection limit of DHEA in urine is about 35 ng/dl. The sensitivity of this method is superior to those of other HPLC methods using a UV detector. Moreover, the peaks of 17-oxosteroids were overlapped by a strong band of UV-absorbing substances in serum or urine extracts and could not be detected. However,

they appeared in the chromatogram measured by a fluorophotometer and could be determined quantitatively.

Good correlation (r = 0.964) was obtained between the values of DHEA sulfate in serum samples determined by the method proposed here and by radioimmunoassay.

The estimation of total urinary neutral 17-oxosteroids serves as a screening test for the diagnosis of adrenal or gonadal disease. However, in order to derive meaningful information, the determination of individual components of this group of steroids is very important. For example, androsterone and etiocholanolone are primary metabolites of testosterone. The increased excretion of these compounds in a male, without proportionate changes of DHEA and 11-oxygenated 17-oxosteroids, is a positive indication of testicular dysfunction. When total 17-oxosteroids are estimated, such specific changes will go unobserved. Therefore, we examined the determination of individual 17-oxosteroids in urinary samples by the method proposed here and compared the results with the total 17-oxosteroid values obtained by a spectrophotometric method after enzymatic hydrolysis of urine samples. As shown in Fig. 5, typical chromatograms obtained from patients are different from each other. Fig. 7A shows the correlation between the total values of 17-oxosteroids obtained by the spectrophotometric method and the sum values of AN, DHEA, and ETIO determined by the HPLC method. The sum values of AN, DHEA, and ETIO correlate well with the total values of 17-oxosteroids. On the other hand, the individual values of AN, DHEA, and ETIO do not correlate with the total values of 17-oxosteroids, as shown in Fig. 7B—D. These results mean that the determination of individual components of 17-oxosteroids in urine samples is very important and the method described in this paper may have clinical potential in the routine assay of 17-oxosteroids in serum and urine samples.

ACKNOWLEDGEMENTS

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STUDIES ON STEROIDS

CLXX. SEPARATION AND DETERMINATION OF BILE ACID 3-SULFATES IN HUMAN BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the simultaneous determination of sulfated bile acids in human bile without prior hydrolysis and solvolysis is described. The sulfate fraction was obtained from a bile specimen by passing it through a Sep-Pak C_{18} cartridge, followed by group separation by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl Sephadex LH-20. Subsequent resolution into the 3-sulfates of unconjugated, glycine- and taurine-conjugated ursodeoxycholate, cholate, chenodeoxycholate, deoxycholate and lithocholate was attained by high-performance liquid chromatography (HPLC) on an SC-02 column. Separation of these sulfates was effected when acetonitrile—0.5% ammonium carbonate (8:31, 8:26 and 8:23, v/v) was used as mobile phase. The sulfated bile acids in human bile were unequivocally identified on the basis of their behaviour in HPLC using mobile phases of various pH values. The present method proved to be applicable to the characterization and quantitation of sulfated bile acids in human bile.

INTRODUCTION

In recent years considerable attention has been focused on the metabolism of unsulfated and sulfated bile acids in man in connection with hepatobiliary diseases [1—4]. The separation and determination of sulfated bile acids has hitherto been performed by gas—liquid chromatography (GLC) with prior hydrolysis and/or solvolysis [3, 4]. This method, however, has the inevitable disadvantages of lack of reliability of the analytical results owing to incomplete deconjugation [5, 6] and formation of artifacts, as well as the loss of information about the conjugated form. In the previous paper of this series we described the high-performance liquid chromatography (HPLC) of the

3-sulfates of unconjugated, glycine- and taurine-conjugated bile acids [7]. The present paper deals with a method for the simultaneous determination of bile acid 3-sulfates in human bile, which involves clean-up with a Sep-Pak C_{18} cartridge, group separation by ion-exchange chromatography on a lipophilic gel, and subsequent resolution into individual sulfated bile acids by HPLC. In addition, characterization of these sulfates by HPLC using mobile phases of various pH values is also described.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus used for this work was a Waters 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model Uvidec-100 II ultraviolet (UV) detector (Japan Spectroscopic Co., Tokyo, Japan) for monitoring the absorbance at 205 nm. The test samples were applied to the chromatograph by a Waters U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. A column (25 cm × 4 mm I.D.) packed with ODS SC-02 (10 μm) (Japan Spectroscopic Co.), an octadecyl-bonded silica, was used under ambient conditions.

Materials

The standard bile acid 3-, 7- and 12-sulfates and glycocholic acid were synthesized in these laboratories by the methods previously reported [8, 9]. All the chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. Sephadex LH-20, and Amberlite XAD-2 and XAD-4 were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden) and Rohm and Haas Co. (Philadelphia, PA, U.S.A.), respectively. Amberlite XAD resins were washed successively with methanol, 6% hydrochloric acid in 70% ethanol, water, 6% sodium hydroxide in 70% ethanol, water and methanol before use. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) (0.8 mequiv./g was prepared in the manner previously reported [10]. A Sep-Pak C_{18} cartridge (Waters Assoc.) was also washed successively with methanol (5 ml) and water (10 ml) before use. Sulfoethyl Sephadex LH-20 (SE-LH-20) was prepared according to the method of Setchell et al. [11].

Procedure for determination of bile acid 3-sulfates in human bile

A bile specimen (100 μl) was diluted with 0.5 *M* phosphate buffer (pH 7.0, 4 ml), heated at 60°C for 1 h and passed through a Sep-Pak C_{18} cartridge. After washing with water (12 ml), unsulfated and sulfated bile acids were eluted with 90% ethanol (4 ml). The eluate was applied to a column (20 × 6 mm I.D.) of PHP-LH-20 (acetate) (110 mg). After removal of neutral compounds by washing with 90% ethanol (4 ml), unsulfated bile acids were separated into the unconjugated, glycine- and taurine-conjugated fractions by stepwise elution with 0.1 *M* acetic acid in 90% ethanol (4 ml), 0.2 *M* formic acid in 90% ethanol (4 ml) and 0.3 *M* acetic acid—potassium acetate in 90% ethanol (pH 6.3, 4 ml) in the manner previously reported [10, 12]. The sulfated bile acids were then eluted with 1% ammonium carbonate in 70% ethanol (4 ml). To the eluate were added glycocholic acid (2.0 μg) and 7α-

acetoxy-12α-hydroxy-3-oxo-5β-cholan-24-oic acid 12-sulfate (40 μg) as internal standards, and the whole was subjected to a SE-LH-20 column (50 mm × 10 mm I.D.) for elimination of inorganic salts. After washing with 70% ethanol (4 ml), the effluent and washing were combined and concentrated in vacuo below 40°C. The residue was redissolved in water (100 μl), a 10—30 μl aliquot of which was subjected to HPLC.

Recovery test for bile acid 3-sulfates added to human bile

The test samples were prepared by dissolving the 3-sulfates (50 μg each of unconjugated and 5 μg each of glycine- and taurine-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate) in human hepatic bile (100 μl). The bile specimen was diluted with 0.5 *M* phosphate buffer (4 ml) and subjected successively to clean-up with a Sep-Pak C_{18} cartridge, group separation on PHP-LH-20, desalting on SE-LH-20 and determination by HPLC in the manner described above.

Extraction of bile acid sulfates by Amberlite XAD resin or Sep-Pak C_{18} cartridge

Amberlite XAD resin. A synthetic mixture of 50 μg each of 3-sulfates of glycine- and taurine-conjugated cholate and chenodeoxycholate was dissolved in water (50 ml), adjusted to pH 2—11 with dilute hydrochloric acid or sodium hydroxide solution and then applied to a column (12.5 cm × 18 mm I.D.) of Amberlite XAD-2 or XAD-4 (10 g). After washing with water (50 ml), the sulfates were eluted with 50—100% ethanol (100 ml) with or without concentrated hydrochloric acid (0.05 ml) or concentrated ammonia (1 ml). The eluate was added with an internal standard and then subjected to HPLC.

Sep-Pak C_{18} cartridge. A synthetic mixture of 50 μg of chenodeoxycholate 3-sulfate, 25 μg each of 3-sulfates of taurochenodeoxycholate, glycine-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate was dissolved in phosphate buffer (pH 7.0, 4 ml) and applied to the cartridge. After washing with water (8 ml), the sulfates were eluted with 90% ethanol. The effluent was fractionally collected (each 0.5 ml), to which was added an internal standard, and then subjected to HPLC.

RESULTS AND DISCUSSION

Group separation of sulfated bile acids on PHP-LH-20

In the previous paper of this series, we demonstrated the separation of unsulfated bile acids into unconjugated, glycine- and taurine-conjugated fractions on a lipophilic ion-exchange gel, PHP-LH-20 [10, 12]. Almé et al. [4] reported the group separation on diethylaminohydroxypropyl Sephadex LH-20 where acetate buffer in 72% ethanol (pH 7.6) was used for the elution of bile acid monosulfates. In this study, 1% ammonium carbonate in 70% ethanol (pH 9.0) was chosen as a suitable eluent for sulfated bile acids. A synthetic mixture of 50 μg each of 3-sulfates of unconjugated, glycine- and taurine-conjugated cholate and lithocholate dissolved in 90% ethanol was applied to a column of PHP-LH-20. After washing with 90% ethanol, unsulfated bile acids were eluted successively with 4 ml each of 0.1 *M* acetic acid in 90% ethanol, 0.2 *M* formic

acid in 90% ethanol and 0.3 *M* acetic acid—potassium acetate in 90% ethanol (pH 6.3). The desired sulfate fraction was then eluted with 1% ammonium carbonate in 70% ethanol and subjected to HPLC. As illustrated in Fig. 1, each of the bile acid 3-sulfates was recovered at a rate of more than 90% in an initial 2-ml effluent. Other 3-sulfates also showed a similar elution pattern.

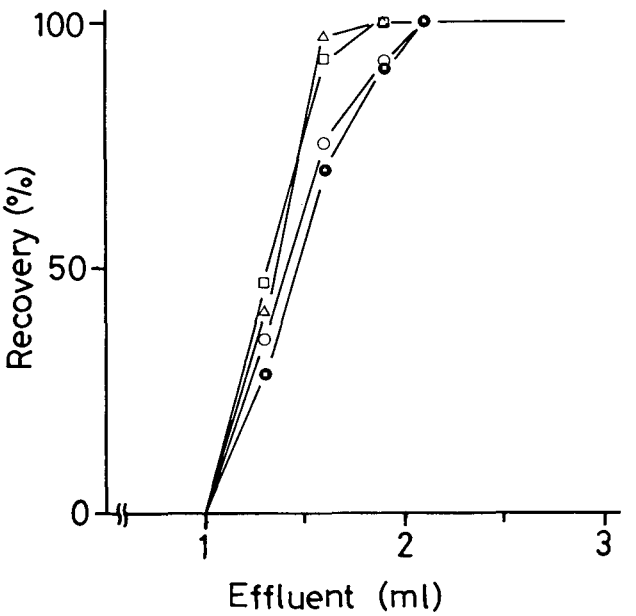

Fig. 1. Cumulative elution curves of bile acid 3-sulfates on PHP-LH-20. (○), Cholate 3-sulfate; (□), glycocholate 3-sulfate; (△), taurocholate 3-sulfate; (●), lithocholate 3-sulfate.

Clean-up procedure for sulfated bile acids in human bile

Amberlite XAD resin is widely used for the separation of polar compounds in biological fluids. Recently, Bradlow [13] reported that steroid conjugates such as dehydroepiandrosterone sulfate are quantitatively eluted with methanol from Amberlite XAD-2 when the conjugates are converted to the triethylamine salts by washing with 0.5 *M* triethylamine sulfate (pH 7.2). By this method, however, sulfated bile acids were not quantitatively recovered. The effect of pH on adsorption and desorption on Amberlite XAD-2 or XAD-4 was investigated with various bile acid 3-sulfates. A synthetic mixture of 50 μg each of 3-sulfates of cholate, glycocholate, taurocholate, chenodeoxycholate, glycochenodeoxycholate and taurochenodeoxycholate was dissolved in water, adjusted to pH 2—11, and then applied to an Amberlite XAD-2 or XAD-4 column. The sulfates eluted with ethanol were separated and determined by HPLC. In the case of Amberlite XAD-4, the sulfates were effectively adsorbed when the solution was adjusted to pH 8—10 (Fig. 2). The use of an acidified eluent was suitable for desorption of the sulfates. The content of ethanol in the eluent was also important (Fig. 3). As for Amberlite XAD-2, the use of pH 4 for the sample solution and pH 9 for the eluent was found most suitable for extraction of sulfated bile acids and this result was almost identical with that

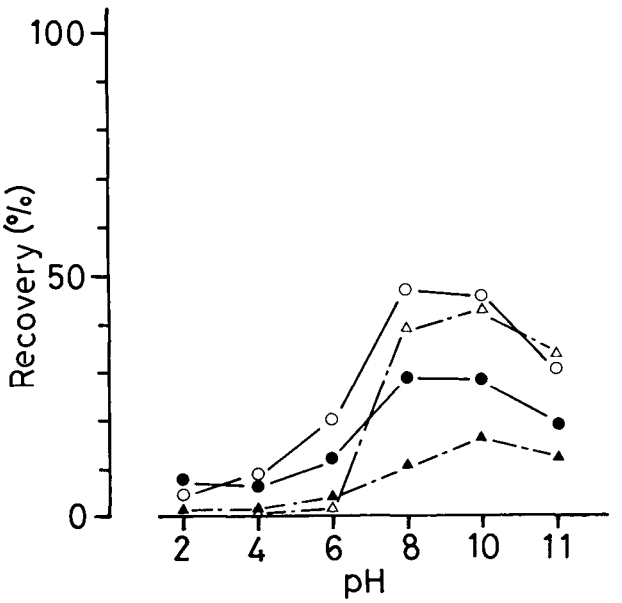

Fig. 2. Effect of pH on the adsorption of bile acid 3-sulfates on Amberlite XAD-4. (○), Glycocholate 3-sulfate; (●), taurocholate 3-sulfate; (△), glycochenodeoxycholate 3-sulfate; (▲), taurochenodeoxycholate 3-sulfate.

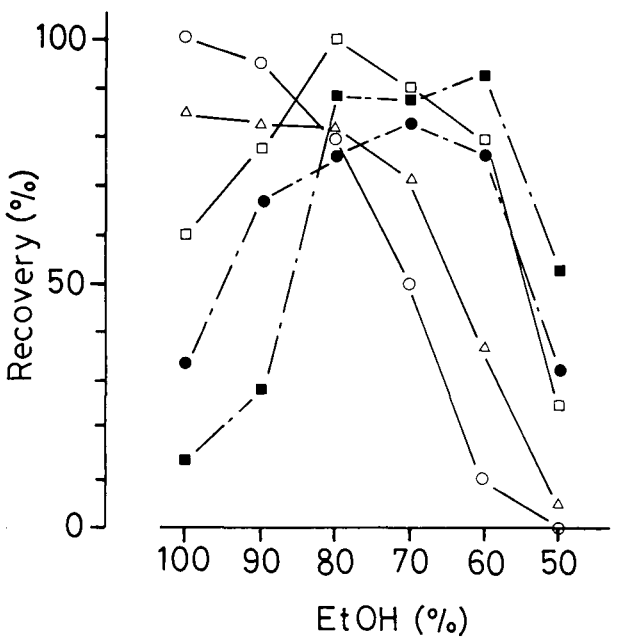

Fig. 3. Effect of ethanol (EtOH) concentration on the desorption of bile acid 3-sulfates on Amberlite XAD-4. (○), Deoxycholate 3-sulfate; (△), glycodeoxycholate 3-sulfate; (□), taurodeoxycholate 3-sulfate; (●), taurochenodeoxycholate 3-sulfate; (■), taurocholate 3-sulfate.

reported previously [4]. However, the recovery rates of the sulfates by these procedures were dependent upon their structures and, hence, the appropriate extraction conditions for all the sulfates could not be established. Moreover, the use of Amberlite XAD resins required removal of interfering inorganic acid or base in the eluent prior to separation on a lipophilic ion-exchange gel [4]. Accordingly, the use of a Sep-Pak C_{18} cartridge [12, 14] for this purpose was then undertaken. A synthetic mixture of 3-sulfates of chenodeoxycholate, taurochenodeoxycholate, glycine-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate was dissolved in phosphate buffer (pH 7.0) and applied to the cartridge. After elimination of inorganic salts by washing with water, the desired sulfates were eluted with 90% ethanol and then determined by HPLC. The sulfated bile acids were recovered at a rate of more than 90% in an initial 2-ml effluent. When 1.5% ethanol was used to remove co-existing polar substances as previously reported [12], a small amount of the polar sulfate was lost. The use of a Sep-Pak C_{18} cartridge was much more efficient and convenient for extraction of sulfated bile acids in biological fluids than Amberlite XAD resin.

Determination of sulfated bile acids in human bile

A standard procedure for the separation and determination of sulfated bile acids in human bile is shown in Fig. 4. The sulfates were separated by HPLC on the ODS SC-02 column under the conditions previously reported [7] with a minor modification. First, 3-sulfates of unconjugated, glycine- and taurine-conjugated ursodeoxycholate and cholate were separated with acetonitrile—0.5% ammonium carbonate (8:31, v/v) and then the 3-sulfates of unconjugated and conjugated chenodeoxycholate and deoxycholate were resolved with acetonitrile—0.5% ammonium carbonate (8:26, v/v). Finally, acetonitrile—0.5% ammonium carbonate (8:23, v/v) was chosen as a suitable mobile phase for the separation of lithocholate 3-sulfates. For quantitation of ursodeoxycholate and cholate 3-sulfates, 7α-acetoxy-12α-hydroxy-3-oxo-5β-cholan-24-oic acid 12-

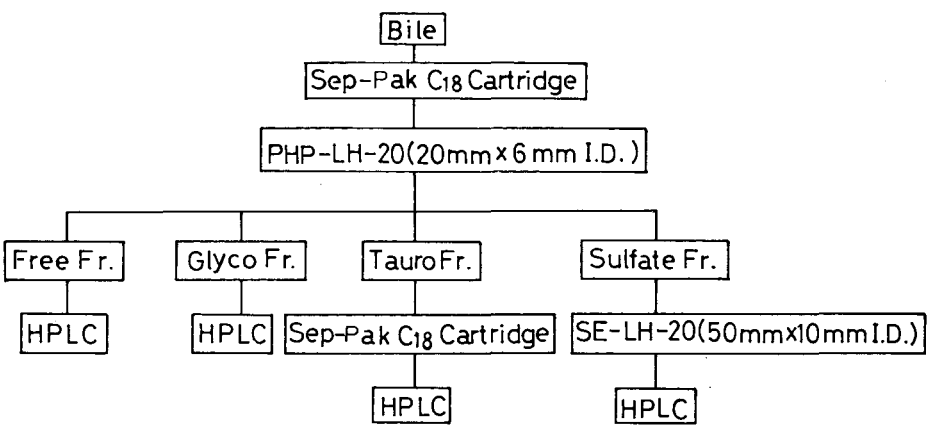

Fig. 4. General scheme for separation and determination of bile acid 3-sulfates in human bile.

sulfate was used as an internal standard, while for other sulfates glycocholic acid was used.

The calibration curve was constructed by plotting the ratio of peak area of each bile acid 3-sulfate to that of the internal standard against the amount of 3-sulfate. The quantitation limits of glycine- and taurine-conjugated and unconjugated bile acid 3-sulfates obtainable by monitoring the absorbance at 205 nm were 0.5, 1.0 and 5.0 μg, respectively. Applying the standard procedure to human bile, 3-sulfates of conjugated bile acids were determined with a satisfactory reproducibility (Table I). The known amounts of representative bile acid 3-sulfates were added to human bile and their recovery rates were estimated. As listed in Table II, almost all bile acid 3-sulfates were recovered at a rate of more than 90%, the only exception being lithocholate 3-sulfate (88%).

TABLE I

REPRODUCIBILITY OF THE PRESENT METHOD FOR DETERMINATION OF BILE ACID 3-SULFATES IN HUMAN BILE

Bile acid 3-sulfate	Found (μg per 0.1 ml, ± S.D.)*	
	G**	T**
Ursodeoxycholate	5.46 ± 0.42	0.88 ± 0.18
Cholate	2.10 ± 0.35	1.33 ± 0.24
Chenodeoxycholate	5.63 ± 0.19	5.59 ± 0.13
Deoxycholate	7.59 ± 0.17	8.20 ± 0.30
Lithocholate	2.38 ± 0.21	1.01 ± 0.28

*n = 9.
**G = glycine conjugate; T = taurine conjugate.

TABLE II

RECOVERY OF UNCONJUGATED AND CONJUGATED BILE ACID 3-SULFATES ADDED TO HUMAN BILE

Bile acid 3-sulfate	Bile	Added (μg per 0.1 ml)	Expected (μg per 0.1 ml)	Found (μg per 0.1 ml)	Recovery (%, ± S.D.*)
Cholate	0	47.0	47.0	45.2	96.4 ± 7.5
Chenodeoxycholate	0	49.1	49.1	48.6	99.0 ± 0.8
Deoxycholate	0	46.7	46.7	45.0	96.4 ± 7.0
Lithocholate	0	34.0	34.0	30.0	88.2 ± 6.2
Glycocholate	0	6.4	6.4	6.1	95.3 ± 5.1
Glycochenodeoxycholate	2.0	4.8	6.8	6.7	98.5 ± 1.9
Glycodeoxycholate	0	4.5	4.5	4.4	97.8 ± 3.8
Glycolithocholate	0	4.0	4.0	3.8	95.0 ± 2.5
Taurocholate	0	5.9	5.9	5.6	94.9 ± 5.1
Taurochenodeoxycholate	1.0	5.3	6.3	6.2	98.4 ± 2.6
Taurodeoxycholate	0	5.3	5.3	5.1	96.2 ± 1.6
Taurolithocholate	0	5.1	5.1	4.9	96.1 ± 2.7

*n = 8.

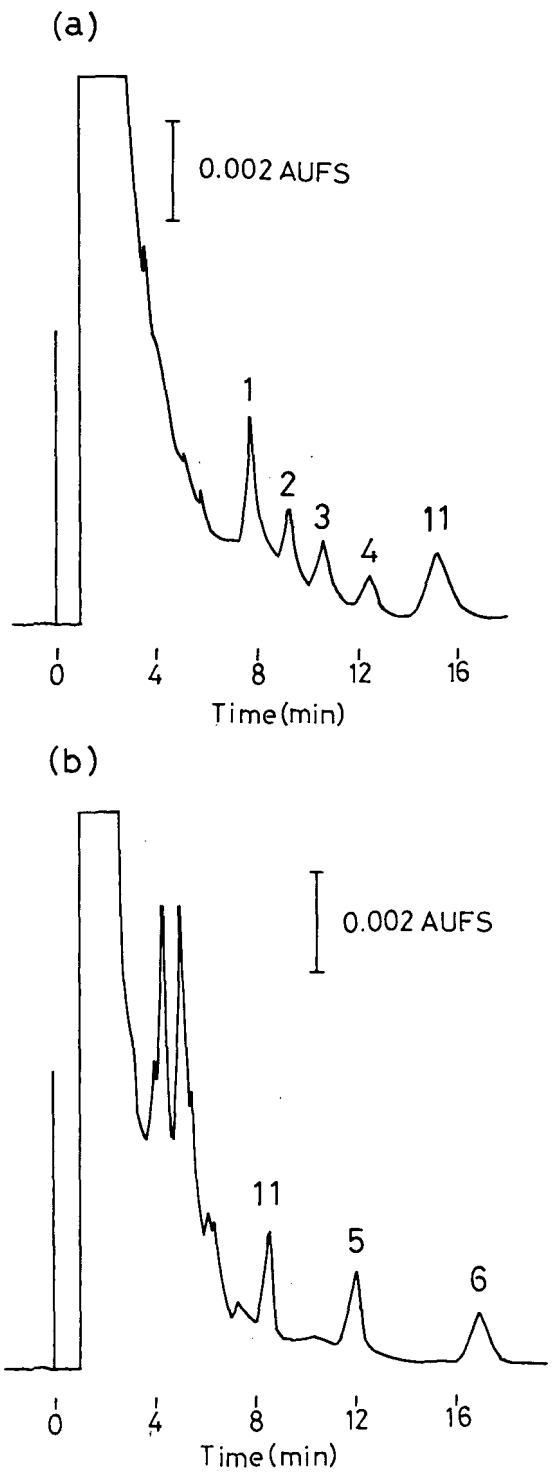
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Fig. 5.

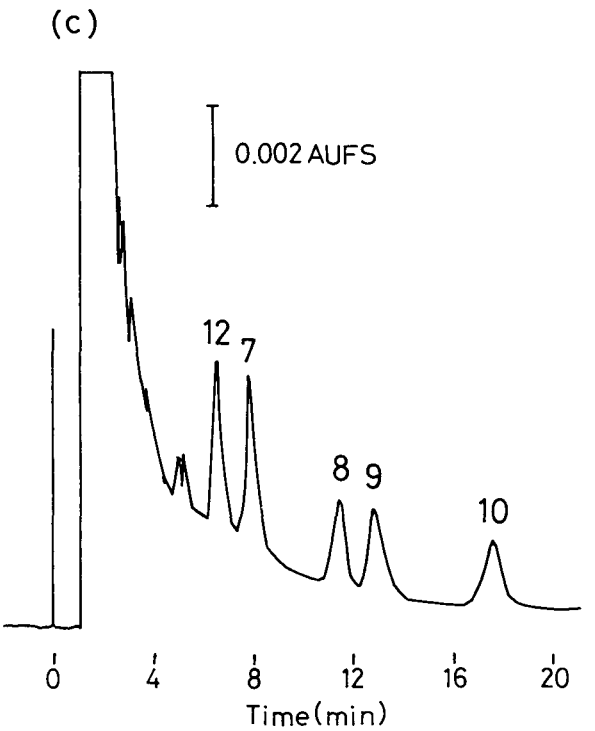

Fig. 5. Separation of bile acid 3-sulfates in human bile by HPLC. Conditions: SC-02 column; mobile phase, acetonitrile—0.5% ammonium carbonate (a) 8:26 (v/v), (b) 8:23 (v/v), (c) 8:31 (v/v), 2 ml/min. 1 = Glycochenodeoxycholate 3-sulfate, 2 = glycodeoxycholate 3-sulfate, 3 = taurochenodeoxycholate 3-sulfate, 4 = taurodeoxycholate 3-sulfate, 5 = glycolithocholate 3-sulfate, 6 = taurolithocholate 3-sulfate, 7 = glycoursodeoxycholate 3-sulfate, 8 = tauroursodeoxycholate 3-sulfate, 9 = glycocholate 3-sulfate, 10 = taurocholate 3-sulfate, 11 = glycocholate (internal standard), 12 = 7α-acetoxy-12α-hydroxy-3-oxo-5β-cholan-24-oic acid 12-sulfate (internal standard).

A chromatogram of bile acid 3-sulfates in human bile which had been processed in the manner described above is illustrated in Fig. 5. Simultaneous determination of bile acid 3-sulfates was carried out with ten bile specimens collected from post-operative patients with obstructive jaundice. The results obtained are listed in Table III. It should be noted that in almost all the cases 3-sulfates of conjugated ursodeoxycholate were found in a larger amount, corresponding to 5—38% of the total bile acid 3-sulfates. No unconjugated bile acid 3-sulfates could be detected even when 1 ml of human bile was subjected to HPLC.

Characterization of bile acid 3-sulfates in human bile

The disadvantage of HPLC in structural elucidation, because the information provided is insufficient, has already been pointed out. For this purpose GLC—mass spectrometry is widely used. This technique, however, is not applicable to the characterization of sulfated bile acids. Recently, a new monitoring system using dual wavelengths has been devised [15, 16]. This method is applicable to compounds having a chromophore but not to sulfated bile acids. In a

TABLE III

AMOUNTS OF BILE ACID 3-SULFATES IN BILE OF PATIENTS WITH OBSTRUCTIVE JAUNDICE

Results are given in μg/ml.

Subject	Cholate		Chenodeoxy-cholate		Deoxycholate		Lithocholate		Ursodeoxy-cholate	
	G*	T*	G	T	G	T	G	T	G	T
A**	29	14	62	46	69	69	10	8	40	9
B***	48	16	55	41	trace	trace	10	13	10	trace
C	18	13	17	17	n.d.	9	n.d.	n.d.	n.d.	n.d.
D***	n.d.§	n.d.	20	10	n.d.	n.d.	n.d.	n.d.	8	n.d.
E	n.d.	n.d.	24	25	n.d.	16	n.d.	n.d.	26	7
F	49	18	39	25	26	17	90	29	63	trace
G	51	13	20	16	n.d.	trace	10	16	25	trace
H	trace	n.d.	33	26	17	17	n.d.	n.d.	30	12
I	11	n.d.	11	12	5	5	n.d.	n.d.	10	17
J	13	n.d.	29	28	48	43	32	14	36	n.d.

*G = glycine conjugate; T = taurine conjugate.
**Pre-operative patient.
***Patient administered ursodeoxycholic acid.
§ n.d. = not detectable.

previous study we investigated the chromatographic behaviours of sulfated bile acids with mobile phases of varying pH and found that they were dependent upon the position of the sulfate and hydroxyl groups and the structure of the side-chain [17]. In the present study, this finding was applied to the structural characterization of bile acid 3-sulfates in human bile. The eluate corresponding to each peak on the chromatogram was collected and, after the addition of an internal standard, subjected to HPLC using three mobile phases of varying pH. As listed in Table IV, relative k' values of bile acid sulfates in bile were identical with those of authentic samples. Moreover, the peak area ratio of each sulfate to the corresponding internal standard showed almost the same value at pH 3.5, 5.5 and 7.5 with a standard deviation of 0.7—6.3 (Table V). These results imply that the present method undergoes no interferences with co-existing substances and is favorable for the determination and structural elucidation of sulfated bile acids in biological fluids.

It is hoped that the availability of an excellent method for the separation and determination of sulfated bile acids without prior hydrolysis and solvolysis may provide more precise knowledge on the metabolic profile of bile acids and may serve in the diagnosis of hepatobiliary diseases.

TABLE IV

RELATIVE k' VALUES OF SULFATED BILE ACIDS IN HUMAN BILE AND STANDARD SAMPLES*

Sulfated bile acid	pH 3.5		pH 5.5		pH 7.5	
	G**	T**	G	T	G	T
Ursodeoxycholate 3-S** (a)	1.95	0.68	0.53	0.70	0.42	0.62
Ursodeoxycholate 7-S	3.70	1.43	1.15	1.53	0.97	1.43
Bile	1.95	0.68	0.53	0.70	0.42	0.62
Cholate 3-S (a)	2.55	1.00	0.78	1.00	0.71	1.00
Cholate 7-S	2.10	0.89	0.66	0.83	0.62	0.84
Cholate 12-S	2.55	1.03	0.76	0.92	0.69	0.91
Bile	2.55	1.00	0.78	1.00	0.71	1.00
Chenodeoxycholate 3-S (b)	3.10	0.88	0.64	0.82	0.57	0.83
Chenodeoxycholate 7-S	3.18	0.96	0.72	0.81	0.67	0.98
Bile	3.10	0.88	0.64	0.82	0.57	0.83
Deoxycholate 3-S (b)	3.54	1.00	0.76	1.00	0.70	1.00
Deoxycholate 12-S	4.18	1.32	0.99	1.13	0.92	1.34
Bile	3.54	1.00	0.76	1.00	0.70	1.00
Lithocholate 3-S (c)	4.43	1.00	0.80	1.00	0.67	1.00
Bile	4.43	1.00	0.80	1.00	0.67	1.00

*The figures express k' values relative to the following internal standards: (a) taurocholate 3-sulfate; (b) taurodeoxycholate 3-sulfate; (c) taurolithocholate 3-sulfate.
**G = glycine conjugate; T = taurine conjugate; S = sulfate.

TABLE V

PEAK AREA RATIOS OF BILE ACID 3-SULFATES IN HUMAN BILE TO INTERNAL STANDARDS

Sulfated bile acid	Internal standard	pH			S.D. (%)
		3.5	5.5	7.5	
Glycoursodeoxycholate 3-S*	Glycocholate 3-S	1.73	1.68	1.74	1.9
Tauroursodeoxycholate 3-S	Taurocholate 3-S	0.25	0.24	0.27	6.3
Glycocholate 3-S	Glycoursodeoxycholate 3-S	0.60	0.66	0.60	5.6
Taurocholate 3-S	Tauroursodeoxycholate 3-S	1.33	1.28	1.32	2.0
Glycochenodeoxycholate 3-S	Glycodeoxycholate 3-S	0.72	0.75	0.76	2.9
Taurochenodeoxycholate 3-S	Taurodeoxycholate 3-S	0.76	0.83	0.78	4.6
Glycodeoxycholate 3-S	Glycochenodeoxycholate 3-S	1.49	1.51	1.47	1.3
Taurodeoxycholate 3-S	Taurochenodeoxycholate 3-S	0.98	0.99	0.99	0.7
Glycolithocholate 3-S	Glycocholate	0.42	0.38	0.40	5.0
Taurolithocholate 3-S	Glycocholate	0.37	0.38	0.39	2.6

*S = sulfate.

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CHROMBIO. 982

DETERMINATION OF PHENYLPYRUVIC ACID IN URINE AND SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A highly sensitive and simple method for the determination of phenylpyruvic acid in urine and serum is described which employs high-performance liquid chromatography with fluorescence detection. Phenylpyruvic acid, after extraction with ethyl acetate, is reacted with 4'-hydrazino-2-stilbazole in aqueous methanol to give the corresponding fluorescent hydrazone which is separated by reversed-phase chromatography on μBondapak Phenyl. The lower limits of detection are 25 and 32 pmol for phenylpyruvic acid in 0.2 ml of urine and serum, respectively. This sensitivity permits the determination of the acid in urine of normal adults and newborn infants.

INTRODUCTION

It is well known that phenylpyruvic acid (PPA) is greatly increased in serum and urine of patients with phenylketonuria. Several methods have been reported for the determination of PPA in biological samples. Spectrophotometric methods [1—5] are not sensitive and selective for PPA. Gas chromatographic methods [6, 7] and gas chromatographic—mass spectrometric methods [8, 9] are sensitive and selective, but not simple to perform. A high-performance liquid chromatographic (HPLC) method with UV detection for the determination of PPA in urine, based on the pre-column derivatization of PPA with 2,3-diaminonaphthalene, has been reported [10]. However, this method is not so sensitive and requires a long time for the derivatization. Although HPLC methods with fluorescence detection have also been described for the sensitive determination of 2-oxo acids based on the derivatization with 4-bromomethyl-7-methoxycoumarin [11] and with N^1-methylnicotinamide chloride

[12], these methods have not been applied to PPA in urine and serum.

Recently, we found that the reaction of PPA with 4′-hydrazino-2-stilbazole (4H2S; fluorogenic reagent for 2-oxo acids and other carbonyl compounds) to give the corresponding hydrazone [13] was enhanced by the addition of methanol to the reaction mixture, the resulting fluorescence being stabilized for a long time. The hydrazone could be separated from the products from other carbonyl compounds present in biological samples by reversed-phase HPLC. We thus developed a highly sensitive and simple HPLC method with fluorescence detection for the determination of PPA in human urine and serum.

EXPERIMENTAL

Materials and reagents

All chemicals were of analytical-reagent grade, unless otherwise noted. 4H2S dihydrochloride was purchased from Tokyo Kasei Ind. Co. (Tokyo, Japan). Double-distilled water and solvents were used. Urine and serum samples of adults were obtained from normal volunteers in our laboratory. Urine samples from newborn infants were supplied from Kyushu University Hospital.

Apparatus

A Mitsumi liquid chromatograph equipped with a 7120 syringe-loading sample injector and a Shimadzu FLD-1 fluorescence detector fitted with a coated mercury lamp (emitting light, ca. 300—400 nm; maximum intensity of the light, 360 nm) and an EM-4 secondary cut-off filter (cutting out the light of wavelengths shorter than 430 nm) was used. The column was μBondapak Phenyl (particle size, 10 μm; 300 × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.). This column can be used for more than 500 injections with only a small decrease in the theoretical plate number when washed with aqueous methanol (1 : 1, v/v) at the flow-rate of 1 ml/min for ca. 10 min after everyday analyses. The fluorescence spectra of the column effluents were measured with an Hitachi MPF-4 spectrofluorimeter in 10 × 10 mm cells. The slit-widths in terms of wavelengths were set at 10 nm in both the exciter and the analyser. The spectra are uncorrected.

Procedure

To 0.2 ml of urine or serum placed in a 10-ml centrifuge tube, 1.0 ml of water and a mixture of benzene and ethyl acetate (1 : 1, v/v) were added. The mixture was shaken for 3 min and centrifuged. The organic layer was discarded, then the aqueous layer was acidified, with ca. 0.15 ml (3 drops) of concentrated hydrochloric acid, to pH 1 or less, followed by addition of 0.5 g of sodium chloride. The mixture was extracted with 1.0-ml portions of ethyl acetate (twice) by 5-min shaking and centrifugation. The extracts were combined and concentrated to dryness in vacuo at room temperature. To the residue, 1.0 ml of water, 0.5 ml of 0.5 *M* ammonium chloride solution (pH 4.0, adjusted with 0.1 *M* hydrochloric acid) and 0.5 ml of 1.2 m*M* 4H2S dihydrochloride solution in methanol (freshly prepared) were added. The mixture was warmed at 50°C for 10 min in the dark to develop fluorescence and cooled in ice—water. Within 2 h, an aliquot (50 μl) of the reaction mixture was applied

to the chromatograph. The mobile phase was a mixture of 0.1 *M* hydrochloric acid, tetrahydrofuran and water (10 : 24 : 64, v/v; pH 2.0) and the flow-rate was 1.0 ml/min. The column temperature was ambient (ca. 25°C). The height of the peak at the retention time of 13 min was used for the quantitation. The amount of PPA was calibrated by means of the standard addition method: 1.0 ml of water added to the sample in the procedure was replaced by 1.0 ml of a PPA standard solution (0.50 nmol/l; prepared with the sodium salt of PPA; stable for more than 6 months at 5°C).

RESULTS AND DISCUSSION

HPLC conditions

Fig. 1 shows the chromatograms obtained with PPA solutions and the reagent blank. The peak observed at the retention time of 13 min is repro-

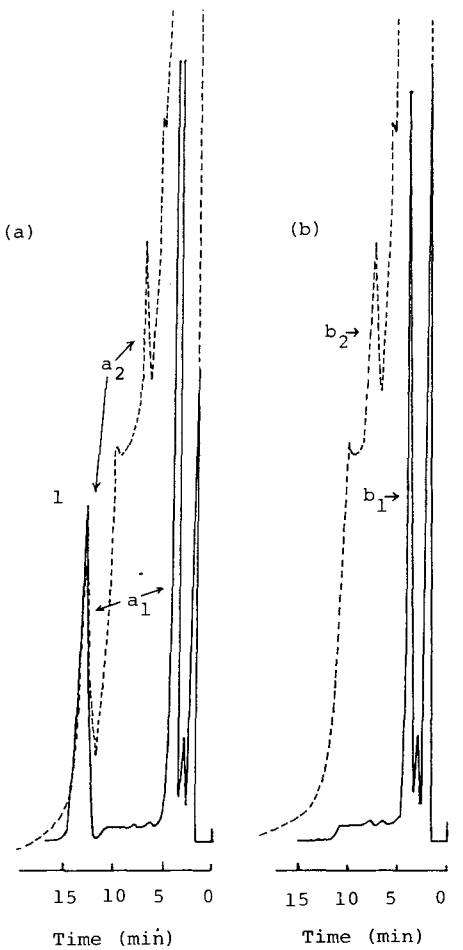

Fig. 1. Chromatograms of (a) 4H2S derivative of PPA, and (b) reagent blank. Aliquots (1.0 ml) of PPA solutions of 20 nmol/ml (a_1) and 0.5 nmol/ml (a_2), and of water for blank, were treated with 4H2S as in the procedure. Detector sensitivity: a_1 and b_1, 1; a_2 and b_2, 32. Peak 1 = PPA.

ducible and there is a linear relationship between the peak height and the amount of PPA under the prescribed conditions. The peak is not interfered with by the blank which is recorded as large peaks when the instrument sensitivity is set at a high level for the determination of PPA at sub-nanomol concentrations (Fig. 1, a_1 and a_2, peak 1).

The concentration of tetrahydrofuran in the mobile phase affects the separation of the peaks. At a concentration greater than 26%, the peak for PPA overlaps those of the blank, while a concentration of less than 20% causes delay in the elution with broadening of the peak; a concentration of 24% was selected for the procedure recommended. The 4H2S derivative of PPA fluoresces most intensely at pH 2 or less with a minimum blank fluorescence, and the column packing μBondapak Phenyl can be used in the limited range of pH 2—8. Therefore, a mobile phase of pH 2.0 was used in the recommended procedure.

Sample solutions for HPLC

Under the HPLC conditions described, 4H2S derivatives of biologically important carbonyl compounds examined (e.g. pyruvic, oxalacetic, 2-oxoglutaric, 2-oxobutyric, 2-oxocaproic, 2-oxoadipic, 2-oxoisovaleric, indolepyruvic, and *p*-hydroxyphenylpyruvic acids, formaldehyde, acetaldehyde, *n*-butylaldehyde, propionaldehyde, benzaldehyde, acetone and diacetyl) eluted much earlier or later than the retention time of 13 min. However, the peak for PPA overlaps that of the derivative of *p*-hydroxybenzaldehyde, which exists usually in a small amount in urine [14]; and the derivatives of isovaleraldehyde and vanillin elute closely to that of PPA (retention times, 12.3 and 12.7 min, respectively). These aldehydes give peak heights of less than 0.2% that of PPA at equimolar concentrations.

A number of other compounds examined (e.g. L-α-amino acids, sugars, aliphatic and aromatic amines, carboxylic acids, aldehydes and ketones, phenols, steroids and many others), all of them of biological importance, gave no peak on the chromatogram when added to urine and serum at a concentration of 100 nmol/ml or greater.

Water-diluted urine and serum are washed with benzene and ethyl acetate prior to the extraction of PPA in the procedure recommended. If this washing is omitted, the peak for PPA overlaps slightly with those due to unknown substances present in urine and serum samples. PPA can be extracted from strongly acidified urine and serum with ethyl acetate in the presence of a saturating concentration of sodium chloride.

The reaction of 2-oxo acid with 4H2S has been carried out in aqueous solution [13]. Methanol added to the reaction mixture enhances the reaction of PPA with the reagent and stabilized the resulting fluorescence (Fig. 2) in the concentration range of 20—30%; 25% was used in the procedure. Dimethylsulfoxide and dimethylformamide caused a decrease in the fluorescence development (Fig. 2). 4H2S was dissolved in the methanol. The reagent gives the most intense fluorescence at a concentration greater than ca. 1 m*M*; 1.2 m*M* was used as a sufficient concentration.

The fluorescence reaction proceeds most effectively at about pH 4. This was achieved by using 0.5 *M* ammonium chloride solution of pH 4.0. Formic acid solution at pH 4.0, when used in place of the ammonium chloride solution as

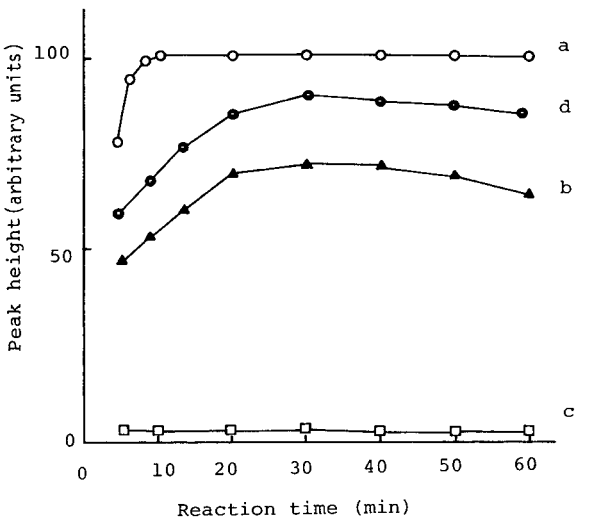

Fig. 2. Effect of solvent and reaction time on the fluorescence development. Portions (1.0 ml) of PPA solution (10 nmol/ml) were treated as in the procedure for the fluorescence development with (a) 4H2S dissolved in methanol, (b) dimethylsulfoxide, (c) dimethylformamide, and (d) water, for various reaction times.

previously described [13], caused some interfering peaks in the chromatogram. Higher temperature allows the fluorescence to develop more rapidly. At the recommended temperature, 50°C, the fluorescence intensity (peak height) reaches a maximum after warming for 10 min or more (Fig. 2). The fluorescence is unstable in daylight, and so the reaction should be carried out in the dark. The resulting fluorescence is stable for more than 2 h at room temperature.

Determination of PPA in urine and serum

Fig. 3 shows typical chromatograms obtained with normal urine and normal serum spiked with PPA according to the procedure. Small peaks observed at the retention time of 23 and 30 min for normal urine and a peak at 26 min for normal serum were unidentified. These peaks do not interfere with the quantitation of PPA in the biological samples.

The fluorescence excitation (maximum, 401 nm) and emission (maximum, 544 nm) spectra of the effluent from peak 1 in Fig. 3a were identical with those of the effluent from peak 1 in the chromatogram for PPA solution (Fig. 1a). A four-times methanol-diluted effluent of peak 1 in Fig. 3a (apparent pH 2.7) had the same fluorescence spectrum as that of peak 1 in Fig. 1a (excitation and emission maxima, 403 and 449 nm, respectively). When a mixture (ca. 1:1, v/v) of both effluents was subjected to HPLC on a LiChrosorb RP-18 (particle size, 5 μm; Japan Merck, Tokyo, Japan) column (150 × 4 mm I.D.; packed as previously described [15]; column temperature, 25°C) with 0.1 *M* hydrochloric acid—tetrahydrofuran—water (10:25:65, v/v) as mobile phase (flow-rate, 0.7 ml/min), a single peak was obtained at the retention time of 8.6 min. These observations indicate that the component of peak 1 in Fig. 3 is due undoubtedly to PPA from the urine sample.

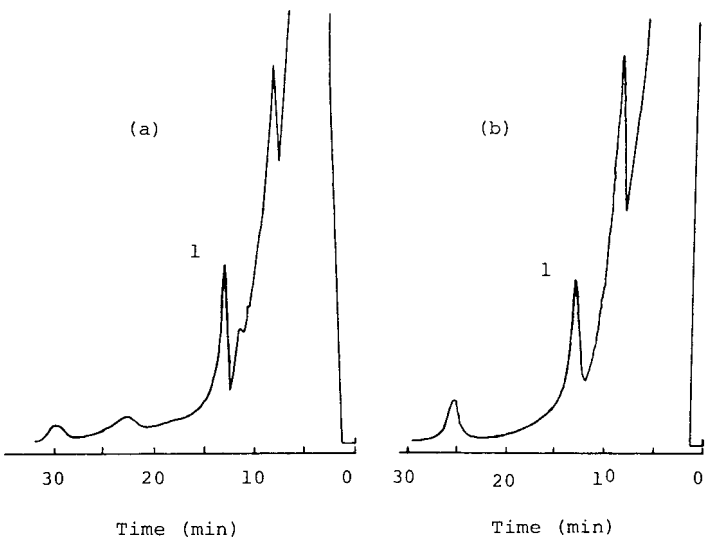

Fig. 3. Chromatograms of 4H2S derivative of PPA in (a) normal urine, and (b) normal serum (free from PPA) spiked with 0.5 nmol/ml PPA, obtained by the recommended procedure. Peak 1 = PPA. The concentration of PPA in urine was 0.58 nmol/ml.

A linear relationship was observed between the peak height and the amount of PPA added to urine or serum up to at least 4 nmol per 0.2 ml. The recoveries of PPA added to 0.2 ml of urine and serum in the amount of 0.5 nmol were 100 ± 3% and 78 ± 4% (mean ± standard deviation, n = 15 each), respectively. The recoveries were calculated from the determined values obtained with the fortified urine and serum samples and a PPA standard solution (0.5 nmol/ml) treated as in the procedure. The lower limits of detection for PPA in 0.2 ml of urine or serum were 25 and 32 pmol, respectively.

TABLE I

URINARY EXCRETION (24 h) OF PPA FROM HEALTHY PERSONS

Adults			Newborn infants*
Age (years)	Sex	PPA (nmol)	PPA (nmol)
22	m	800	1.48
22	m	500	1.54
23	m	920	4.36
28	m	550	3.31
29	f	990	8.59
30	f	970	4.05
30	m	860	5.54
30	m	520	13.26
32	m	500	19.15
33	m	450	2.74
Mean ± S.D.		710 ± 209	6.40 ± 5.44

*All female subjects, 2 days old.

The precision of the method was examined by performing ten separate determinations on urines containing 490 and 220 pmol per 0.2 ml PPA, and sera (free from PPA) spiked with 500 and 200 pmol PPA per 0.2 ml. The standard deviations were 15 and 8 pmol per 0.2 ml for PPA in urine, and 26 and 13 pmol per 0.2 ml for PPA in serum, respectively.

The concentration of PPA in normal urines of adults (22—33 years old, n = 10) and newborn infants (2 days old, n = 10) determined by this method were 0.68 ± 0.29 pmol per 0.2 ml and 0.66 ± 0.43 pmol per 0.2 ml (mean ± standard deviation), respectively. The amount of PPA in 24-h urines of these subjects are shown in Table I. PPA could not be detected in sera of normal adults (22—33 years old, n = 10) by this method. The results are the same as those reported by other workers [5, 16]. The concentration of PPA in sera of patients with phenylketonuria is very high (more than 25 nmol/ml [5]) and so can be easily determined by this method.

This study has provided the first HPLC method that permits the determination of PPA in normal urines. The method is simple to perform and may therefore be applied for routine use.

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CHROMBIO. 1008

MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR FOR DIRECT INJECTION ANALYSIS OF CATECHOLAMINES IN BODY FLUIDS

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SUMMARY

A new micro high-performance liquid chromatographic system has been developed, which determines catecholamines from directly injected human urine without any sample pretreatment. The system consists of a mixing junction for adjusting the sample to pH 8.5, a micro alumina precolumn for enriching catecholamines in the sample, and a dual electrochemical detector for selectively detecting catecholamines based on their electrochemical reversibility. The system is able to operate with directly injected body fluids and to determine simultaneously all of the four catecholamines with high precision.

INTRODUCTION

Catecholamines play an important role in the central nervous system and in neurological diseases. Consequently, their separation and determination have received considerable attention, and electrochemical methods are proving very useful in such studies [1—5]. Dual electrochemical detectors, having two working electrodes, for high-performance liquid chromatography (HPLC) are especially attractive for selective detection of electroactive species [4, 5]. Micro high-performance liquid chromatography (MHPLC), using packed columns of bore size less than 1 mm, is a very suitable technique for analysis of trace samples of biomedical origin [6]. An amperometric detector based on a thin-layer electrochemical cell with one working electrode suitable for MHPLC was recently described, and successfully utilized for the determination of aminophenol isomers separated by a micro ODS column [7]. In the present work, a thin-layer electrolytic cell with two working electrodes was designed and con-

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structed for use as the detector for MHPLC. A twin electrode detector was used to detect catecholamines selectively, based on their electrochemical reversibility in many electroactive eluates.

In the development of analytical methods using liquid chromatography for biological samples (for example, urine, serum, saliva, plasma), one of the most time-consuming steps, which introduces considerable sources of error, is sample pretreatment and enrichment prior to injection into the chromatograph. Approaches towards on-column sample enrichment for direct injection of body fluids in HPLC have been reported [8—10]. So far, the determination of catecholamines by direct injection of untreated body fluids into a micro liquid chromatograph has not been reported. Thus, we adapted the idea of the use of a precolumn as a protecting device on the micro separation analytical column, following preconcentration of the catecholamines by adsorption on a very small alumina column.

This paper describes a novel analytical system for the analysis of catecholamines by MHPLC with dual electrochemical detection, with micro precolumn sample enrichment, and for direct urine injection without the classical sample pretreatment steps.

EXPERIMENTAL

Sample pretreatment

Typical classical steps in the pretreatment of human urine for subsequent HPLC runs for determining catecholamines with electrochemical detection are the following: urine (5 ml) → stabilize → buffer (pH 6.5) → adsorb (ion-exchange resin) → wash → elute → stabilize → buffer (pH 8.6) → adsorb (alumina) → wash → dry → extract → HPLC [1]. Nearly all steps are susceptible to errors, and waste time and laboratory capacity. The application of the proposed MHPLC system, with micro precolumn and dual electrochemical detector, reduces the number of steps significantly; for example, urine (50--100 μl) → MHPLC.

MHPLC system with micro precolumn and dual electrochemical detector

The direct injection analytical system is shown schematically in Fig. 1. Three micro feeders (Azuma Denki Co., Model MF-2), micro syringes (Terumo Co., Model CAN-1.00) and three-way valves were used to feed the mobile phase, buffer solution and water. A sample injector (Rheodyne Co., Model 7125) with 100-μl sample loop and a six-way valve (Rheodyne Co., Model 50177M) were used for sample injection and alternative connection of the micro precolumn with the sample enrichment system and chromatographic system, respectively. The design of the twin electrode thin-layer electrolytic cell for the dual electrochemical detector and connection with the micro separation column are shown in Fig. 2. The thin-layer cavity was constructed of two fluorocarbon resin blocks separated by a PTFE sheet 50 μm thick and 2 mm wide. Two working electrodes were made with glassy carbon disks of 3 mm diameter contained in one of the blocks. The reference electrode, silver/silver chloride electrode, was held in a cylindrical hole in the other block. A stainless-steel tube served both as the counter electrode and the exit line. A dual potentiostat

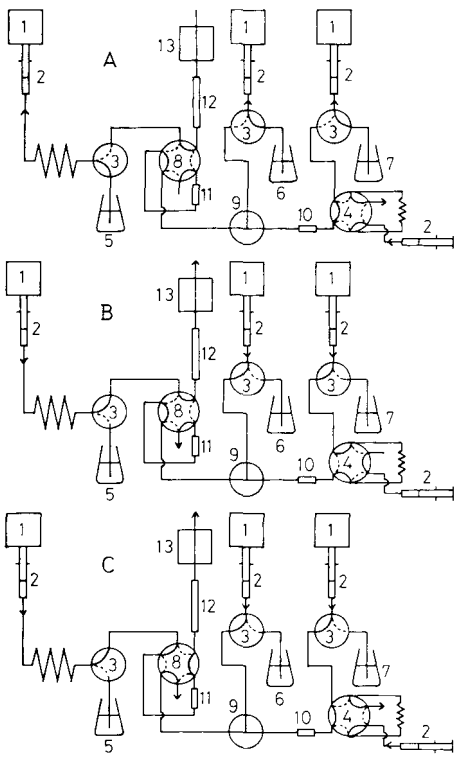

Fig. 1. Block diagram and flow-chart of the MHPLC system with micro precolumn and dual electrochemical detector. 1 = micro feeder, 2 = micro syringe, 3 = three-way valve, 4 = sample injector, 5 = mobile phase, 6 = buffer solution, 7 = water, 8 = six-way valve, 9 = mixing joint, 10 = solution filter, 11 = micro precolumn, 12 = micro separation column, 13 = twin electrode thin-layer electrolytic cell.

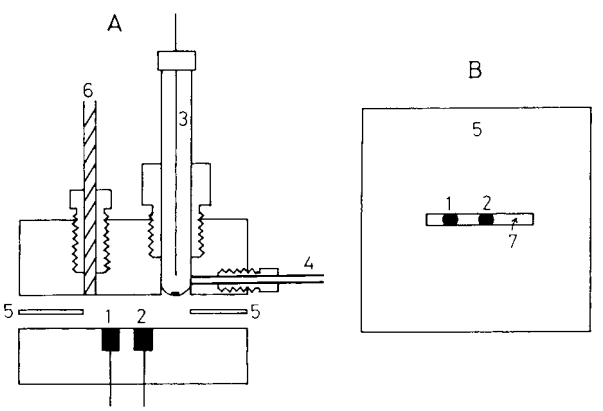

Fig. 2. Construction of twin electrode thin-layer electrolytic cell and connection with micro separation column: (A) side view of cell; (B) top view of spacer. 1, 2 = working electrode (glassy carbon), 3 = reference electrode (Ag/AgCl), 4 = counter electrode (stainless-steel tube), 5 = spacer (PTFE sheet), 6 = micro separation column, 7 = hole.

(Nikko Keisoku Co., Model DPGS-2) was employed to control independently the potentials of the two working electrodes and to measure the currents. The anodic and cathodic chromatograms were simultaneously recorded on a dual-pen recorder (Yokogawa Co., Model 3056).

The micro separation column for analysis was filled by the technique described earlier [11] with ODS (Yanagimoto Co., Yanapak ODS, 5 μm) in a PTFE tube 15 cm × 0.5 mm I.D. The micro precolumn for enrichment was made by packing alumina (E. Merck, LiChrosorb Alox T, 5 μm) in a PTFE tube 2 cm × 0.5 mm I.D. The solution filter (typical dimensions 1 cm × 0.5 mm I.D.) was made by packing with fine quartz wool.

Reagents

Analytical reagent grade chemicals were used without further purification. All solutions were prepared from distilled and deionized water. For standard samples, noradrenaline, adrenaline, dopamine and *l*-dopa were dissolved in a phosphate buffer of pH 3 to prepare the stock solutions. The mobile phase for analysis was Britton—Robinson buffer (pH 1.8) containing 0.5 m*M* 1-heptanesulfonic acid, sodium salt, as the ion-pair reagent. The buffer solution for pretreatment of the micro precolumn and pH adjustment of the sample was Tris buffer (pH 8.8) containing 0.25% EDTA (disodium salt) and 0.05% $NaHSO_3$ for stabilizing catecholamines.

Procedures

The flow-chart for the direct injection MHPLC system is shown in Fig. 1. At the positions of each valve as shown in part A, the following two procedures are first performed. The mobile phase, buffer solution and water are filled in each respective micro syringe. The first sample of human urine is taken with a 100-μl micro syringe and injected into the sample loop of the sample injector. By switching each valve as in Fig. 1 part B, the procedures for conditioning the micro precolumn followed by sample enrichment and for conditioning the micro separation column are performed at the same time. The micro precolumn is conditioned with the buffer solution of pH 8.8 delivered at a flow-rate of 33 μl/min. The sample is delivered by the water at a flow-rate of 33 μl/min for enrichment by the micro precolumn, solid particles in the sample being removed through the solution filter, and the sample is mixed with a flow of the buffer solution of pH 8.8 in the mixing joint to adjust the sample to pH 8.5. The sample is injected into the micro precolumn for 15 min with a mixed flow of the water and the buffer solution, and then the micro precolumn is washed for a further 15 min with only a flow of water by stopping the flow of the buffer solution. The flow-line from the mixing joint to the micro precolumn was made of a PTFE tube 12 cm × 0.5 mm I.D. to mix the sample with the buffer solution completely. Parallel to the enrichment procedure, the micro separation column is conditioned with the mobile phase delivered at a flow-rate of 8.3 μl/min. Next, by switching the six-way valve, as in Fig. 1 part C, the mobile phase is introduced into the micro separation column through the micro precolumn. In this procedure, the adsorbed compounds are eluted from the micro precolumn and simultaneously separated by the micro separation column. Parallel to the chromatographic procedure, the next sample is taken by

switching the valve of the sample injector again. After the adsorbed compounds are completely eluted from the micro precolumn, the next sample enrichment is performed during the separation process of catecholamines by switching again each valve in Fig. 1 part B. There was no need to change the alumina precolumn in a series of at least 100 analyses with direct injection of the urine samples.

Selective detection of catecholamines

Consider a reversible or quasi-reversible redox couple. The anode and cathode of the twin electrode thin-layer cavity are set at potentials where the reductant is oxidized and the oxidant is reduced, respectively. The reductant of the reversible or quasi-reversible species is oxidized at the anode placed upstream and the product of this electrode reaction is re-reduced at the cathode placed downstream, while the reductant of the irreversible species is not re-reduced at the cathode. It should, therefore, be noted that only the reversible and/or quasi-reversible species are selectively detected at the cathode.

The separated catecholamines are introduced into the twin electrode thin-layer electrolytic cell, in which the anode and cathode are set at the potentials (V vs. Ag/AgCl) of (+) 0.80 and (+) 0.20, respectively. The catecholamines are selectively detected by monitoring the reduction current at the cathode. The reduction current was measured with one pen of the dual-pen recorder through the electric filter having the time constant of ca. 1 sec to cut out high-frequency noise.

The potentials of the anode and cathode suitable for the selective detection of catecholamines were selected by measuring the cyclic semiderivative of current vs. electrode potential curves by means of semidifferential electroanalysis [12, 13]. The details will be described elsewhere.

RESULTS AND DISCUSSION

Quantitation of catecholamines

The MHPLC system with micro precolumn and dual electrochemical detector was used for the quantitative analysis of catecholamine mixtures. Typical chromatograms of the four catecholamines obtained from a 100-μl injection of a solution of 30 ng/ml of each of noradrenaline, adrenaline and dopamine plus 60 ng/ml of *l*-dopa using the micro ODS column are shown in Fig. 3, in which parts A and B are, respectively, the anodic and cathodic chromatograms. It should be noted that the negative and positive direction peaks correspond to the anodic and cathodic responses, respectively. All the above four catecholamines gave both anodic and cathodic chromatographic peaks. This indicates that their electrode reactions in the Britton—Robinson buffer of pH 1.8 containing 0.5 m*M* 1-heptanesulfonic acid sodium salt used for the mobile phase are reversible or quasi-reversible. The peak separation is good enough and both the anodic and cathodic responses were linear with the amounts of catecholamines injected, as shown in Table I. The procedure of sample enrichment with the alumina precolumn of 2 cm × 0.5 mm I.D. was found to be linear up to the amount injected of ca. 40 ng of each of the four catecholamines. It should be mentioned that the percentage enrichment of *l*-dopa with the micro alumina

TABLE I

RELATIONSHIPS BETWEEN ANODIC AND CATHODIC PEAK HEIGHT AND AMOUNT OF CATECHOLAMINES BY MHPLC WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR

Potentials (V vs. Ag/AgCl): anode (+) 0.80, cathode (+) 0.20; flow-rate of mobile phase, 8.3 μl/min.

Species		Relationship*	Correlation coefficient
Noradrenaline	Anodic	$y = -15.71x + 6.55$	0.999
	Cathodic	$y = 7.33x - 4.39$	0.996
Adrenaline	Anodic	$y = -5.28x + 1.06$	0.997
	Cathodic	$y = 2.41x - 0.77$	0.994
Dopamine	Anodic	$y = -4.82x + 3.88$	0.994
	Cathodic	$y = 2.37x - 1.93$	0.996
l-Dopa	Anodic	$y = -0.21x + 0.37$	0.987
	Cathodic	$y = 0.09x - 0.15$	0.993

*y = peak height measured in nA; x = amount of catecholamine measured in ng.

precolumn in this system is small compared with that of the other catecholamines, as seen in Fig. 3 and Table I. This would be improved by selecting a more suitable buffer solution for adjusting the sample to pH 8.6.

The cathodic responses were found to be about 44, 45, 48 and 42% for noradrenaline, adrenaline, dopamine and *l*-dopa, respectively, of the corresponding anodic responses under the experimental conditions used; i.e. mobile phase flow-rate 8.3 μl/min, anode potential (+) 0.80 V and cathode potential (+) 0.20 V vs. Ag/AgCl. The ratios of cathodic to anodic responses for the four catecholamines were substantially constant in the range of cathode potentials from (+) 0.20 to 0 V under an anode potential of (+) 0.80 V vs. Ag/AgCl. The background current on the cathode tended to increase with decreasing cathode potential. Therefore, the cathode potential of (+) 0.20 V vs. Ag/AgCl was selected for the selective detection of catecholamines in this study.

The cathodic responses reached a high level of precision, as shown in Table II. The relative standard deviations for repetitive determination of catecholamines in the MHPLC system with micro precolumn and dual electrochemical detector were 0.6, 0.9, 1.8 and 5.1% for noradrenaline, adrenaline, dopamine and *l*-dopa, respectively.

Catecholamines in human urine

Typical chromatograms for the determination of catecholamines in 100 μl of human urine directly injected without any pretreatment in the MHPLC system are shown in Fig. 4a and b. Parts A and B are, respectively, the anodic and cathodic chromatograms. Peaks 1, 2, 3 and 4, due to noradrenaline, adrenaline, dopamine and *l*-dopa, are the expected endogenous compounds in the

TABLE II

PRECISION FOR DETERMINATION OF CATECHOLAMINES BY MHPLC WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR

Sample: 100 μl of 40 ng/ml each of noradrenaline, adrenaline and dopamine plus 200 ng/ml of *l*-dopa. Potentials (V vs. Ag/AgCl): anode (+) 0.80, cathode (+) 0.20.

Number	Cathodic peak height (inches)			
	Noradrenaline	Adrenaline	Dopamine	*l*-Dopa
1	3.32	1.04	0.97	0.35
2	3.32	1.06	0.99	0.31
3	3.36	1.05	0.96	0.34
4	3.35	1.04	0.95	0.33
Mean	3.34	1.05	0.97	0.33
Relative S.D.	0.6	0.9	1.8	5.1

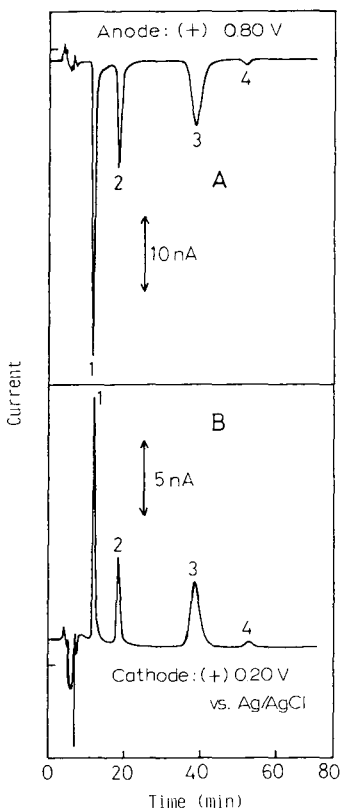

Fig. 3. Typical chromatograms of standard catecholamines by the MHPLC system with micro precolumn and dual electrochemical detector: (A) anodic response, (B) cathodic response. Peaks: 1 = noradrenaline, 2 = adrenaline, 3 = dopamine, 4 = *l*-dopa. Sample: 100 μl of a standard solution of 30 ng/ml each of noradrenaline, adrenaline and dopamine plus 60 ng/ml of *l*-dopa. Potentials (V vs. Ag/AgCl): anode (+) 0.80, cathode (+) 0.20.

urine. Their peaks were verified using the standard samples. Of particular interest in part A are the peaks appearing as the shoulder of noradrenaline in Fig. 4b and as the background of *l*-dopa in Fig. 4a. The compound or compounds responsible for these peaks and the peaks appearing after *l*-dopa in part A were not identified. By recording the cathodic current, it was shown that there were essentially no cathodic peaks corresponding to the unknown anodic peaks (see part B), suggesting that the compound or compounds producing anodic peaks are irreversibly oxidized. On the other hand, the compound responsible for the peak appearing after adrenaline in part A of Fig. 4 seems to be one of the metabolites of the catecholamines, because the corresponding cathodic peak appeared in the cathodic chromatograms. It is clear that the proposed dual electrochemical detector can selectively detect catecholamines from many electroactive species co-existent in the urine on the basis of their electrochemical reversibility.

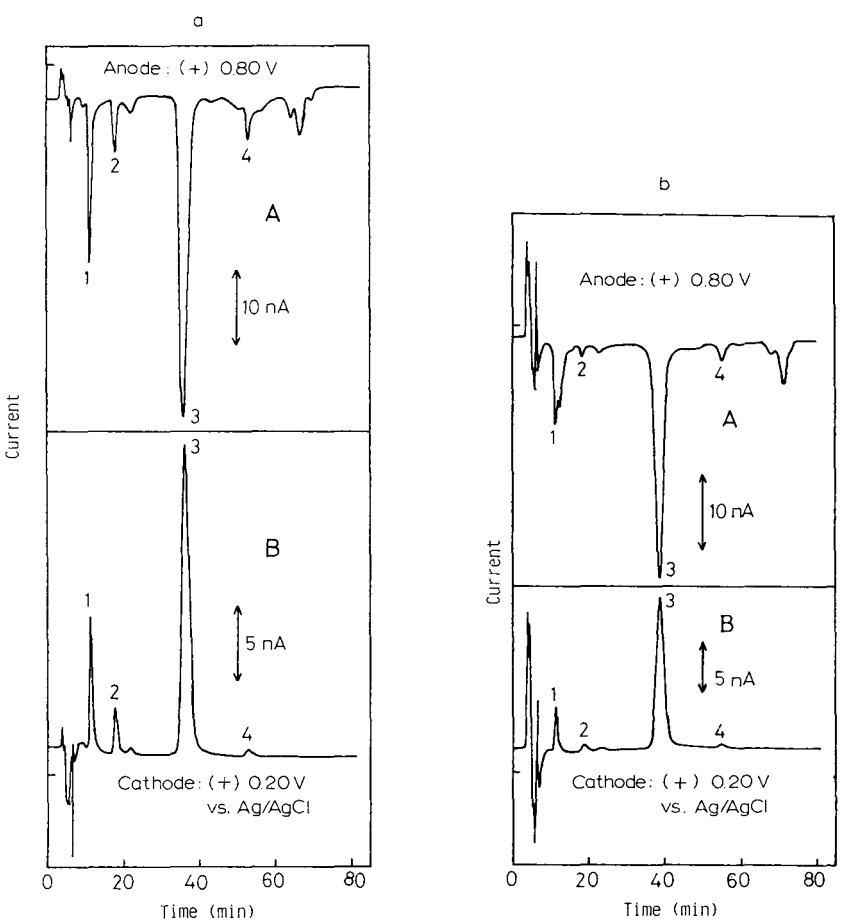

Fig. 4. Typical chromatograms of catecholamines in directly injected human urine: (A) anodic response; (B) cathodic response. Peaks: 1 = noradrenaline, 2 = adrenaline, 3 = dopamine, 4 = *l*-dopa. Potentials (V vs. Ag/AgCl): anode (+) 0.80, cathode (+) 0.20. Sample: 100 μl of human urine.

Human urine from five healthy individuals was analyzed from the linear regression equations in Table I using the cathodic chromatograms. The results are shown in Table III. The concentrations for noradrenaline, adrenaline and dopamine in Table III are within the range of results reported in the literature for normal human urines using HPLC methods with a single electrochemical detector [1] and with a fluorimetric detector [14]. Values for normal urine levels of *l*-dopa do not appear to be available in the literature. This seems to be due to the lack of sensitivity for *l*-dopa in the fluorimetric detection and that the classical pretreatment of urine for electrochemical detection involves ion-exchange procedures before adsorption on alumina, *l*-dopa not being enriched [1]. The values in Table III are somewhat smaller than those obtained using the classical manual method with single electrochemical detection. This indicates that single electrochemical detection tends to overestimate the true values because of lack of selectivity in detection.

The present system appears to be the first method which simultaneously determines all four of these catecholamines in human urine directly injected into the micro liquid chromatograph. Total analysis time for one sample was approximately 1.5 h. One hour is required for the analysis of each additional sample. This time can not be said to be shorter than in the classical manual method, because the manual method may process more samples on a batch-wise basis per working day. However, the sample through-put time in this automated system will be able to be shortened by improving the speed of MHPLC and by arranging for more precolumns to be operated at the same time.

Although this entire article refers to catecholamine analysis in urine, it seems that the system is applicable to other body fluids such as plasma, serum and saliva.

TABLE III

ANALYTICAL RESULTS OF CATECHOLAMINES IN URINE FROM HEALTHY INDIVIDUALS

Sample number	Concentration (ng/ml)			
	Noradrenaline	Adrenaline	Dopamine	*l*-Dopa
1	17	16	101	70
2	17	10	94	65
3	10	7	80	63
4	16	11	86	70
5	16	9	133	60

CONCLUSIONS

The MHPLC system with micro precolumn and dual electrochemical detection is applicable to body fluid samples such as urine without any pretreatment, for the simultaneous determination of four catecholamines with high precision at low concentration. Three processes in this system provide maxi-

mum selectivity for catecholamines. Initially, the compounds are extracted via liquid—solid adsorption on an alumina precolumn. Secondly, separation is effected by means of reversed-phase liquid chromatography. Finally, detection is selectively accomplished by dual electrochemical detection based on their electrochemical reversibility.

Characteristic features of this system are: automated sample pretreatment, direct injection of body fluids (urine, etc.), no internal standard required, high precision, only small amounts of materials for precolumn and separation column and reagents for mobile phase, etc., required. The possibility of further development of this system for clinical purposes seems clear.

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POST-COLUMN DERIVATIZATION OF GUANIDINO COMPOUNDS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING NINHYDRIN

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SUMMARY

A new method for the high-performance liquid chromatography of guanidino compounds using ninhydrin as the fluorescence reagent is described. Use of organic solvent is not required since ninhydrin is highly soluble in aqueous media, and the problem of precipitation formation occurring in the phenanthrenequinone method was avoided. Creatine was also assayed in the present procedure. Separation of ten guanidino compounds was completed within 30 min using a small-size (38 × 4.2 mm I.D.) strong cation-exchange column.

INTRODUCTION

The level of guanidino compounds such as guanidinoacetic acid [1—4] and methylguanidine [2—7] rises significantly in the plasma of uremic patients. Liquid chromatography has recently been used for the separation and determination of these guanidino compounds. Several workers [8, 9] have reported the assay of plasma guanidines employing a modified automatic amino acid analyser. However, this analytical method is rather time-consuming and the colorimetry shows poor sensitivity.

Yamada and Itano [10, 11] have reported that micro-amounts of arginine and arginine-containing peptides can be detected fluorimetrically using 9,10-phenanthraquinone (PQ). This reaction was applied to the microdetermination of guanidines by Sakaguchi et al. [12]. Yamamoto and co-workers [13, 14] have recently developed a high-performance liquid chromatographic (HPLC) method for the fluorimetric determination of guanidino compounds in physiological fluids using PQ as the reagent for the post-column derivatization. Although this method is highly sensitive, PQ is practically insoluble in water, so the derivatization reagent should be prepared by dissolving PQ

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in dimethylformamide. The derivatization reagent often causes precipitation which blocks the chromatographic tubing after it is mixed with the column effluent.

The present paper describes a new HPLC system using ninhydrin [15—19], which is highly water-soluble, for the development of fluorescence from guanidino compounds. A rapid separation of guanidino compounds using a small-size column is also described.

EXPERIMENTAL

Chemicals

Guanidinosuccinic acid, guanidinobutyric acid, guanidinopropionic acid, guanidinoacetic acid, and methylguanidine hydrochloride were all obtained from Sigma (St. Louis, MO, U.S.A.). Creatine, creatinine, L-arginine hydrochloride, sodium citrate, sodium hydroxide, sodium chloride, perchloric acid (60%), boric acid, and ninhydrin were purchased from Wako Pure Chemical (Osaka, Japan). All chemicals used were of analytical reagent grade.

Elution buffers and reagent solutions

The eluent buffer solutions are listed in Table I. The pH of each eluent buffer is adjusted with perchloric acid or 1.0 *N* sodium hydroxide. All eluent buffers, 0.75 *N* sodium hydroxide solution and 0.6% ninhydrin solution are prepared using glass-redistilled water, and passed through a 0.22-μm microfilter (Fuji Photo Film, Tokyo, Japan) prior to use.

TABLE I

COMPOSITION OF ELUENTS

	Eluents					
	First (5 min)	Second (8 min)	Third (2 min)	Fourth (14 min)	Fifth (2 min)	Sixth (3 min)
pH	3.5	5.0	6.0	11.4		
Na^+ concentration (*N*)	0.15	0.35	0.15	0.85		
Sodium citrate $2H_2O$ (g/l)	14.7	34.3	34.3	34.3		
Perchloric acid (60%) (ml/l)	10.5	11.0	3.0	—		
Boric acid (g/l)	—	—	—	6.2		
Sodium hydroxide (g/l)	—	—	—	4.0		
					0.2 *N* NaOH	H_2O

Chromatographic system

Fig. 1 shows the flow diagram of our chromatograph. A single plunger pump (Sanuki Industry, Tokyo, Japan) served to deliver the eluent with the constant flow-rate of 0.7 ml/min. The eluent-selecting valve was controlled by an SGR-1A step gradient programmer unit (Shimadzu Seisakusho, Kyoto, Japan), and pumped through a valve universal injector (Sanuki Industry, Tokyo, Japan). An ISC-05/S0504 packed column (strong cation-ex-

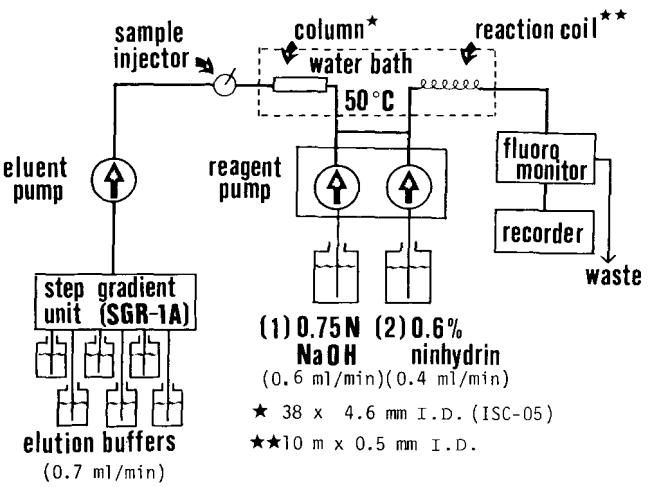


Fig. 1. Flow diagram of the HPLC system.

change resin; 5-μm particle size; 38 mm × 4.2 mm I.D.; Shimadzu Seisakusho) was used for the separations. It was operated at 50 ± 0.05°C using a C-600 thermo-unit (Taiyo Scientific Industry, Tokyo, Japan). A double plunger pump (Sanuki Industry) served to deliver the alkaline solution and the ninhydrin solution at constant flow-rates of 0.6 ml/min and 0.4 ml/min, respectively.

The column effluent was first mixed with 0.75 *N* sodium hydroxide in a T-junction, and then mixed with 0.6% ninhydrin solution in another T-junction. The mixture was then allowed to flow through a PTFE-tubing reaction coil (10 m × 0.5 mm I.D.) where it was heated to 50 ± 0.05°C in a water-bath.

The fluorescence intensity of the effluent was measured using an RF-500 LC spectrofluoromonitor (Shimadzu Seisakusho). The excitation and emission wavelengths were 395 nm and 500 nm, with slit widths of 20 nm and 40 nm, respectively. The excitation lamp was a xenon discharge lamp (Wacom R & D Corp.).

Operation of the chromatograph for analysis

The guanidino compounds were separated with a strong cation-exchange column using a stepwise pH gradient. The first buffer was pumped through the column for 5 min, then the second buffer for 8 min, the third buffer for 2 min and the fourth buffer for 14 min; then the column was washed successively with 0.2 *N* sodium hydroxide for 2 min and with water for 3 min. Ten guanidino compounds (from taurocyamine to methylguanidine) were analysed within 30 min using this system.

Preparation of samples

To 200 μl of plasma were added 100 μl of 30% trichloroacetic acid solution and the mixture was centrifuged at 1000 *g* for 10 min. Then the pH of the supernatant solution was adjusted to 2.0 with 0.4 *N* sodium hydroxide and 150 μl of this deproteinized sample were used for the analysis.

RESULTS AND DISCUSSION

Effects of pH and ionic strength on the retention times of guanidino compounds

Fig. 2 shows the effect of the pH and ionic strength of 0.35 *N* sodium citrate buffer on the retention times of various guanidino compounds. Guanidinosuccinic acid (GSA), creatine (CT) and guanidinoacetic acid (GAA) were not favorably separated by gradient lowering of the pH (Fig. 2A). On the other hand, these three guanidino compounds were satisfactorily resolved by decreasing the ionic strength of the eluent buffer as shown in Fig. 2B. Accordingly, 0.15 *N* sodium citrate buffer (pH 3.5) was used for the separation (Table I).

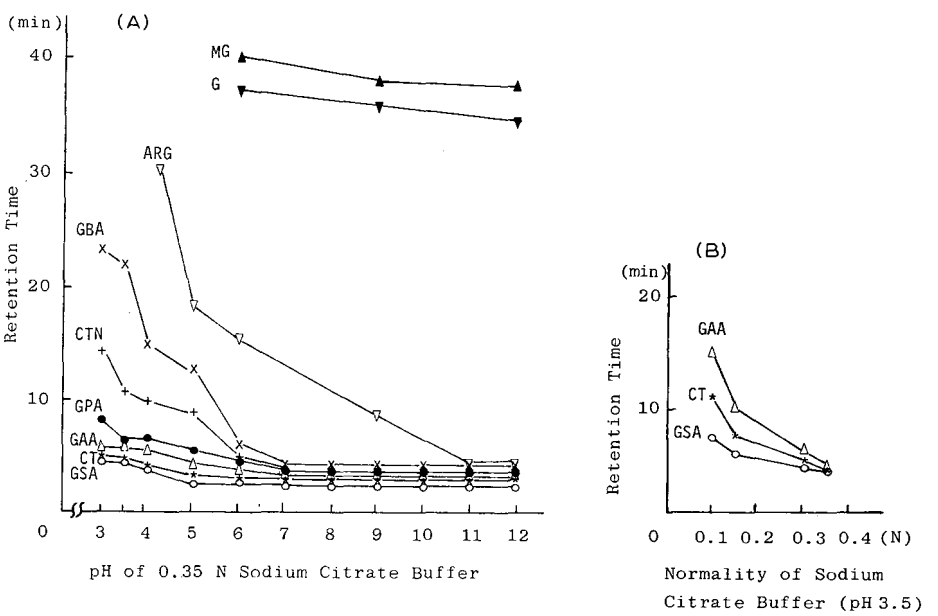


Fig. 2. (A) Effect of the pH of the elution buffer on the retention times of various guanidino compounds. (B) Effect of the ionic strength of the elution buffer on the retention times of various guanidino compounds.

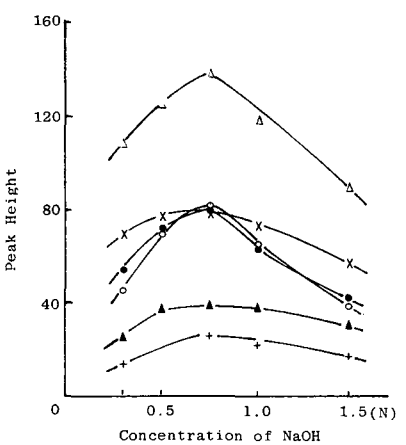
The retention time of guanidinopropionic acid (GPA) decreased markedly around pH 3.0—4.0 and was constant above pH 5.0. The values for creatinine (CTN) and guanidinobutyric acid (GBA) sharply decreased around pH 5.0—6.0 and were approximately constant above pH 6.0. Guanidine (G) and methylguanidine (MG), strongly basic guanidino derivatives, were not eluted within 30 min with 0.35 *N* sodium citrate buffer (pH 11.4). G and MG were eluted with 0.85 *N* sodium citrate buffer, containing 0.62% (w/v) boric acid (pH 11.4), showing the retention times of 15 min and 18 min, respectively. The conditions for the stepwise gradient elution were set as shown in Table I on the basis of these results. All the guanidino compounds tested were eluted with sodium citrate buffers within 29 min, and the column was washed with 0.2 *N* sodium hydroxide and water after the separation.

Fluorescence properties of ninhydrin derivatives of guanidino compounds

Ninhydrin has been reported to combine with guanidine, monosubstituted guanidines, and N,N-disubstituted guanidines to give highly fluorescent addition products in strongly alkaline media [15—17]. This reaction has also been used for the determination of creatine [18]. The five-membered ring of ninhydrin is cleaved immediately after the addition of alkali to produce *o*-carboxyphenylglyoxal [18], which then condenses with guanidines. Accordingly, amino acids do not interfere with the reaction when the ninhydrin solution is previously made alkaline. The present system first delivers sodium hydroxide solution and then ninhydrin solution to the column effluent. Ninhydrin is converted by the action of alkali into *o*-carboxyphenylglyoxal [16] which does not react with amino acids.

The excitation maxima of ninhydrin derivatives of guanidines in the effluent from the column were at 305 and 395 nm, and the single emission peak had a maximum at 500 nm. The fluorescence at 395 nm was approximately twice as intense as that excited at 305 nm. These data agreed well with those reported by Conn and Davis [16]. The excitation and emission wavelengths of the fluoromonitor were therefore set at 395 nm and 500 nm, respectively.

Reaction conditions for the post-column derivatization were examined by injecting 50 μl of standard solution of guanidino compounds. Fig. 3 shows the fluorescence intensity of the derivatized guanidino compounds against the alkali concentration. All the guanidino compounds gave the maximum fluorescence intensity at the sodium hydroxide concentration of 0.75 *N*. Fig. 4 demonstrates that the maximum fluorescence intensity was shown at the ninhydrin concentration of 0.6%. The effect of temperature on the reaction of the derivatized guanidino compounds with ninhydrin was studied in



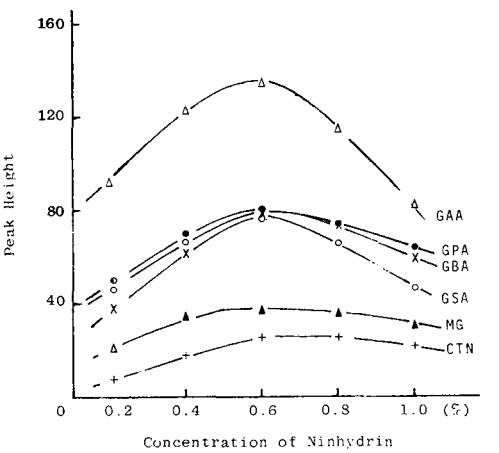


Fig. 3. Effect of alkali concentration on the fluorescence intensity of various guanidino compounds (1 nmol each). ○, GSA; △, GAA; ×, GBA; +, CTN; ●, GPA; ▲, MG (for abbreviations, see text).

Fig. 4. Effect of ninhydrin concentration on the fluorescence intensity of various guanidino compounds (1 nmol each). For abbreviations, see text.

the range of 30°C to 70°C. Fig. 5 shows that the optimum temperature for these compounds except creatine was 50°C. Creatine gave a slightly lower peak at 50°C whereas it gave maximum peak height at 40°C. However, this does not affect the assay because the ninhydrin reagent is highly sensitive to creatine.

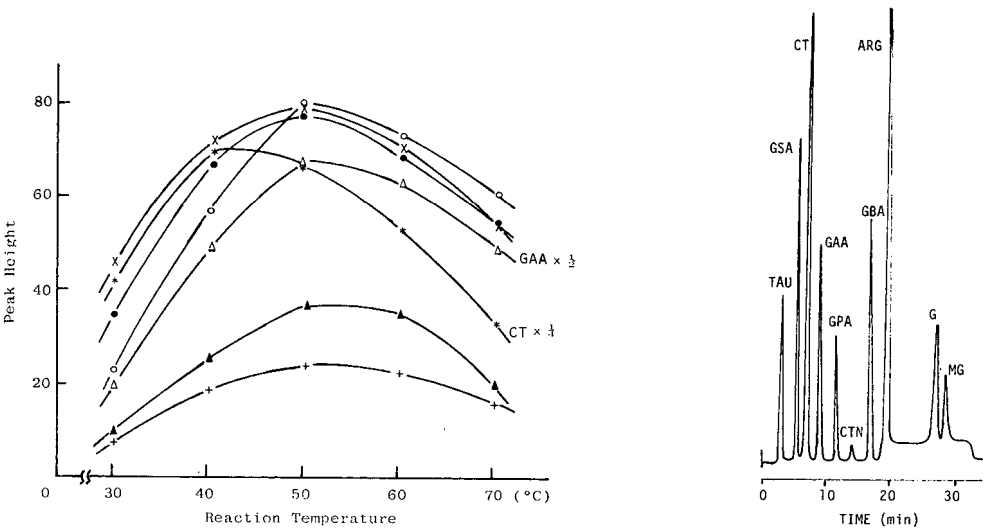


Fig. 5. Effect of reaction temperature on the fluorescence intensity of various guanidino compounds (1 nmol each). ○, GSA; *, CT; △, GAA; ×, GBA; +, CTN; ●, GPA; ▲, MG (for abbreviations, see text).

Fig. 6. Chromatogram of a standard mixture of guanidino compounds. TAU = taurocyamine (1 nmol); GSA = guanidinosuccinic acid (1 nmol); CT = creatine (0.5 nmol); GAA = guanidinoacetic acid (0.5 nmol); GPA = guanidinopropionic acid (0.5 nmol); CTN = creatinine (5 nmol); GBA = guanidinobutyric acid (1 nmol); ARG = arginine (2 nmol); G = guanidine (2 nmol); MG = methylguanidine (0.5 nmol).

Chromatographic separation and quantitative response

A typical separation of a standard solution of guanidino compounds is demonstrated in Fig. 6. GSA, CT, GAA, GPA, CTN, GBA, arginine (ARG), G and MG were all completely resolved, and the entire analysis required 35 min including the column-washing procedure.

The limits of detection for guanidino compounds, determined by the peak height at twice the noise level, are as follows: GSA, 5 pmol, CT, 1 pmol; GAA, 10 pmol; GPA, 5 pmol; CTN, 1 nmol; GBA, 1 pmol; ARG, 50 pmol; G, 5 pmol; MG, 5 pmol.

Standard curves for these guanidino compounds are depicted in Fig. 7. Fluorescence responses are linear for these seven guanidino compounds up to at least 5 nmol. The lower limit of the assay varies from 10 pmol (GSA, GPA, GBA and MG) to 50 pmol (GAA).

Excellent reproducibility was observed for the determination of all the guanidino compounds listed in Table II.

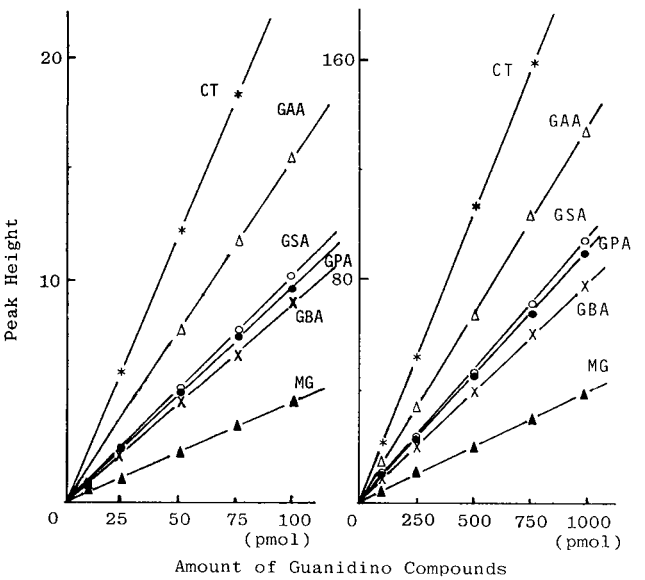


Fig. 7. Standard curves for guanidino compounds. For abbreviations, see text.

TABLE II

DAY-TO-DAY PRECISION OF THE PRESENT METHOD FOR AQUEOUS SOLUTIONS OF VARIOUS GUANIDINO COMPOUNDS

Injection volume was 50 μl. n = 8 in all cases.

Compound*	Concentration (nmol per 50 μl)	C.V. (%)
GSA	0.136	0.57
CT	1.360	2.54
GAA	0.170	3.29
GPA	0.105	1.57
CTN	3.570	1.65
GBA	0.344	2.16
ARG	4.900	1.36
G	0.342	1.59
MG	0.059	2.63

*For abbreviations, see text.

Excellent recoveries of these compounds from human serum were observed with satisfactory reproducibility, as listed in Table III.

Analysis of human plasma samples

Fig. 8 shows a chromatogram of a plasma sample from a person in normal health. The following compounds were assayed: CT, 0.24 mg/dl; GAA, 15 μg/dl; CTN, 1.20 mg/dl; ARG, 2.06 mg/dl.

Fig. 9 shows a chromatogram of a plasma sample from a chronic glomerulonephritis patient. The following guanidines were identified by comparing

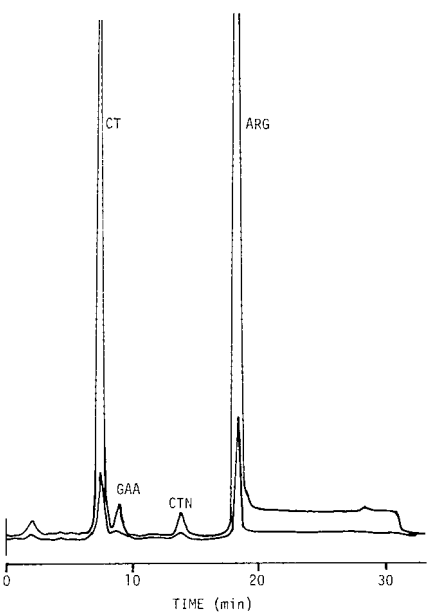
TABLE III

RECOVERIES OF GUANIDINO COMPOUNDS FROM HUMAN SERUM

150 μl of deproteinized samples were injected. n = 9 in all cases.

Compound*	Added (nmol)	Within-day	
		Recovery ($\bar{X}$) (%)	C.V. (%)
GSA	0.272	92.1	2.30
CT	1.00	90.9	3.11
GAA	0.340	95.5	3.17
GPA	0.289	96.3	0.06
CTN	18.0	96.2	1.20
GBA	0.081	91.3	0.25
ARG	5.00	96.7	1.32
G	3.00	96.6	1.35
MG	2.00	93.9	1.74

*For abbreviations, see text.



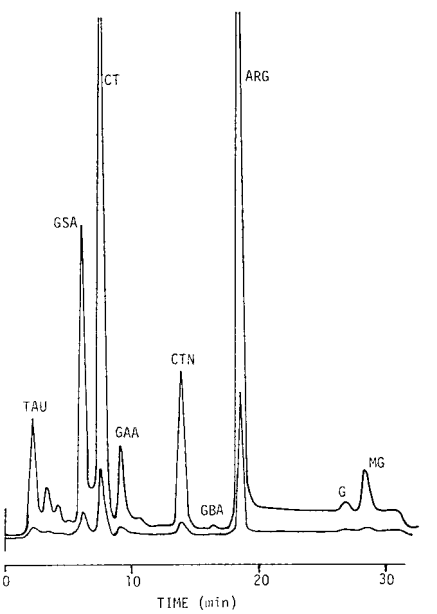


Fig. 8. Chromatogram of a plasma sample from a person in normal health. For abbreviations, see text.

Fig. 9. Chromatogram of a plasma sample from a chronic glomerulonephritis patient. For abbreviations, see text.

retention times with those of standard solutions: GSA, 0.475 mg/dl; CT, 0.26 mg/dl; GAA, 49 μg/dl; CTN, 10.55 mg/dl; ARG, 2.56 mg/dl; G, 43.3 μg/dl; MG, 98.2 μg/dl.

The present method is as sensitive as the PQ method. Creatine, which is

not detected by the PQ method, can be sensitively detected. The present method, therefore, offers more-detailed metabolic information.

Ninhydrin is readily soluble in water and its aqueous solution can be used as the fluorescent reagent. Accordingly, the present method does not require any organic solvent and no precipitation occurs during the chromatography. In addition, the use of a short column, ISC-05, facilitated more rapid separation of guanidino compounds compared with the conventional methods. Conclusively, the ninhydrin method is more practical and widely applicable than the PQ method and it is recommended to be adopted in clinical laboratories.

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HISTAMINE IN TISSUE: DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER CONDENSATION WITH *o*-PHTHALDIALDEHYDE

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SUMMARY

Histamine was determined by reversed-phase high-performance liquid chromatography in perchloric acid extracts after condensation with *o*-phthaldialdehyde. Fluorescence was monitored at 360 nm excitation and 450 nm emission wavelengths after elution with mixtures of 0.1 *N* acetic acid containing 0.1% pentanesulfonic acid and acetonitrile. The detection limit was 0.9 pmol of histamine. The histamine content was determined in rat whole brain, medulla oblongata, dorsal and ventral spinal cord, dorsal and ventral skin of the hind paw, stomach, ileum, rectum, lung and a hind-quarter perfusate, and compared to published data. The advantages of the described method over other methods are (A) rapid analysis in an automated system, (B) no selective extraction procedure is necessary, and (C) interfering substances are easily separated from the histamine fluorophore.

INTRODUCTION

The fluorimetric method for the estimation of histamine in biological samples is based on the reaction with *o*-phthaldialdehyde in alkaline medium to form a fluorescent product which is converted to a more fluorescent and stable product by acidification [1]. Much effort has been given to avoiding interference from other naturally occurring substances, mainly histidine and spermidine [2—4] by specific purification techniques such as ion-exchange chromatography [5—9] or selective extraction into isoamyl alcohol [10] prior to the reaction with *o*-phthaldialdehyde. Thin-layer chromatography as well as paper, thin-layer and gel electrophoresis [11, 12], or high-speed liquid chromatography [13], have also been used to identify the histamine fluorophore from amongst other, interfering substances.

With the development of novel chromatographic methods another tool

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has become available to circumvent such laborious extraction procedures. Recently, high-performance liquid chromatography (HPLC) has been employed for the separation of various biogenic amines after condensation with *o*-phthaldialdehyde [14]. This method involves extensive sample clean-up and stepwise gradient elution over 80 min. The present paper describes a simple and rapid method for the determination of histamine after condensation with *o*-phthaldialdehyde using HPLC under isocratic elution conditions.

EXPERIMENTAL

Reagents and materials

L-Histamine dihydrochloride, L-histidine hydrochloride (pyrogen-free and histamine-free), sodium imidazole-4-acetate and spermidine trihydrochloride were obtained from Serva (Heidelberg, G.F.R.); *o*-phthaldialdehyde was from E. Merck (Darmstadt, G.F.R.), and sodium 1-pentanesulfonate from Fluka (Buchs, Switzerland). All other reagents were of analytical grade (E. Merck). All solvents were degassed in an ultrasonic bath prior to use.

Stock solutions

Standards of L-histamine, L-histidine, imidazoleacetic acid, and spermidine in concentrations of 1 mg ml^{-1} were dissolved in 0.2 *N* perchloric acid. *o*-Phthaldialdehyde was dissolved in methanol (1% w/v).

Apparatus, column, and mobile phase

The HPLC system consisted of an Altex 110A pump with pulse dampener, a Kontron ASI-45 autosampler with a Rheodyne injection valve, a reversed-phase column (Waters μBondapak C_{18}, 300 × 3.9 mm, particle size 10 μm), and a Kontron SFM 23 fluorescence detector. Chromatogram recordings and all calculations were performed on a Shimadzu CR1A integrator.

The mobile phase consisted of 0.1 *N* acetic acid containing 0.1% pentanesulfonic acid; acetonitrile concentrations ranged from 15 to 25% (Fig. 1). In extracts containing high concentrations of interfering substances, such as those from nervous tissue, the separation of the histamine fluorophore was optimised by use of a more polar elution medium (15% acetonitrile). The retention time of the histamine fluorophore was about 12 min, the time of the HPLC analysis was 30 min. To avoid overloading of the column by non-polar substances which adhere to the column, particularly after several sample injections, the chromatography was interrupted 15 min after each sample injection and 500 μl of acetonitrile were injected. Impurities adhering to the column were washed out with the front peak of acetonitrile. Further equilibration was carried out for 13 min prior to the injection of the next sample. In all tissue samples listed in Table I, except nervous tissue, the concentrations of interfering substances were found to be so low that a sufficient separation of the histamine fluorophore was achieved by the use of a non-polar elution medium (mobile phase containing 20—25% acetonitrile). In this instance, the retention time of the histamine fluorophore was 5—6 min and the time of the HPLC analysis could be shortened to 15 min. Column wash during the automated analysis was performed by injection of 500 μl

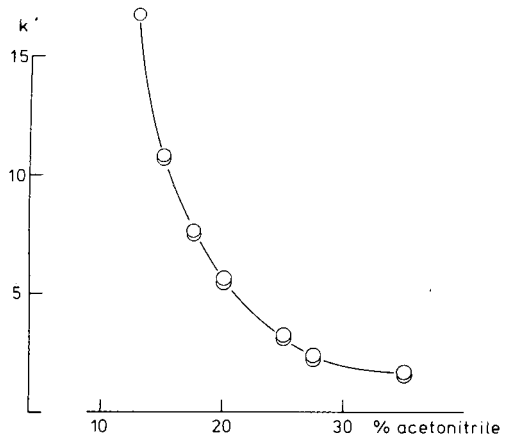

Fig. 1. k' values for the histamine—*o*-phthaldialdehyde fluorophore in 0.1 *N* acetic acid, 0.1% pentanesulfonic acid—acetonitrile mixtures at a flow-rate of 2.5 ml min^{-1}, fluorescence detection at 360 nm excitation and 450 nm emission wavelengths.

TABLE I

HISTAMINE CONTENT OF DIFFERENT RAT TISSUES COMPARED WITH PUBLISHED DATA

Data are expressed as the mean ± S.E.M., *n* as indicated, references in the square brackets.

Tissue	*n*	Histamine (nmol g^{-1})			
		HPLC	Radioenzymatic assay	Fluorimetric assay	Bioassay
Whole brain	5	0.75 ± 0.03	0.72 [17] 0.54 [16]	0.47 [21] 0.68 [20] 0.50 [21] 0.41 [22] 0.43 [24]	0.65 [4] 0.48 [18] 0.51 [23]
Medulla oblongata	5	0.50 ± 0.08		0.27 [24]	
Dorsal spinal cord	5	0.45 ± 0.01			
Ventral spinal cord	5	0.34 ± 0.03			
Hind paw, dorsal skin	10	195 ± 38	198.9 [17]		590.4 [18]
Hind paw, ventral skin	10	164 ± 11			
Stomach, antrum	6	106 ± 8	320.6 [17]		326.1 [10]
Stomach, fundus	6	104 ± 15	59.9 [17]		137.2 [18]
Ileum	6	71 ± 5			50.0 [18]
Rectum	6	75 ± 5			22.7 [18]
Lung	6	76 ± 9			153.5 [10] 32.7— 99.0 [18]
Hind-quarter perfusate	10	1.05 ± 0.20			0.82 [19]

of acetonitrile after ten samples followed by an equilibration period of 13 min prior to the injection of the next sample.

No satisfactory results in the separation of the histamine fluorophore were

obtained by the use of heptanesulfonic acid instead of pentanesulfonic acid or without ion-pair reagent, due to tailing and peak broadening.

Extraction of tissues

Sprague—Dawley rats of either sex, weighing about 250 g, were killed by cervical dislocation. The brain, the medulla oblongata, the spinal cord, the hind-paw skin, the stomach, a portion of the ileum (10 cm in length, close to the ileocoecal junction), and the rectum were excised. Tissue samples were frozen in liquid nitrogen, weighed, and pulverised using a Braun—Melsungen Dismembrator (1 min, 10 mm amplitude). The powder was taken up in approximately 5—10 volumes of 0.2 *N* perchloric acid and dispersed by ultrasonication for 1 min. The samples were centrifuged (1000 *g*, 10 min) and the supernatants stored at —20° C.

Extraction of perfusates

Perfusion of the isolated hind-quarter of the rat was performed according to the method of Erjavec et al. [15]. Sprague—Dawley rats of either sex (200—300 g) were used. The aorta and the vena cava inferior were cannulated at the level of the iliolumbar arteries and perfused with oxygenated Krebs—Ringer solution containing gelatine at a flow-rate of 2.2 ml min^{-1} (for details see ref. 15). The venous outflow was collected in periods of 5 min at 0° C. The samples were acidified by the addition of 17 µl of concentrated perchloric acid (70%, w/v) per ml of sample (final concentration 0.2 *N*). Protein precipitates were removed by centrifugation (1000 *g*, 10 min). The supernatants were stored at —20° C.

Estimation of histamine

Samples of 1 ml volume were made alkaline by the addition of 0.4 ml of 1 *N* sodium hydroxide. Derivatisation was performed by adding 0.3 ml of *o*-phthaldialdehyde stock solution followed by vigorous mixing. This *o*-phthaldialdehyde concentration was found to be sufficient for quantitative derivatisation in all investigated tissue extracts under the described conditions. This was checked using an internal standard of 3630 pmol of histamine. The samples were allowed to react with the *o*-phthaldialdehyde for 4 min. Then the fluorophore was converted to a more fluorescent and stable product by acidification with 0.1 ml of 3 *N* hydrochloric acid. Since the spermidine standard and all tissue samples contained a precipitate after acidification, all samples were centrifuged (1000 *g*, 10 min); 500 µl of the supernatant were directly injected into the HPLC system. Fluorescence was monitored at 360 nm excitation and 450 nm emission wavelengths [1]. The detection limit was 0.9 pmol histamine per 500 µl of injected sample at a signal-to-noise ratio of 3:1.

RESULTS AND DISCUSSION

Analysis of standards

As shown in Fig. 2A, the standards of L-histamine used produced three peaks after condensation: a major peak at 12.3 min retention time and two

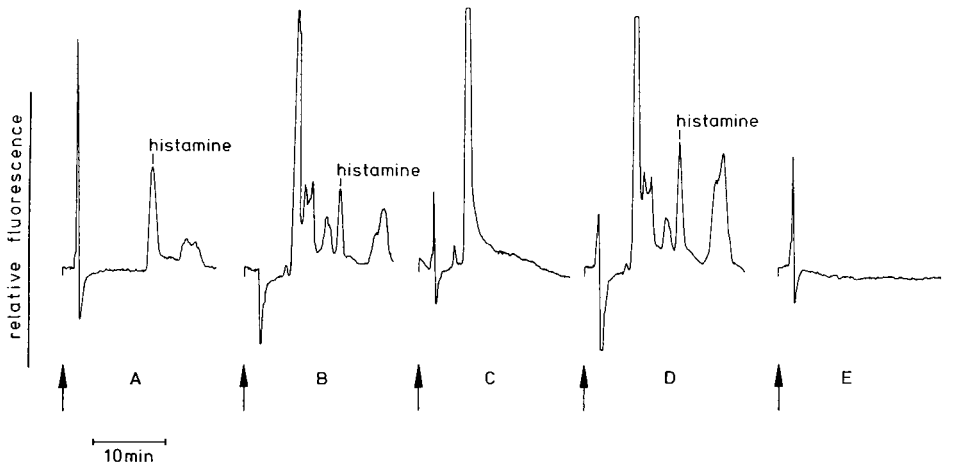

Fig. 2. Chromatograms of standards: (A) L-histamine (25.0 pmol); (B) L-histidine (16.1 nmol); (C) spermidine (19.4 nmol); (D) mixture of histamine (12.5 pmol), L-histidine (8.1 nmol), and spermidine (9.7 nmol); (E) solvent. All substances were diluted in 0.2 *N* perchlorid acid. Injection volume was 500 μl. The mobile phase consisted of 0.1 *M* acetic acid, 0.1% pentanesulfonic acid and 15% acetonitrile at a flow-rate of 2.0 ml min^{-1}. Attenuation for these chromatograms: position "high" of the fluorimeter, integrator input 32 mV full scale. The arrows indicate the injection of samples.

smaller ones with retention times of 16.8 and 18.0 min. The peak areas of the two minor peaks were proportional to the peak area of the main peak at all concentrations investigated. The minor peaks seemed to be by-products formed by the condensation procedure since they occurred even in biological samples. All calculations refer to the major peak of fluorescence which was linearly dependent on the histamine concentration up to 2270 pmol ml^{-1} (linear regression, $r^2 = 0.99$).

The condensation product of L-histidine showed at least eight peaks (Fig. 2B). The main peak which seemed to correlate with the L-histidine content had a retention time of 6.9 min. Three other peaks corresponded to histamine and histamine by-products as inferred from internal standardisation, indicating that the standard L-histidine contained impurities of histamine. However, the histamine content of the standard substance was found to be less than 0.05%. Standards of spermidine showed a single peak at a retention time of 7.3 min (Fig. 2C). Standards of imidazoleacetic acid, even in concentrations of 20 nmol per sample, showed no fluorescence by this method.

The peaks of histidine and spermidine may overlap each other but the histamine fluorophore is always separated from all of these peaks (Fig. 2D). The reproducibility of the assay was tested by eighteen repeated injections of 18.8 pmol of histamine obtained from a single derivatisation. The first injection was performed not earlier than 30 min after derivatisation because a slight initial decline of fluorescence was observed within this period. The following seventeen injections were made at intervals of 30 min. The mean of the calculated peak areas showed a standard deviation of 8.3%. This indicates reproducibility of the analysis and the stability of the histamine fluorophore for at least 9 h, which agrees with the results of Davis et al. [14].

Analysis of tissue and perfusate extracts

In agreement with others [3, 4, 10, 11] we found the ratio of histamine to interfering substances, mainly spermidine, to be highest in brain and other nervous tissues. Kremzner and Pfeiffer [3] reported that rat brain contains 1000 times more spermidine than histamine. Michaelson [4] reported a spermidine to histamine ratio of 500:1 in nervous tissues. However, even under these unfavourable conditions, the histamine fluorophore could be completely separated from interfering substances like spermidine and histidine using a more polar elution medium (mobile phase containing 15% acetonitrile), as shown in Fig. 3A. The retention time of the histamine fluorophore was about 12 min, and the last peak obtained had a retention time of 25 min.

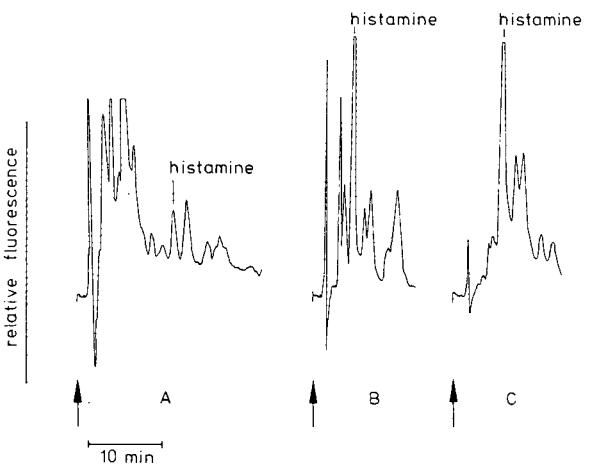

Fig. 3. Chromatograms of rat tissue samples and perfusates of the isolated rat hind-quarter. (A) Medulla oblongata (8.1 pmol), mobile phase contained 15% acetonitrile, flow-rate 2.0 ml min^{-1}, integrator input 32 mV full scale. (B) Perfusate of the rat hind-quarter (20.5 pmol), mobile phase contained 20% acetonitrile, flow-rate 2.5 ml min^{-1}, integrator input 32 mV full scale. (C) Dorsal skin of the hind paw (2.9 nmol), mobile phase contained 20% acetonitrile, flow-rate 2.5 ml min^{-1}, integrator input 128 mV full scale. The arrows indicate the injection of samples.

All other tissues were found to contain far fewer interfering substances than nervous tissue. A sufficient separation of the histamine fluorophore from interfering substances was achieved with a less polar elution medium (mobile phase containing 20—25% acetonitrile). Under these conditions the duration of the HPLC analysis could be shortened to 15 min (Fig. 3B and C).

The content of histamine in some rat tissues and in the perfusates from the isolated rat hind-quarter measured by HPLC is within the range of values obtained by other methods (Table I). The detection limit of 0.9 pmol enabled the measurement of histamine even in a crude homogenate of the rat medulla oblongata, as shown in Fig. 3A.

The described HPLC method for the determination of histamine in biological samples has the following advantages over other methods including gas chromatography—mass spectrometry [25] and radioenzymatic assays [16,

17]: (A) analyses can be carried out rapidly in an automated system after a simple sample preparation and derivatisation with *o*-phthaldialdehyde; (B) interfering substances are easily separated from the histamine fluorophore; (C) no specific extraction procedure is necessary; and (D) no loss of histamine seems to occur.

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GEL CHROMATOGRAPHY OF HEPARIN

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SUMMARY

It is assumed that heparin is a heterogeneous substance. In order to further investigate the purification of heparin, a column chromatographic technique for the fractionation of heparin is described using various diameters of bead form cross-linked dextran gels and an automated apparatus. It was observed that Sephadex G-50 resulted in the separation of three well formed peaks and provided superior resolution compared to all other gels. One of the peaks, representing 51% of the original material, possessed strong anticoagulant activity as measured by the recalcification time, partial thromboplastin time, thrombin time and the anti-X_a test. This peak also possessed strong metachromasia after electrophoresis as well as having a very potent anticoagulant effect in vivo. This technique may have a significant role in the purification of this agent from tissue sources.

INTRODUCTION

Heparin, whose main use as a drug is for the prevention and control of thromboembolism, is a poly-dispersed mixture of highly electronegative chains with molecular weights ranging from 3,000 to 40,000 daltons; the isolation of the component that possesses the anticoagulant activity has been sought after for many years.

A recent study has shown that thirteen fractions could be obtained by partition fractionation and may provide a tool for isolating and characterizing heparins [1]. Yet, the use of electrofocusing [2] has fractionated heparin into 21 components of different molecular weights; however, this has been disputed [3, 4]. Gel chromatography was and still is being used in attempts to purify this agent [5—19]. In general, these investigations using the Sephadex type gels have shown that heparin gave rise to one peak or to a broad peak that had multiple shoulders.

The main purpose of this study was to systematically examine and compare the chromatography of heparin by employing gel chromatography using various diameters of bead form cross-linked dextran gels. Coagulation tests

and electrophoresis on agarose gel were used to analyze the isolated fractions. It was shown that column chromatography, employing Sephadex G-50, resulted in the resolution of several well formed peaks; one of the peaks possessed strong anticoagulant activity.

EXPERIMENTAL

Materials

Sodium heparin (Abbott, North Chicago, IL, U.S.A.), was used in these experiments. It was a white amorphous powder with a nitrogen content of 2.1%. The potency was measured at 169 units per mg. Reagents for the various clotting tests were: human thrombin (Ortho Diagnostics, Raritan, NJ. U.S.A.); 0.02 *M* calcium chloride (Difco Labs., Detroit, MI, U.S.A.); activated cephaloplastin reagent (Actin™, Dade, Miami, FL, U.S.A.); factor X_a (Sigma, St. Louis, MO, U.S.A.); agarose (Indubiose A-45, L'Industrie Biologique Française, Gennevilliers, France); toluidine blue (Fisher Scientific, Pittsburgh, PA, U.S.A.); barbital buffer, pH 8.6; Tris · HCl buffer, pH 8.0. The plasma for the in vitro clotting tests was obtained from rats; blood was collected into 1/10 volume of 0.1 *M* sodium citrate and centrifuged immediately to obtain the plasma.

Gel types

Sephadex (Pharmacia, Uppsala, Sweden) G-type for gel chromatography was employed throughout the experiments. These were G-200, G-150, G-100, G-75, G-50 and G-25. The first four types had a dry bead diameter of 40—120 μm; the last two had a dry bead diameter of 50—150 μm. The dry gel was always mixed with 200 ml of 0.086 *M* Tris·HCl buffer, pH 8.0. The amounts of dry gel employed were: G-200 and 150, 2.5 g; G-100, 3.5 g; G-75, 4.5 g; G-50, 6.5 g and G-25, 11 g. After swelling and degassing, the wet gels were then used for the preparation of the columns. The size of the columns was 1 × 60 cm.

Column development

Spectrum analysis was done in a Unicam SP 800B spectrophotometer in order to determine the optimum wavelength for following the elution of peaks from the columns; maxima were found at 218, 255 and 275 nm. A Spectrochrom 130 (Beckman) analyzer was used for almost all of the chromatographic runs [20, 21]; the effluents from the columns were alternatively measured at the 218, 255 and 275 nm wavelengths employing both 1 and 0.25 cm pathlengths and recorded on a logarithmic scale. Heparin (200 mg) was dissolved in distilled water and charged to the column. The washing solution for the columns was 0.086 *M* Tris·HCl, pH 8.0 containing 1 *M* sodium chloride; the columns were jacketed to maintain the temperature at 25°C. The flow-rate of the washing solution was approximately 7.5 ml/h and 2.5-ml fractions were collected. It was not possible to develop the G-200 columns in the Spectrochrom 130. Instead, the fractions from these gel columns were obtained using a Buchler fraction collector; absorption of each fraction was then determined in the Unicam SP 800B spectrophotometer. The quantita-

tion of the peaks was carried out by two methods, the first by determining the area under the curve and the second by cutting out the individual peaks from the chromatogram and then weighing them; the total was then equal to 100% and each peak was a percentage of this. There was very little difference between the two methods and the averages of both methods were used to quantitate the peaks of the chromatograms.

Coagulation assay of heparin

The anticoagulant activity of each fraction was measured by several techniques. Because of the large amounts of heparin used for chromatography, the eluted fractions had to be diluted before the clotting tests were performed. The diluted samples were then mixed with 0.45 ml of rat plasma and the anticoagulant potential determined using the activated partial thromboplastin time, recalcification time, thrombin time [22] and factor X_a inhibition assay [23].

Electrophoresis

This technique consisted of coating microscopic slides (7.5 × 2.5 cm) with 0.9% agarose. Two slits were made at 2 cm from the cathode end of the slide. A 3-μl aliquot of each fraction obtained from the column was then applied to the slits and electrophoresis started immediately using barbital buffer, pH 8.6; the electrophoresis time was 25 min at 130-V using a Gelman apparatus. After electrophoresis, the slides were fixed, stained with toluidine blue and the spots measured [10].

In vivo activity of isolated peaks

After in vitro testing of the fractions of each peak from G-50 columns, they were combined and peaks I, II and III were concentrated in vacuo from the frozen state and tested in triplicate for anticoagulant activity in vivo. A plastic cannula was inserted into the femoral artery of rats; the blood in the cannula was kept from clotting by the use of citrate. Approximately 0.25 mg of each peak was dissolved in 1 ml of saline and administered by tail vein; other rats given similar amounts of non-chromatographic heparin served as controls. Samples of blood were collected over a 5-h period and tested for anticoagulant activity using the thrombin time. Before each blood sample was tested, sufficient amount of blood was discarded from the cannula to eliminate the citrate effect; more blood was taken and mixed with 0.1 volume of 0.1 *M* citrate and the thrombin time determined.

RESULTS AND DISCUSSION

There have been many attempts to purify heparin by gel chromatography and in only a few instances [5, 10, 13, 15, 17—19] have chromatograms from the chromatography on non-fractionated commercial heparin been shown. The present investigation gave us an opportunity to compare the chromatography of commercial heparin by a systematic approach using various types of cross-linked dextran gels.

Only one peak was found when G-200 was used in the column; all the

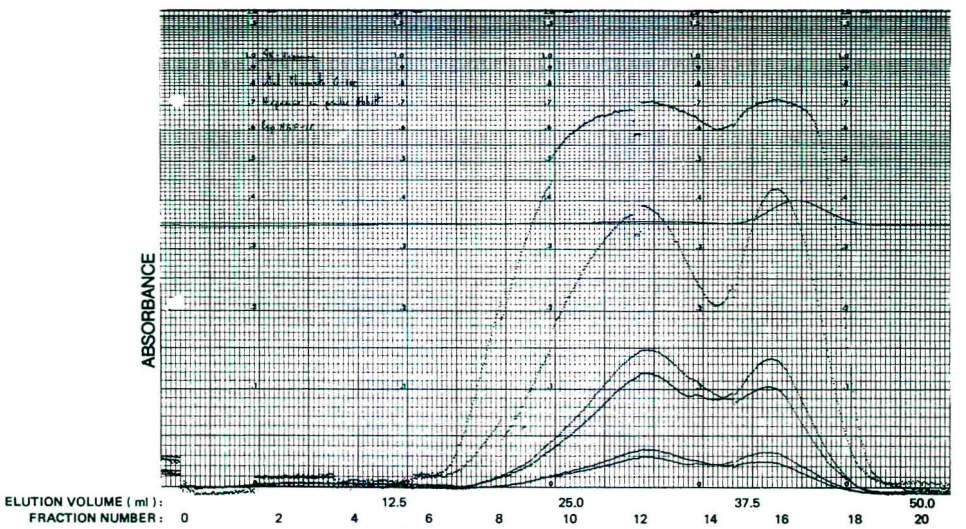

Fig. 1. Chromatogram from a gel G-100 column; 200 mg of heparin were applied to 3.5 g of gel. Column size was 1 × 60 cm. The different tracings on the recording paper represent absorption readings on a logarithmic scale at 218, 255 and 275 nm wavelengths for both the 1.0 and 0.25 cm pathlengths.

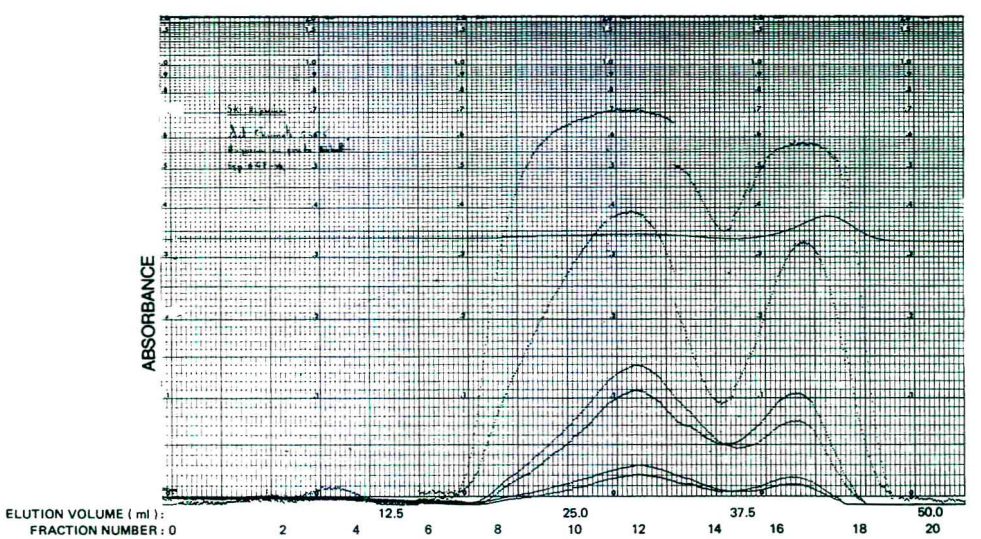

Fig. 2. Chromatogram from a gel G-75 column; 200 mg of heparin were applied to 4.5 g of gel. Column size was 1 × 60 cm. The different tracings on the recording paper represent absorption readings on a logarithmic scale at 218, 255 and 275 nm wavelengths for both the 1.0 and 0.25 cm pathlengths

fractions from this peak possessed anticoagulant and metachromatic activity and did not show any difference to the original material. When G-150 was substituted for G-200 in the columns, one peak was again obtained and gave similar results in clotting and electrophoretic analysis as to those found with G-200. However, as can be seen in Fig. 1, chromatography employing G-100

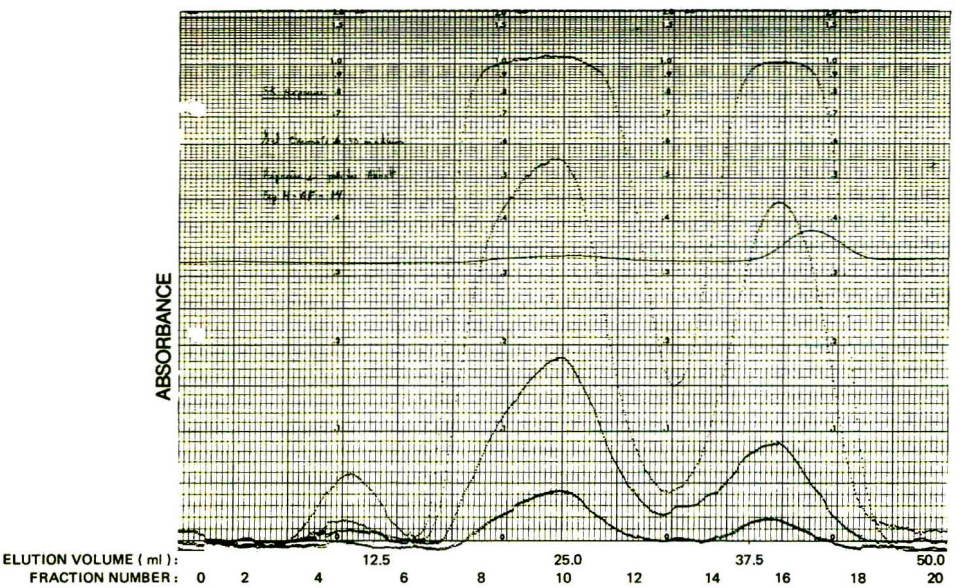

Fig. 3. Chromatogram from a gel G-50 column; 200 mg of heparin were applied to 6.5 g of gel. Column size was 1 × 60 cm. The different tracings on the recording paper represent absorption readings on a logarithmic scale at 218, 255 and 275 nm wavelengths for both the 1.0 and 0.25 cm pathlengths.

began to resolve the heparin material; two peaks were found which is similar to the observations of Walton et al. [5] and Laurent et al. [13]. The fractions from both these peaks possessed anticoagulant and metachromatic activity. However, the second peak was much less active in both of these parameters.

Figs. 2 and 3 show the results of G-75 and G-50 respectively. It can be seen that resolution could still be improved; with the G-50 columns, it was possible to isolate three distinct peaks (identified as peaks I, II and III). The results of the various clotting tests and metachromatic activity of the different fractions of a G-50 column are shown in Table I. The first peak (peak I) which represented 3% of the charged material and consists of the larger molecular weight species was devoid of anticoagulant and metachromatic activity. The last peak (peak III) which consisted of 46% of the applied sample is due to the smaller molecular weight material; it has weak metachromatic and anticoagulant activities. The weak activities observed at the beginning of peak III may be due to the slight lag between the spectrophotometer readings and the collection of the samples and could be part of the second peak. It has been shown that heparin with a molecular weight of less than 6000 daltons has little effect in thrombin neutralization but has high anti-factor X_a activity; just the opposite was found when the molecular weight is greater than 25,000 daltons [15, 24, 25]. The fractions which appeared at the beginning of peak III possessed more anticoagulant potential as measured by the X_a assay than by the activated partial thromboplastin time.

Peak II proved to be the most interesting; this peak contained 51% of the

TABLE I

COAGULATION AND METACHROMATIC ANALYSIS OF FRACTIONS FROM A G-50 COLUMN

Peak	Fraction No.	Activated partial thromboplastin time (sec)	Recalcification time (sec)	Thrombin time (sec)	Factor X_a inhibition assay (sec)	Metachromasia*
I	1	20.1	50.4	17.3	22	—
	2	22.3	50.7	19.4	21	—
	3	21.2	49.2	18.7	23	—
	4	20.5	48.1	17.2	20.4	—
	5	22.1	50.2	16.0	20.4	—
	6	21.8	50.5	17.8	21	—
II	7	23.7	52.6	18.3	23.1	—
	8	23.7	70.3	19.5	24	+/—
	9	25.6	100.8	30.9	28.9	+
	10	100.8	250.9	55.5	200	4+
	11	72.1	200.8	45.8	200	4+
	12	31.4	140.4	40.1	200	3+
	13	28.3	150.8	42.5	200	2+
III	14	24.2	80.5	33.4	103	+
	15	23.8	60.4	28.3	48	+
	16	21.1	50.1	25.7	30	+
	17	22.1	48.3	20.6	25.5	+
	18	20.2	50.4	22.2	25.0	+
	19	21.2	52.5	19.4	25.2	+/—
	20	20.3	48.1	20	23.3	—
Control		18—21	50—57	17—19	20—23	

*Intensity of metachromasia was measured semiquantitatively after electrophoresis; — = no reaction and 4+ = a strong reaction.

applied heparin. It possessed strong anticoagulant activity as measured by the activated partial thromboplastin time, recalcification time, thrombin time and the anti-factor X_a assay. All these tests were used to measure the heparin anticoagulant activity because of the persistent controversy as to which is the best test that relates the true heparin concentration in patients who are receiving heparin therapy [26, 27]. However, it is generally accepted that heparin treatment should be monitored by clotting tests in order to prevent complications of bleeding on one hand and thrombosis on the other. What difference or advantages that the material from peak II may have in patients receiving antithrombotic therapy will require further investigations. However, preliminary studies in rats have shown that peak II does have a very potent anticoagulant effect. After intravenous injection, it was found that the thrombin time was markedly prolonged (more than 300 sec) at 2 h. This was not observed if we used similar amounts of unfractioned heparin; peaks I and III also failed to show any anticoagulant activity under the same conditions. Chromatographic runs using G-25 were inferior to that of G-50.

Our chromatography results are different from those obtained by Graham and Pomeroy [18] or Mulloy and Johnson [19] who used distilled water to develop their columns. It is the opinion of the latter investigators that fractionation on suitable gels using sodium salts at sufficient concentration gives a separation essentially on a molecular weight basis but this might not be the case if just water is used [19].

It is concluded that chromatography on Sephadex G-50 in the present system results in the optimum isolation of a heparin component that is highly active as measured by several assay methods and therefore should be of use in the preparation of this agent from tissue sources.

ACKNOWLEDGEMENT

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VALPROIC ACID AND SEVERAL METABOLITES: QUANTITATIVE DETERMINATION IN SERUM, URINE, BREAST MILK AND TISSUES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY USING SELECTED ION MONITORING

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SUMMARY

A method has been developed for the simultaneous quantitative determination of valproic acid (2-propylpentanoic acid) and its metabolites 2-propyl-2-pentenoic acid (*trans*), 2-propyl-3-pentenoic acid (*trans*), 2-propyl-4-pentenoic acid, 3-hydroxy-2-propylpentanoic acid, 4-hydroxy-2-propylpentanoic acid, 5-hydroxy-2-propylpentanoic acid, 3-oxo-2-propylpentanoic acid, and 2-propylglutaric acid. All compounds were extracted at pH 5.0 with ethyl acetate. The concentrated extracts were trimethylsilylated and the resulting mixtures analyzed by a gas chromatography—mass spectrometry—computer system operated in the selected ion monitoring mode. Linear calibration curves were obtained in the concentration ranges studied (0.1—20 μg/ml for metabolites, 0.1—150 μg/ml for valproic acid). The recoveries of the drugs were between 92 and 97%. The relative standard deviations were between 3.9 and 8.1% (analysis of multiple 10-μl samples of patient urine). The lower detection limits were found to be between 2.8 and 18 ng/ml using 200-μl serum samples. The derivatized extracts were stable for at least one week.

Applications of the method described include studies of placental transfer of valproic acid and metabolites in the human, the elimination of these substances by the neonate, their transfer via mother's milk, and their levels in mouse brain.

INTRODUCTION

Valproic acid (VPA, 2-propylpentanoic acid*) is an anticonvulsant drug which is widely used for the control of a broad spectrum of epileptic seizures; for recent reviews see refs. 1 and 2. VPA is predominantly cleared by metabolic pathways and a number of metabolites have been identified [3—8]. Although numerous procedures have been developed for the determination of VPA [9—18], most of which rely on gas chromatography, quantitative methods exist for the assay of only a few VPA metabolites [4, 6, 7]. Also, these procedures require relatively large samples and are not sufficiently sensitive for the complete analysis of metabolites in human blood or urine samples.

Furthermore, some of the metabolites may be biologically active; and knowledge of their kinetics may therefore significantly contribute to the understanding of the pharmacological activity of VPA and possibly also of the side-effects observed.

We have therefore developed procedures for the simultaneous assay of VPA and the metabolites depicted in Fig. 1. The procedures were of sufficient sensitivity and selectivity for the analysis of small samples ($\leqslant$ 200 μl or mg) of serum, urine, saliva, breast milk, brain and fetal tissues. Analysis was performed by a gas chromatography—mass spectrometry—computer system (GC—MS—computer) operated in the selected ion monitoring mode (mass fragmentography). The high selectivity of the method enabled us to use very simple sample-handling procedures, thus minimizing the danger of labile substances decomposing.

MATERIALS AND METHODS

Chemicals and reagents

The metabolites of VPA (for their structures and abbreviations of their names see Fig. 1) have been prepared by chemical synthesis. The 3-hydroxy, 4-hydroxy, 5-hydroxy and 3-keto metabolites were synthesized as described previously [4, 6]. The 2-en (*trans*) metabolite was prepared by dehydrobromination of 2-bromo-2-propylpentanoic acid with N,N-diethylaniline. The 3-en (*trans*) metabolite, contaminated with 2-en (*trans*), was formed as a side-product of the above synthesis and purified by fractional distillation. The 4-en metabolite was synthesized by decarboxylation during boiling of a solution of 2-allyl-2-propylmalonic acid in a toluene—pyridine mixture. Details of these procedures will be published elsewhere. The internal standard used (2-ethyl-2-methylcaproic acid) was obtained from Fluka (Neu-Ulm, G.F.R.), acetonitrile and pyridine ("dried") were from Merck (Darmstadt, G.F.R.), ethyl acetate (Nanograde) was from Promochem (Wesel, G.F.R.), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) from Pierce (Günter Karl OHG, Geisenheim, G.F.R.), and β-glucuronidase—arylsulfatase from Boehringer (Mannheim, G.F.R.).

*Although VPA is often administered as the sodium salt and, furthermore, is present at physiological pH mostly in its dissociated form, this compound is conventionally referred to as "acid".

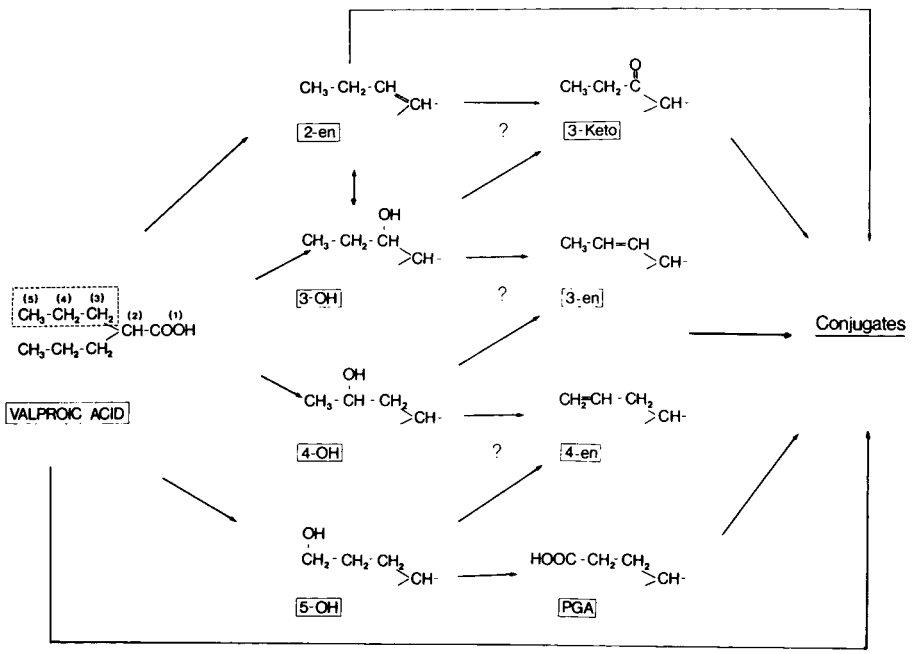

Fig. 1. Tentative metabolic scheme for valproic acid. Abbreviations: VPA = valproic acid (2-propylpentanoic acid); 2-en = 2-propyl-2-pentenoic acid (*trans*); 3-en = 2-propyl-3-pentenoic acid (*trans*); 4-en = 2-propyl-4-pentenoic acid; 3-OH = 3-hydroxy-2-propylpentanoic acid; 4-OH = 4-hydroxy-2-propylpentanoic acid; 5-OH = 5-hydroxy-2-propylpentanoic acid; 3-keto = 3-oxo-2-propylpentanoic acid; PGA = 2-propylglutaric acid. VPA, 2-en (*trans*) and 3-en (*trans*) are present in urine predominantly as conjugates and can be hydrolyzed at pH 5.0 with glucuronidase—arylsulfatase.

Hydrolysis of conjugated metabolites

A sample (2—200 μl) of urine, serum, breast milk or tissue homogenate was pipetted into a disposable 1.5-ml Eppendorf microtube and diluted to 200 μl if necessary; 50 μl of 1 *N* NaH_2PO_4 (adjusted to pH 5.0) and 30 μl of β-glucuronidase—arylsylfatase (5 U/ml) were added. This mixture was slowly agitated at 37°C for 1 h and then processed as described below.

Extraction procedure

A sample (2—200 μl, depending on availability) of serum, urine, breast milk, or tissue homogenate was pipetted into a disposable 1.5-ml Eppendorf microtube and diluted to 200 μl if necessary. Then 50 μl of 1 *N* NaH_2PO_4 buffer (adjusted to pH 5.0) and 1 ml of ethyl acetate containing the internal standard (1 μg) were added. The tube was shaken for 15 min and then centrifuged for 1 min in a 5012 Eppendorf centrifuge. An 800-μl portion of the supernatant organic phase was transferred to a 1.5-ml disposable glass reaction vial and, following the addition of 100 μl of acetonitrile, preconcentrated to 100—200 μl by a stream of nitrogen. The extraction was repeated using 1 ml of ethyl acetate. The combined extracts were evaporated at 20°C under a stream of nitrogen to a final volume of 10—20 μl. The sample was trimethylsilylated by

adding 30 μl of pyridine and 30 μl of MSTFA at room temperature. After at least 30 min, aliquots of 1—3 μl were injected into the GC—MS system.

GC—MS—computer analysis

A Perkin-Elmer F-22 gas chromatograph was coupled via a Watson-Biemann separator to a Varian MAT CH-7A mass spectrometer. A glass column (2 m × 6 mm O.D. × 2.5 mm I.D.; Bodenseewerk Perkin-Elmer, Uberlingen, G.F.R.) packed with 3% OV-17 on 120—140 mesh Gas-Chrom Q was used (Applied Science Labs.). The trimethylsilylated samples were injected at a column temperature of 80°C. After an initial period of 1 min the column temperature was raised to 140°C at a rate of 12.5°C/min. During this period, the derivatives to be measured eluted from the GC column. The temperature was then raised quickly (30°C/min) to 250°C to elute the accompanying substances, and held there for 1 min. During this time, the selected ion records were plotted and the peak height ratios calculated. After the GC column had cooled down to the initial temperature, the next sample was injected. Four samples were analyzed in this way within 1 h.

The mass spectrometer was controlled by the SS-100 Varian data system and operated in the selected ion monitoring mode. The following ions were selected (see Fig. 1 for the structures of the metabolites): *m/e* 183 (3-keto), *m/e* 185 (5-OH, PGA), *m/e* 199 [4-en, 3-en (*trans*), 2-en (*trans*), 4-OH], *m/e* 201 (VPA), *m/e* 215 (internal standard), and *m/e* 275 (3-OH). The results were displayed on a Tektronix 4010 terminal and plotted on a Tektronix hardcopy unit.

Quantitation

Standard calibration graphs were obtained by the analysis of 200-μl portions of drug-free human serum or urine, to which known amounts of VPA and the metabolites indicated in Fig. 1 had been added. These samples were processed as described above. The stored samples were kept frozen at −30°C.

RESULTS AND DISCUSSION

We have evaluated a number of solvents and their mixtures for the extraction of the drugs at various pH values. It was found that ethyl acetate extraction at pH 5.0 led to good yields (Table I) without decomposition of any of the metabolites. At the same pH the conjugates could also be released by glucuronidase—arylsulfatase treatment. Therefore, hydrolysis of the conjugates and extraction of the aglycones could be performed at the same pH, further simplifying the experimental procedures. During the concentration of the extracts, loss of volatile compounds was avoided by the addition of acetonitrile as well as by the incomplete evaporation of the extracts (see Experimental).

Some of the metabolites could not be chromatographed without decomposition. Therefore, various derivatization procedures were evaluated and it was found that each of the drugs could be derivatized by trimethylsilylation into derivatives with excellent GC properties. The derivatization reaction was performed by simply adding the reagents. After a reaction time of 0.5 h (or overnight) the mixture was directly injected into the GC—MS system. The

TABLE I

RECOVERIES, RELATIVE STANDARD DEVIATIONS AND LOWER DETECTION LIMITS

Compound	Recovery of isolation procedure (%)	Lower detection limit* (ng/ml)	Relative standard deviation (%)**
VPA	97	3.6	4.5
2-en	94	2.9	
4-en	96	6.1	
3-OH	92	18.0	8.1
4-OH	96	5.5	4.8
5-OH	96	2.8	3.9
3-keto	96	3.0	5.6
PGA	95	4.6	7.6

*Signal-to-noise ratio = 2 using 200-μl serum samples.
**Ten 10-μl urine samples (daily dose of the epileptic patient: 1.5 g of VPA) were analyzed as described. Only the unconjugated metabolites were measured. Therefore, *trans*-2-en, *trans*-3-en, and 4-en, which are present in urine predominantly as conjugates, are not listed. For the concentrations of the drugs present see the legend to Fig. 4B.

derivatives were found to be stable for at least a week (see below). The hydroxylated metabolites did not form lactones as side-products during the isolation and derivatization procedure.

The mass spectra of the trimethylsilylated derivatives generated by electron impact are shown in Fig. 2. All intense ions were evaluated, but those indicated in Fig. 2 were selected in the final method by the criteria of favorable baselines and detection limits. The ion selected for the internal standard (*m/e* 215) was 14 mass units higher than that for VPA.

The 4-OH metabolite was present in the synthesized sample, as well as in all patient samples encountered, as both diastereoisomeric forms, which were separated by the GC column (see Fig. 4). Since both forms yielded identical mass spectra, the total amount of the 4-OH present was calculated by adding both peaks (ion 199). The diastereoisomeric forms of the 3-OH were not separated by GC and eluted in a slightly broadened peak.

In the case of the 2-en metabolite, a pure standard of the *trans* isomer was available, and this isomer was predominantly present in patient serum and urine samples.

The oxo function of the 3-keto metabolite enolized during trimethylsilylation to yield *cis* and *trans* isomers which were not separated on the GC column used (see Fig. 4). Difficulties during quantitation were not encountered by this enolization.

The reproducibility of the method was evaluated by the analysis of urine samples of an epileptic patient treated with VPA. The relative standard deviations (between 3 and 8%) are listed in Table I. The lower detection limits, using 200-μl samples, were found to be in the low ng/ml range (Table I). Larger sample volumes further increased the sensitivity of the method.

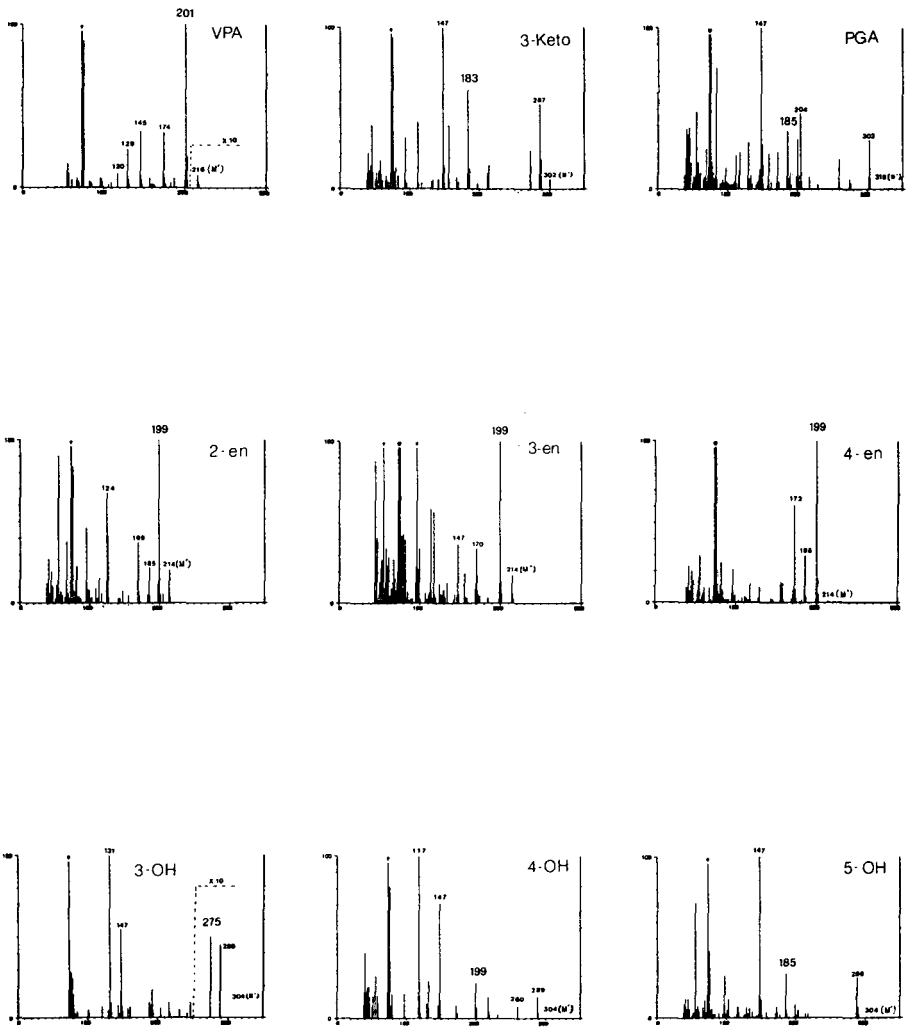

Fig. 2. Electron-impact mass spectra of the trimethylsilylated derivatives of VPA and metabolites. The abbreviated names of the compounds are spelled out in the legend of Fig. 1. The ions selected for mass fragmentography are indicated. All hydroxy and carboxy groups are trimethylsilylated. The 3-keto metabolite carries two trimethylsilyl groups, one on the carboxy and one on the hydroxy group (latter formed via enolization of the oxo function).

Fig. 3 shows the plots of the peak height ratios vs. amounts of drug and metabolites added [0.1—20 μg/ml (g) for the VPA metabolites and 0.1—150 μg/ml (g) for VPA]. The slopes of such plots (Fig. 3) indicate linear dependence of the peak height ratios vs. drug concentrations. The square of the correlation coefficient (r^2) exceeded 0.99.

The stability of the derivatives used was evaluated by the repeated analysis of the same set of standard serum samples. The data listed in Table II indicate that the derivatives were stable for at least a week if the samples were tightly capped and stored at 4°C.

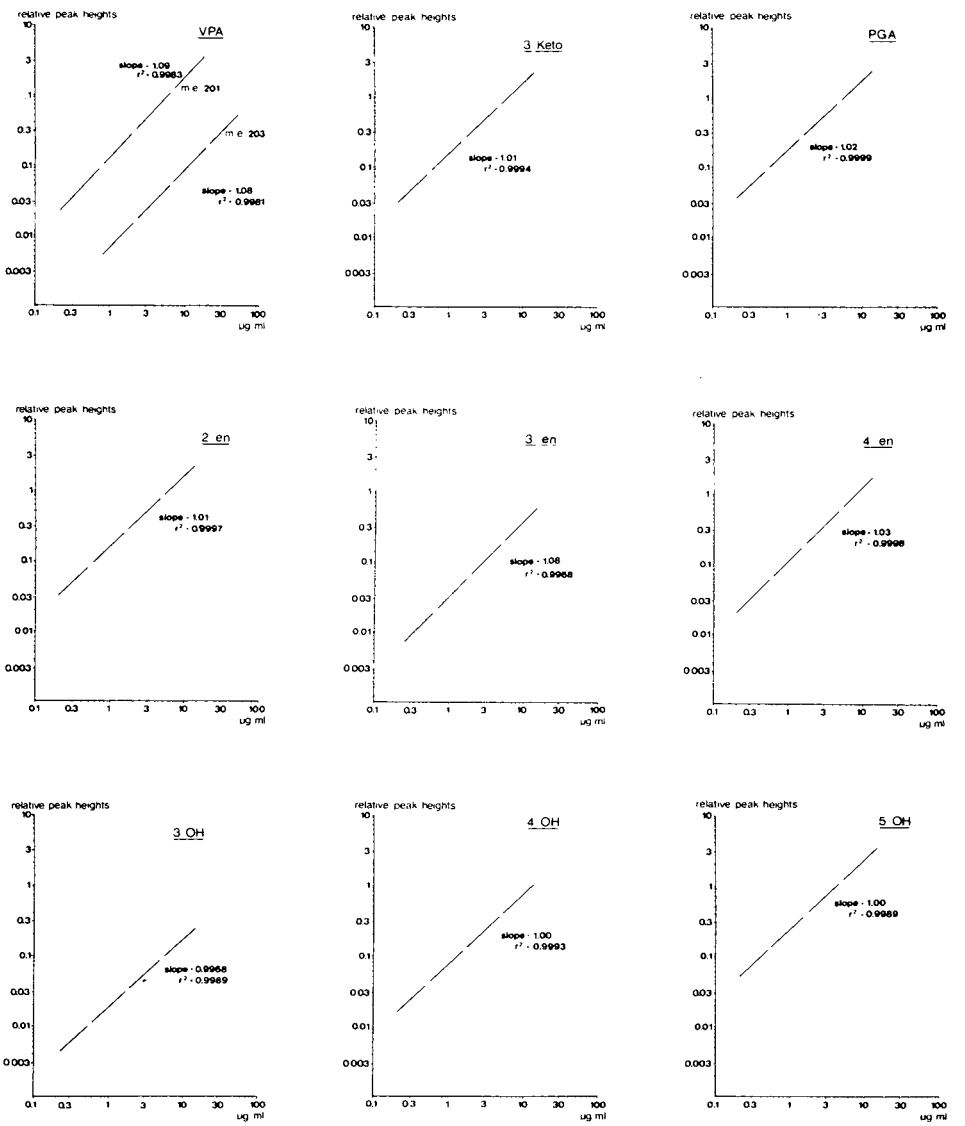

Fig. 3. Double logarithmic plots of the peak heights of the selected ions from trimethylsilylated VPA and metabolites (relative to the peak height of the trimethylsilylated internal standard used) vs. amounts of VPA and metabolites in spiked serum samples.

In spite of the simplicity of the sample-handling procedures, VPA and metabolites could be assayed in small samples of urine (Fig. 4B), serum (Fig. 4A), breast milk, and tissues due to the sensitivity and selectivity of the GC—MS method. Our method has now been in continuous operation for over a year. We have predominantly studied the metabolic profiles of VPA in human serum and urine, placental transfer, neonatal pharmacokinetic and transfer via mother's milk of VPA and metabolites as well as transfer of these substances into brain.

TABLE II

REPEATED ANALYSIS OF THE SAME SET OF STANDARD SERUM SAMPLES

Known amounts of VPA and metabolites were added to serum (0.1—30 μg/ml) for the preparation of calibration curves. GC—MS analysis was performed following the extraction and derivatization procedures. The samples were then stored at 4°C and re-analyzed three and seven days later. The slopes of the experiments on the three different days were used to document stability of the derivatives as well as reproducibility of the assay. The correlation coefficient exceeded 0.99 for all compounds.

Compound	Slope of calibration data				
	Day of analysis			Mean	Relative standard deviation (S.D. × 100/mean)
	0	3	7		
VPA	0.089	0.092	0.093	0.091	2.2
2-en	0.163	0.168	0.171	0.167	2.4
4-en	0.156	0.163	0.170	0.163	4.3
3-OH	0.023	0.022	0.021	0.022	4.5
4-OH	0.113	0.112	0.110	0.112	1.3
5-OH	0.170	0.165	0.162	0.167	2.6
PGA	0.105	0.100	0.095	0.100	5.0

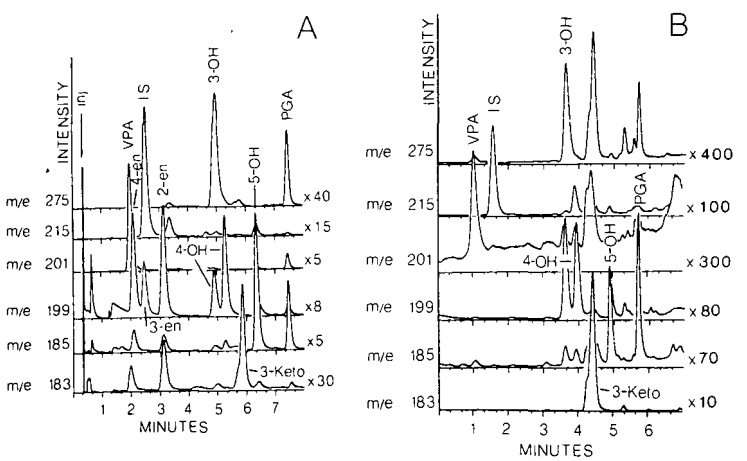

Fig. 4. Selected ion records of (A) a 200-μl serum sample to which VPA and metabolites had been added (3 μg/ml) and (B) of a 10-μl urine sample of a pregnant epileptic woman treated with VPA monotherapy (1.5 g of VPA per day). The concentrations found are: VPA, 4.0 μg/ml; 3-OH, 32.0 μg/ml; 4-OH, 32.1 μg/ml; 5-OH, 10.4 μg/ml; 3-keto, 184 μg/ml; PGA, 25.8 μg/ml. The conjugates (for example, of VPA and 2-en) had not been released by enzymatic treatment of the sample, which explains the absence of the 2-en and 3-en and the low levels of VPA.

It was found that 3-keto and 2-en (*trans*) were the major VPA metabolites in the serum of epileptic patients undergoing VPA therapy, while the 3-keto and the VPA conjugate were the major urinary metabolites. All other VPA metabolites measured were also present in serum and urine, albeit in lower

concentrations [19, 20]. Placental transfer studies indicate that VPA and some metabolites are present in cord blood in higher concentrations than in maternal blood [20]. These findings are rather unusual because most drugs are found in cord blood in concentrations that are equal to or lower than maternal blood levels. The reason for fetal accumulation of VPA and metabolites is not known. The half-lives of VPA and the two main metabolites (40—50 h) in the neonates [20] were much longer than those of adult epileptic patients (8—16 h).

Breast feeding can be performed with apparent safety because only approximately 3% of the maternal VPA levels and 7% of the maternal 3-keto levels were found in breast milk.

The methods described are also applicable without modification to the analysis of brain tissue homogenates for a study of brain levels of VPA and metabolites [21]. Also, our method can be directly applied to the analysis of VPA pharmacokinetics in patients during steady-state therapy; following administration of 1,2-^{13}C-labelled VPA the kinetics of this drug and metabolites can be studied in detail in blood and urine [19].

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QUANTITATIVE DETERMINATION OF NEFOPAM IN HUMAN PLASMA, SALIVA AND CEREBROSPINAL FLUID BY GAS—LIQUID CHROMATOGRAPHY USING A NITROGEN-SELECTIVE DETECTOR

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SUMMARY

A sensitive and selective gas—liquid chromatographic method for the determination of nefopam in human plasma, saliva and cerebrospinal fluid has been developed. The method includes the selective extraction of nefopam and the internal standard, orphenadrine, from biological fluids by a double extraction procedure. The extracted nefopam and internal standard are analyzed by a gas chromatograph equipped with a 3% OV-17 glass column and a nitrogen—phosphorus flame ionization detector (NPFID) operated in the nitrogen mode. The detector provides the needed high sensitivity and also selectivity due to the inherent characteristics of NPFID to discriminate against non-nitrogen containing materials. Five nanograms nefopam per ml plasma or saliva are routinely quantitated with a 1-ml sample or as little as 2 ng per ml cerebrospinal fluid with a 3-ml sample. The intra-day reproducibilities, expressed as the relative standard deviation, are 5, 2 and 3% at 10, 35 and 75 ng/ml plasma levels, respectively. The accuracies expressed by relative error at these levels are 12, −4 and −2%, respectively. The inter-day reproducibility is demonstrated by the small relative standard deviation, 2%, of the slopes from ten plasma standard curves run on ten different days. In various clinical studies in humans the method has been successfully applied to the study of single-dose pharmacokinetics of nefopam and the monitoring of nefopam concentrations in saliva and cerebrospinal fluids.

INTRODUCTION

Nefopam, 3,4,5,6-tetrahydro-5-methyl-1-phenyl-1H-2,5-benzoxazoxine hydrochloride, also Acupan® or Ajan®, (I), is a new analgesic having a unique heterocyclic structure. The compound, first synthesized by Klohs et al. [1], was introduced in Mexico in 1975 and the German Federal Republic in 1976. Recently, it has entered the market in the United Kingdom, Belgium, and some South American countries and was recently approved for marketing in France.

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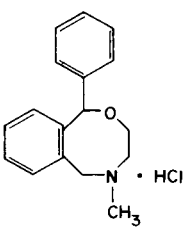
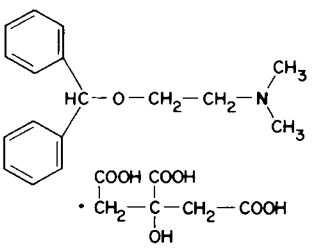
A number of studies concerning the analgesic properties of I have been reported [2—6].



I
(Nefopam)



Internal Standard
(Orphenadrine Citrate)

Prior to in vivo metabolic studies it was necessary to establish a sensitive, chemically specific assay to follow the physical translocation and chemical biotransformation of I. The recommended therapeutic dose of this basic drug is low, and the initial studies in humans indicated that the plasma concentrations of I are usually in the low ng/ml range. Two gas—liquid chromatographic (GLC) methods for the determination of I in plasma have been reported [7, 8]. These methods involve detection of I in plasma by the flame ionization detector. One method has a sensitivity of 20 ng/ml with a 2-ml sample and the second method 10 ng/ml with a 5-ml sample. The aim of the present investigation was to improve the sensitivity and expand the method to other biological fluids such as saliva and cerebrospinal fluid, thus providing a tool for broader investigation of the pharmacokinetics of I at the lower concentrations present at later time periods post dosing as well as in other biological fluids. Because I is a nitrogen-containing compound, and there are many literature reports citing the use of a nitrogen-selective detector for plasma drug quantitation [9—13], it seemed probable that the use of a nitrogen-selective detector could increase the sensitivity of an assay method for I. This paper presents the results of this investigation with a nitrogen-specific detector. A similar GLC method using the same approach was developed concurrently by a group collaborating with this laboratory [14].

EXPERIMENTAL

Reagents

All solvents were Nanograde® and all reagents were analytical reagent grade. Aqueous solutions of 0.1 *N* and 0.5 *N* sodium hydroxide, and 0.1 *N* hydrochloric acid were prepared in distilled water.

Blank plasma

Human plasma was obtained from volunteers who had fasted overnight and had not been on any medication for the previous week. The donors were also asked to refrain from caffeine-containing food and beverages for at least 24 h prior to donating blood.

Gas chromatography

A Hewlett-Packard Model 5840A gas chromatograph equipped with a Model 18847A dual nitrogen—phosphorus flame ionization detector was used. The glass column (180 cm × 2 mm I.D.) was silanized and packed with 3% OV-17 on 80—100 mesh Chromosorb W HP and conditioned overnight at 240°C.

The operating conditions were: injection port temperature, 225°C; detector temperature, 300°C; oven temperature, 190°C isothermal; carrier gas (helium) flow-rate, 30 ml/min; hydrogen flow-rate, 3 ml/min; air flow-rate, 80 ml/min. The detector voltage (d.c.) was set at 16—18 V depending on the age of the bead. An oven temperature program was maintained following each injection: 8.5 min isothermal heating at 190°C, then 30°C/min from 190—240°C and 3 min isothermal heating at 240°C. A 4-min period for cooling and stabilization was maintained between injections. Under these conditions typical retention times of I and the internal standard were 7.6 and 4.5 min, respectively.

Standard solutions

All stock solutions of I and the internal standard were made in methanol. Standard solutions containing 100, 50, 25, 10 and 5 ng of I (free base) per 0.1 ml were made by diluting a 10 μg/ml primary standard solution. The 10 μg/ml stock solution of orphenadrine was diluted to give an internal standard solution of 40 ng (free base) per 0.1 ml.

Extraction of I

Plasma. To a 15 × 125 mm culture tube with a polyethylene-lined screw cap add 1 ml of experimental plasma and 0.1 ml of methanol. Along with the samples, prepare six standards in blank plasma by adding 0, 5, 10, 25, 50, and 100 ng of I in 0.1 ml of methanol to 1 ml of blank plasma. Add 0.1 ml of internal standard solution, 3 ml of 0.1 *N* sodium hydroxide and 5 ml of benzene in that order to all tubes. The tubes are shaken for 10 min on a reciprocal mechanical shaker (horizontal position) and centrifuged for 5 min at 1670 *g*. Then 4.5 ml of the top (benzene) layer are transferred to a tube containing 3 ml of 0.1 *N* hydrochloric acid, and the tubes are shaken for 10 min and centrifuged for 5 min.

The benzene is aspirated and discarded, and 1 ml of 0.5 *N* sodium hydroxide and 5 ml of benzene are added to each tube, and the tubes are shaken for 10 min and centrifuged for 5 min. Then 4.5 ml of the benzene layer are transferred to a 12-ml conical extraction tube and evaporated to dryness at 60°C under a nitrogen stream. The residue is redissolved in 100 μl of methanol and mixed for 10 sec with a vortex mixer. Then 10-μl aliquots of each sample are injected into the gas chromatograph. The benzene can be replaced with toluene in this procedure.

Cerebrospinal fluid. Because of the extremely low concentrations of I in the cerebrospinal fluid (CSF), 3 ml of CSF are generally analyzed. When less than 3 ml of CSF sample is available, distilled water is added to make up the total volume of 3 ml. The extraction and analytical procedures are the same as that for plasma.

Saliva. One milliliter or less saliva sample is analyzed in the same manner as plasma; when less than 1 ml is used, distilled water is added to make up the total volume to 1 ml.

Calculation

The area of each I and internal standard peak was determined by an HP Model 18850A integrating GLC terminal.

Area ratios between I standards and the internal standard were plotted against concentration of I. A straight line was fitted by the least-squares method, and its slope and intercept at the area ratio axis were determined. The mathematical expression of the standard curve is:

$$\text{area ratio} = A\ (\text{I concentration}) + B \tag{1}$$

where A is the slope of the line, and B is the intercept of the line at the area ratio axis. Unknown samples were calculated from the following equation:

$$\text{I concentration in unknown sample} = \frac{\text{area ratio} - B}{A} \tag{2}$$

RESULTS AND DISCUSSION

Chromatography

Baseline separation of I and the internal standard was achieved using the OV-17 column, with no significant interference with I or the internal standard by any endogenous material present in normal human plasma. Typical GLC tracings of extracts from blank human plasma, plasma from human blood dosed with I, and authentic I and internal standard are shown in Fig. 1. The retention times, under the experimental conditions are 7.6 and 4.5 min for I and the internal standard, respectively. The reason for needing this apparent over-separation is that caffeine (retention time = 5.7 min) is eluted between I and the internal standard. In a clinical study where caffeine-containing food or beverages could not be controlled, the size of the caffeine peak varied widely depending on the time of ingestion of caffeine-containing food and beverages and the time of blood sampling. When the caffeine peak is extremely large, it can affect the accuracy of measurement of I at the 5 ng/ml level. Since there are some materials which are eluted substantially slower than I, in order to avoid interference with the next sample by these memory peaks, the column temperature is programmed up to drive off these materials quickly before the next injection.

Limited tests with toluene as the solvent for extraction show identical results in comparison with the standard procedure in which benzene is the solvent. Thus, benzene can be substituted by toluene in this method to eliminate the potential exposure of the analyst to any possible benzene toxicity. In the work of Schuppan et al. [7], diethyl ether was used for extraction. The use of benzene or toluene in the current procedure eliminates the extra precautions taken for the potential explosion of diethyl ether.

At the last step of the extraction procedure where the extract of I was evaporated to dryness and methanol was added to recover I before injection, it is important to add enough methanol to assure the total recovery of I. Incomplete recovery or excessive methanol (diluting the sample) will result in decreased sensitivity, since a smaller sample is presented to the detector. By using ^{14}C-labelled I, it was determined that 100 μl of methanol gives total re-

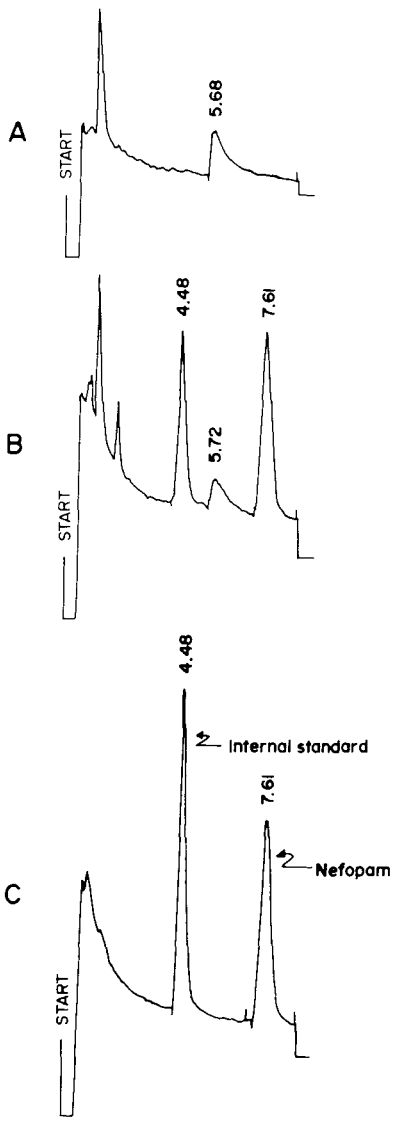


Fig. 1. Chromatograms from human plasma. (A) Blank plasma; (B) plasma of subject dosed with nefopam with the internal standard added, nefopam, 67 ng/ml, internal standard, 40 ng/ml; (C) mixture of authentic nefopam and internal standard, amount injected, nefopam, 5 ng, internal standard, 4 ng.

covery of I under the described experimental conditions.

It was also observed that there is some solvent effect on the nefopam response. For the same amount of nefopam injected in different volumes of solvent, the area responses are different; the larger the solvent volume, the lesser the area per ng of I. Also, for the same solution, when various volumes are injected the resulting responses are not directly proportional; the larger the volume the lesser the response per ng of I. Thus, it is strongly advisable that a

fixed volume of all I standards and unknowns is analyzed to eliminate the potential solvent effect. In our standard procedure a relatively large volume of the sample, 10 μl, is injected because the increase in overall sensitivity with the relatively large amount of I injected more than offsets the loss of response due to the solvent effect.

Selectivity

Two known metabolites of I, desmethyl nefopam and nefopam N-oxide were investigated for possible interference with this method. Even though the direct injection of the N-oxide gave a peak at the retention time of I, presumably due to the conversion of the N-oxide to I on the column, when as much as 100 ng of N-oxide was added to the blank human plasma and extracted following the general procedure, no discernible peak appeared at the retention time of I. N-Oxide, a relatively neutral compound, is most likely left behind during the extraction procedure. Desmethyl nefopam is eluted more slowly than I. Furthermore, when 100 ng of desmethyl nefopam were added to the blank human plasma and treated similarly, only a small peak was detected, probably due to extensive adsorption of the secondary amine on the column. The small response and the longer retention of desmethyl nefopam do not constitute an interference with I.

Extraction recovery

The extraction recovery of I from plasma was determined by a slightly modified extraction procedure in which the internal standard was added after the extraction was completed. The standard curve for this particular experiment was obtained by analyzing various concentrations of I with internal standard present, but without extraction. The extraction recovery check from human plasma was done in triplicate at five concentrations: 5, 10, 25, 50, and 100 ng/ml. The recovery obtained over this concentration range is 87—105% (Table I).

TABLE I

EXTRACTION RECOVERY OF I FROM HUMAN PLASMA (n = 3)

I added (ng/ml)	I detected (mean ± S.D., ng/ml)	Mean recovery (%)
5	4.3 ± 0.3	87
10	10.5 ± 0.4	105
25	23.7 ± 5.2	95
50	52.5 ± 7.1	105
100	97.2 ± 4.1	97

The extraction recovery variations between replicates are relatively wide in some instances. This further emphasizes the importance of both the selection of an internal standard having similar physical properties to I and addition of the internal standard directly to the plasma. The occasional lack of constant extraction recovery of I can be compensated for by the use of orphenadrine as an internal standard; thus, quantitative analysis can be assured.

Sensitivity

Five ng of I per ml of plasma are routinely quantified with a 1-ml sample. The sensitivity (defined as least amount quantifiable) for detection of I in plasma can be further increased by the use of larger samples. However, based on our experience with data obtained following a single therapeutic dose, a 5 ng/ml sensitivity is adequate. For CSF, because of the low concentration of I, a multiple-ml sample has to be used. A sensitivity of 2 ng/ml CSF with a 3-ml sample can be realized. For saliva, the concentration of I in most of the samples analyzed is higher than its corresponding plasma level; therefore a sensitivity of 5 ng/ml with a 1-ml sample is sufficient. In order to quantitate nefopam at 5 and 10 ng/ml levels, the d.c. voltage of the nitrogen detector has to be optimized. However, too high a d.c. voltage will shorten the life span of the alkali bead. Some preliminary data indicated that at low concentration levels, the use of peak height ratios for calibration is slightly better than our current routine method involving area ratios.

Linearity

Six or seven single standards (0—100 ng) were run daily with the unknown samples. The mathematical expression of the least-squares line is $Y = 0.0229$ (I) $- 0.0280$. The correlation coefficient is 0.9995 and the coefficient of determination is 0.9990 indicating good linear proportionality between concentration of I and detector response.

Precision and accuracy

The intra-day precision and accuracy of this method were checked by carrying samples at three concentration levels (10, 35, and 75 ng/ml) in replicates of five through the entire procedure. The detected concentrations were calculated from a standard curve constructed from duplicate standards. The precision was determined by comparing the results between the five replicate samples at each concentration level with the mean and expressed as the relative standard deviation (Table II). The mean detected concentrations were 11.4, 33.6, and 73.7 ng/ml, respectively. The standard deviations were 0.6, 0.7, and 2, and the relative standard deviations were 5, 2, and 3%, respectively.

The accuracy of this method is indicated by the small mean error between the detected and actual values for the samples described above. The mean errors were 1.4, −1.4, and −1.3 ng/ml for the 10, 35, and 75 ng/ml concentrations, respectively. The corresponding relative errors were 12, −4, and −2%. Since this method is intended to analyze samples from biological studies, the precision and accuracy levels are more than adequate.

Ten standard curves for human plasma covering a 5—100 ng/ml range were generated during the analysis of plasma samples from a metabolic study in humans over a period of 19 days. The slope for each standard curve is tabulated in Table III. The slopes ranged from 223×10^{-4} to 237×10^{-4} with a mean and standard deviation of $(230 \pm 5) \times 10^{-4}$. The day-to-day reproducibility of the human plasma standard curve is excellent as indicated by the very small (2%) relative standard deviation of the slopes.

TABLE II

PRECISION AND ACCURACY OF DETERMINATION OF I (n = 5)

I added (ng/ml)	I detected (mean ± S.D., ng/ml)	Relative S.D. (%)	Mean error (ng/ml)	Relative error (%)
10	11.4 ± 0.6	5	1.4	12
35	33.6 ± 0.7	2	−1.4	−4
75	73.7 ± 2	3	−1.3	−2

TABLE III

SLOPES OF THE LINEAR REGRESSION LINES OF THE PLASMA STANDARD CURVES FROM TEN DIFFERENT DAYS

The ten standard curves covered a 5—100 ng/ml range. Mean ± S.D. = 230 ± 5; relative S.D. = 2%.

Day	Slope × 10^4
1	230
2	233
6	236
7	229
8	229
9	236
13	237
14	225
16	226
19	223

Application of the method

Plasma. This method has been extensively applied to the study of single-dose plasma pharmacokinetics in normal, healthy human volunteers, and the monitoring of plasma levels of I in patients following a repeated dosing regimen. One example of these applications is described here.

A normal, healthy human volunteer was given a single, intramuscular (deltoid muscle), 30-mg dose of I. Ten-ml heparinized blood samples were taken before medication and 1, 2, 3, 5, 7, 9, 11, 13, 15, 24, 36, and 48 h after dosing. The plasma levels of I in these samples were determined by the method described above. The resulting plasma concentrations of I vs. time after dose were plotted on a log—linear scale (Fig. 2). The highest plasma levels were detected 1 h post dosing (first sampling time) indicating the rapid absorption of I from the injection site. The plasma levels of I from 1 to 15 h decline mono-exponentially with an estimated plasma half-life ($t_{½}$) of 4.3 h. The last quantifiable time point ($\geqslant$ 5 ng/ml) was at 15 h, which is about four half-lives. Thus, the method is more than adequate for the measurement of the plasma pharmacokinetics of nefopam in humans following a single 30-mg intramuscular dose.

Saliva. For certain drugs plasma to saliva concentrations remain constant over a wide concentration range [15] and thus, the saliva concentration reflects the plasma concentration. In this situation, saliva level measurement provides a

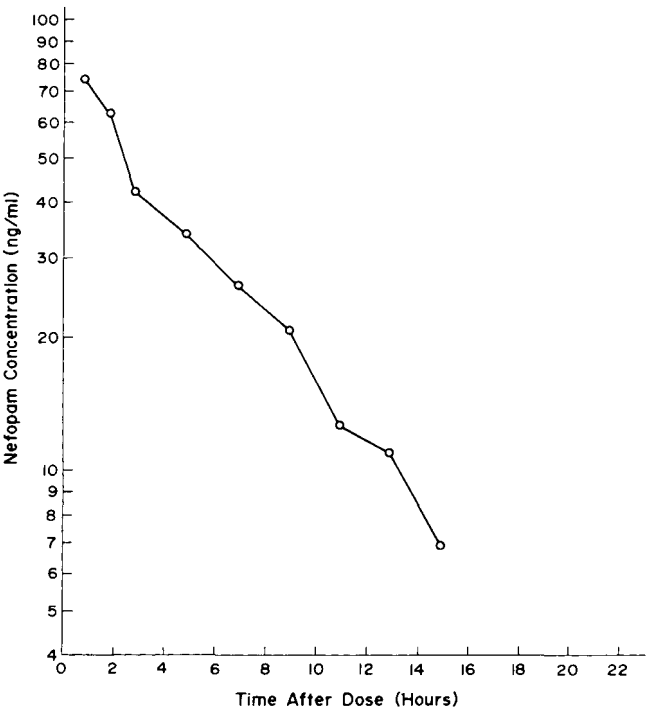


Fig. 2. Plasma nefopam levels in a normal healthy human volunteer following a single intramuscular 30-mg dose of nefopam.

non-invasive means for monitoring drug levels in the body. I was investigated to determine if such a constant plasma—saliva relationship exists. In the intramuscular study described above, saliva samples were also taken before medication and at 1, 2, and 3 h. Saliva samples were obtained by paraffin stimulation and expectoration into a disposable sputum-collecting cup. The first two expectorations were discarded and then a 3-min saliva collection was taken. It was demonstrated that a standard curve (5—100 ng/ml) in saliva is identical to that in distilled water, and in all subsequent saliva analyses, a standard curve of I in distilled water was used for the calibration of unknowns. Saliva samples from eight subjects were analyzed. The results are shown in Table IV. The results indicated that I is present in saliva and in most of the samples the saliva/plasma ratios were larger than one. However, they were variable. Thus, the saliva concentration of I does not reflect the plasma levels accurately, and at best approximates the plasma levels.

Cerebrospinal fluid. CSF was obtained from patients with pain due to neurological disease; these patients had been treated with I, 60 mg tid orally for three weeks [17]. The CSF was taken for diagnostic purposes and part of the sample was analyzed for I. Because of the difficulties in obtaining blank CSF for the daily standard curves, it was first demonstrated with a limited amount of CSF that the I standard curve in CSF was identical to that in distilled water, and then during each routine analysis run of unknowns, a distilled water standard curve was used to calibrate the concentrations of I in

TABLE IV

PLASMA AND SALIVA CONCENTRATIONS OF I IN HUMANS FOLLOWING A SINGLE INTRAMUSCULAR 30-mg DOSE OF I

Subject No.	Sampling time (h post dosing)	Plasma (ng/ml)	Saliva (ng/ml)	Saliva/plasma
1	1	74	108	1.5
	2	62	96	1.6
	3	42	109	2.6
2	1	75	176	2.4
	2	50	85	1.7
	3	54	51	0.9
4	1	43	216	5
	2	33	105	3.2
	3	32	79	2.5
8	1	89	361	4.1
	2	53	213	4.0
	3	53	169	3.2
3	1	60	290	4.8
	2	59	148	2.5
	3	58	90	1.6
5	1	46	107	2.3
	2	37	60	1.6
	3	28	27	1.0
6	1	49	113	2.3
	2	34	37	1.1
	3	28	25	0.9
7	1	46	70	1.5
	2	27	36	1.3
	3	25	27	1.1

the CSF. The detailed results of the CSF and plasma concentration relationship are being prepared for publication [17]. In general the CSF concentration of nefopam in humans is very low. These CSF concentrations generally were in the range of expected unbound plasma nefopam concentrations.

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CHROMBIO. 985

SIMULTANEOUS DETERMINATION OF CHLOROQUINE AND ITS DESETHYL METABOLITE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY

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(First received March 16th, 1981; revised manuscript received May 21st, 1981)

SUMMARY

A gas chromatographic method for the simultaneous determination of chloroquine and its metabolite desethylchloroquine in plasma is described. Chloroquine, desethylchloroquine and internal standard are extracted as bases with *n*-hexane—pentanol (90:10) and then back-extracted to an acid aqueous phase. The aqueous phase is made alkaline and after re-extraction into chloroform and evaporation of the chloroform, acylation with trifluoroacetic anhydride is performed. Separation is achieved on an OV-17 column at 250°C. Chloroquine and desethylchloroquine can be determined down to 0.1—0.2 μmol/l (30—60 ng/ml), with a coefficient of variation of 12%, using a nitrogen detector. The method shows good correlation (r = 0.98) with a recently developed liquid chromatographic method.

INTRODUCTION

Chloroquine (CQ) is one of the most frequently used antimalarial agents and is furthermore used as an effective drug in the treatment of rheumatoid arthritis. Desethylchloroquine (CQM) has been identified in urine as the main metabolite of CQ using thin-layer chromatography [1, 2] and by gas chromatography—mass spectrometry [3]. A relationship between serum levels of CQ and the frequency of side-effects has been shown in the treatment of rheumatoid arthritis with CQ [4]. Side-effects may be seen for a plasma concentration of CQ exceeding about 0.8—1.0 μmol/l. A number of different analytical methods for the determination of CQ have been proposed. These include fluorimetry [5], gas—liquid chromatography [6] and spectrodensitometry [7]. These methods do not adequately quantify CQ and CQM separately. A sensitive assay for the determination of plasma levels of CQ and CQM by high-performance liquid chromatography (HPLC) has recently

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been presented [8], which made it possible to study the bioavailability of CQ and its pharmacokinetics in man [9].

In this paper, a selective gas chromatographic method for the simultaneous determination of CQ and CQM using a nitrogen detector is described. It can be used for routine determinations of CQ and CQM levels in patients undergoing chronic treatment with chloroquine as an alternative to the perhaps more demanding HPLC method. The method is documented with respect to precision, recovery at different plasma levels, and clinical cross-testing with a liquid chromatographic method.

MATERIALS AND METHODS

Chemicals and reagents

Chloroquine, desethylchloroquine and 7-iodo-4-(1-methyl-4-diethylaminobutylamino)quinoline, used as the internal standard (IS), were kindly supplied by Sterling-Winthrop, Skärholmen, Sweden. The molecular structures are shown in Fig. 1. *n*-Hexane, 1-pentanol and chloroform were of analytical grade from Merck (Darmstadt, G.F.R.). Trifluoroacetic anhydride (TFA) purum was from Fluka (Buchs, Switzerland). All other chemicals were of analytical or equivalent grade and were used without further purification. The glass utensils used were cleaned with nitric acid (2 mol/l) in an ultrasonic bath, followed by a rinse with deionized, Milli Q filtered water (Millipore, Bedford, MA, U.S.A.).

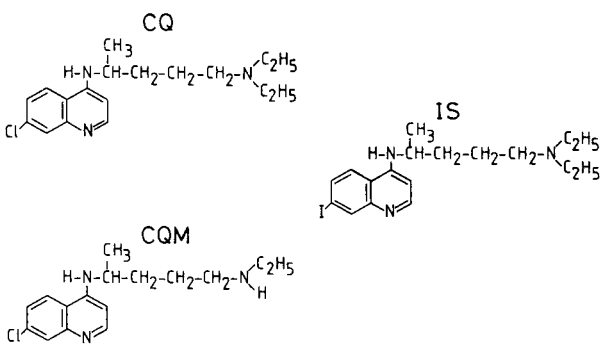

Fig. 1. Chemical structures of chloroquine (CQ), desethylchloroquine (CQM) and internal standard (IS).

Gas chromatography

The analyses were performed on a Perkin-Elmer 990 gas chromatograph connected to a Perkin-Elmer nitrogen—phosphorus detector. The glass column (2 m × 2 mm I.D.) was silanized and packed with 3% OV-17 on Gas-Chrom Q, 80—100 mesh, from Applied Science Labs. (State College, PA, U.S.A.). Nitrogen, purified with a molecular sieve, was used as carrier gas at a flow-rate of 40 ml/min and the column temperature was 250°C. The injector and detector were both held at 300°C. The flow-rates of hydrogen and air to the detector were 5 ml/min and 70 ml/min, respectively.

Mass spectrometry

A Varian MAT 44 S mass spectrometer was used, coupled to a Varian 3700 gas chromatograph equipped with a glass column filled with the same packing as above. The ionization energy was 70 eV.

Extraction and derivatisation procedure

A 2.0-ml aliquot of the plasma sample, or urine diluted 1:20, and 0.100 ml of the internal standard solution (50 μmol/l) were made alkaline with 1.0 ml of sodium hydroxide (1 mol/l) and extracted for 15 min with 5.0 ml of *n*-hexane—1-pentanol (90:10). After centrifugation for 10 min, 4.0 ml of the organic phase were transferred to another tube and 5.0 ml of hydrochloric acid (0.2 mol/l) were added. The tube was shaken for 15 min and centrifuged. Then 4.0 ml of the aqueous phase were made alkaline by addition of 0.5 ml of sodium hydroxide (5 mol/l), and extracted with 5.0 ml of chloroform for 15 min. After centrifugation 4.0 ml of the chloroform phase were evaporated to dryness. The residue was dissolved in 50 μl of ethyl acetate and 10 μl of TFA were added. The tube was incubated for 15 min at 50°C, followed by evaporation to dryness. The residue was dissolved in 25 μl of ethyl acetate, and 2 μl were injected into the gas chromatograph.

Calibration curves were prepared each day by analysis of samples with known amounts of CQ and CQM added to blank plasma. Curves in the concentration range of 0.2—2 μmol/l are shown in Fig. 2.

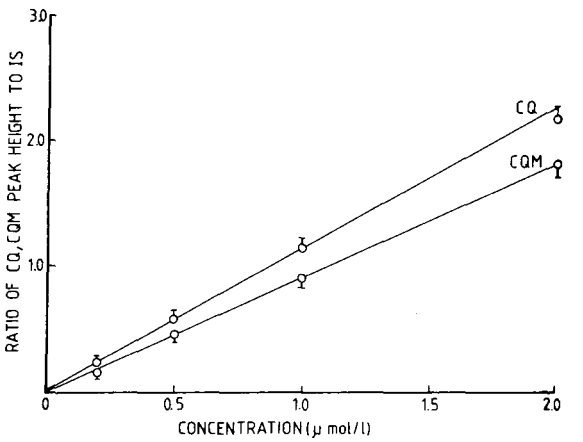

Fig. 2. Calibration curves of chloroquine (CQ) and desethylchloroquine (CQM) obtained after extraction from plasma. Each point represents the mean value of three replicate determinations, and the length of the vertical bars denotes the corresponding standard deviation.

Precision

The precision of the method was determined by analysing pooled plasma.

Extraction efficiency

CQ and CQM were added in the concentration range 0.2—1.0 μmol/l to a pool of plasma from patients undergoing chloroquine therapy or to a phos-

phate buffer (pH 7.0) containing 154 mmol/l sodium chloride. The samples were then analyzed by the present method.

Selectivity

The selectivity of the gas chromatographic determination of CQ and CQM was verified by comparison with an ion-pair liquid chromatographic method with fluorescence detection [8].

RESULTS AND DISCUSSION

Extraction conditions

Distribution conditions for CQ, CQM and internal standard in buffered aqueous solutions using *n*-hexane—1-pentanol (90:10) or chloroform as organic phase have been studied [10]. Quantitative extraction as bases (99%) for equal phase volumes is obtained when the pH of the aqueous phase is >10. For practical reasons, a mixture of *n*-hexane—1-pentanol was chosen in the first extraction step since the organic phase will then be the upper phase and transfer to a new test tube is thus facilitated. The back-extraction to an acid aqueous phase is quantitative for pH <5. Repeated extraction is necessary to obtain a sufficiently pure extract for subsequent work.

Derivatisation conditions

Several stationary phases (OV-7, OV-17, QF-1 and OV-210) were tried in order to separate the tertiary amine CQ from CQM, which is a secondary amine. However, none of these phases adequately separated CQ and CQM. By performing a TFA derivatisation, the retention time is changed for CQM but not for CQ and the internal standard, as is illustrated in Fig. 3. This figure

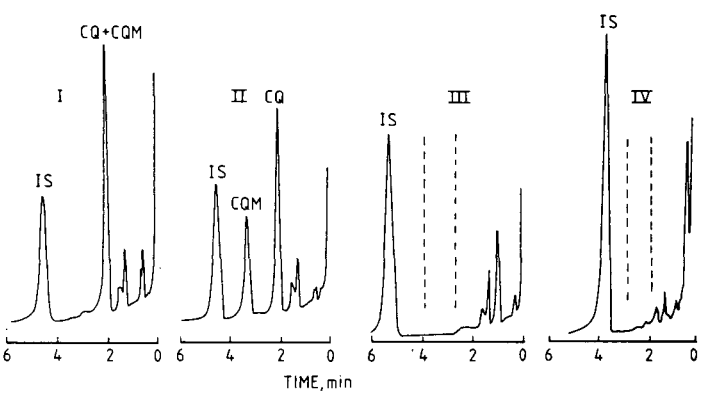

Fig. 3. Gas chromatograms of plasma samples containing chloroquine (CQ), desethylchloroquine (CQM) and internal standard (IS). (I) Plasma sample without TFA derivatisation containing CQ 0.44 μmol/l, CQM 0.37 μmol/l and internal standard. (II) The same plasma sample as in I after TFA derivatisation. (III) Plasma blank with internal standard. Retention times for CQ and CQM at dotted lines. (IV) Urine blank with internal standard. Retention times for CQ and CQM at dotted lines.

also shows that neither plasma nor urine contain endogenous substances that interfere.

The mass spectrum of TFA-CQM is presented in Fig. 4. The molecular ion (*m/z* 387) and the fragmentation pattern indicate that only one trifluoroacetyl group is introduced into the metabolite. The fact that the chromatographic behaviour of CQ and IS is unaffected by the derivatisation procedure indicates that it is the nitrogen on the side-chain which reacts with TFA. This is further corroborated by the mass spectrum of TFA-treated CQ which contained no peak with *m/z* exceeding 319.

The formation of the TFA-CQM derivative is complete within 15 min at 50°C and the product is stable under the reaction conditions for at least 4 h. After evaporation of the excess reagent, the derivative was found to be stable for more than one week at room temperature.

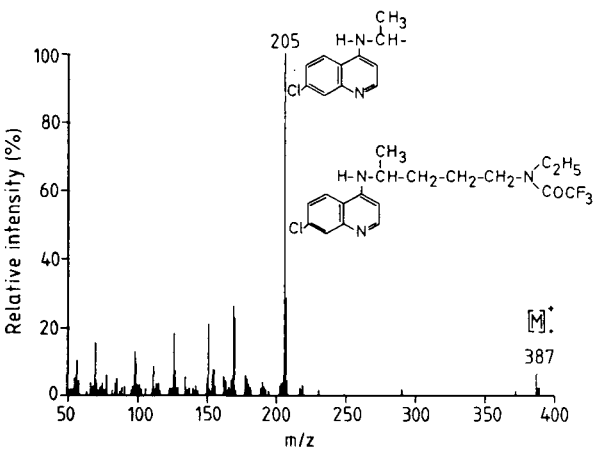

Fig. 4. Mass spectrum of trifluoroacetylated desethylchloroquine. Ions below *m/z* 50 are omitted. Ionization energy = 70 eV.

Sensitivity, precision and recovery

It was possible to determine 0.2 μmol/l CQ and CQM with a relative standard deviation of 12%. Results are shown in Table I. Repeated injections of

TABLE I

PRECISION OF THE METHOD USING POOLED PLASMA

	Concentration (μmol/l)	R.S.D.* (%)	*n*
Chloroquine	0.79	5.8	7
	0.22	11.6	7
Desethylchloroquine	0.77	6.5	7
	0.22	12.3	7

*R.S.D. = relative standard deviation.

the same extract of CQ and TFA-CQM gave a coefficient of variation of 2.1% (n = 10) for both CQ and CQM at the 0.35 μmol/l level. The recovery of CQ and TFA-CQM was about 50% at the 2 μmol/l level. It was estimated from a comparison between the peak heights of extracted plasma standards and those from known amounts of the compounds injected directly into the gas chromatograph. The observed recovery is close to the value (51%) calculated from the extraction procedure. The recoveries of CQ and CQM from spiked plasma samples and an aqueous medium are equal. As shown in Fig. 5, the standard addition plots have the same slopes for both matrices.

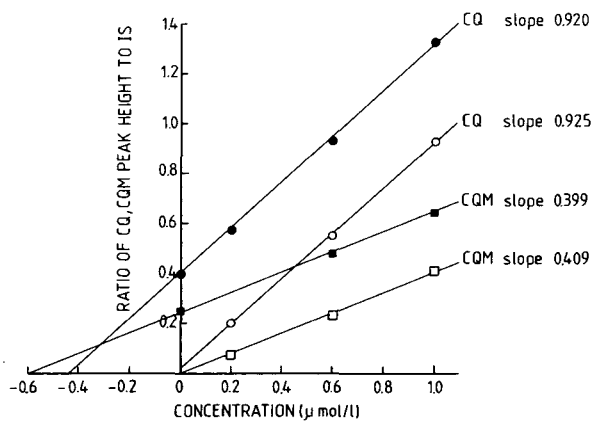

Fig. 5. Comparison of the recovery of chloroquine (CQ) and desethylchloroquine (CQM) from plasma and aqueous solution. Each point represents the average of two determinations. (•), CQ added to a plasma pool from patients; (○), CQ added to a phosphate buffer (pH 7.0) containing 154 mmol/l sodium chloride; (▪), CQM added to a plasma pool from patients; (□), CQM added to a phosphate buffer (pH 7.0) containing 154 mmol/l sodium chloride.

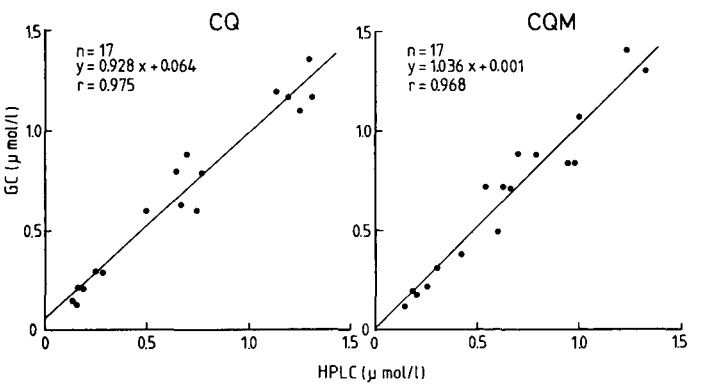

Fig. 6. Comparison of results obtained by liquid chromatography (x) and gas chromatography (y) for chloroquine (CQ) and desethylchloroquine (CQM) in plasma. Samples were taken from patients undergoing chloroquine therapy.

Stability of plasma samples

The influence of storage time on the CQ and CQM levels were determined from ten plasma samples stored for about 11 months at −20°C. No degradation of the compounds was observed within the precision of the gas chromatographic method (± 5% of the actual plasma levels).

Selectivity of the present method

The results of the selectivity are shown in Fig. 6. Since the HPLC and gas chromatographic methods use different methods for extraction, chromatography and detection, the high correlation coefficient demonstrates the good selectivity of the present method.

We have shown experimentally that the following frequently used drugs for treatment of rheumatic diseases — phenylbutazone, naproxen, prednisolone, salicylazosulphapyridine, ibuprofen, indomethacin and salicyclic acid — do not interfere with the present method.

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CHROMBIO. 977

DETERMINATION OF CANNABIDIOL IN PLASMA BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A procedure was developed for the analysis of cannabidiol (CBD) in blood plasma. Tetrahydrocannabidiol was used as an internal standard and was added prior to extraction. The plasma extracts were derivatized with pentafluorobenzyl bromide and the product purified on a mini-column of Florisil. The pentafluorobenzyl derivatives were then analyzed by gas chromatography on a 5% OV-225 column using an electron-capture detector. A detection limit of 50 ng CBD per ml of plasma was observed. The procedure was used to study the plasma level of CBD after its oral and intravenous administration to monkeys.

INTRODUCTION

Accurate methods for the quantitative determination of cannabinoids in biological samples have been in demand for the past several years. (—)-*trans*-Δ^9-Tetrahydrocannabinol (Δ^9-THC) has received the most attention in this area since it was originally reported to be the major component of most *Cannabis* samples and has psychotomimetic activity. The methodologies employed for the quantitative detection of Δ^9-THC in biological samples include derivatization followed by single-column gas chromatography (GC) using an alkaline flame ionization detector [1,2], derivatization followed by dual-column GC using an electron-capture detector [3], derivatization followed by high-performance liquid chromatographic separation and GC detection with an elec-

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tron-capture detector [4], gas chromatography—mass spectrometry (GC—MS) methods [5,6], radioactive tracer methods [7—9] and radioimmunoassay methods [10,11].

Cannabidiol (CBD) is the most abundant cannabinoid in hashish and in the fiber type *Cannabis* [12]. In addition, CBD has interesting biological activities. One of its more important actions is the anticonvulsant activity seen in laboratory animals which suggests that CBD may be a useful antiepileptic agent in humans [13—15]. Studies are currently underway to establish the activity of CBD in humans [16]. In spite of these developments, there are no reports in the literature for the analysis of CBD in blood other than tracer methods [17—19]. Thus a simple analytical procedure is found necessary for determining CBD levels in biological fluids. Reported here are the procedures developed for the quantitative analysis of plasma samples for CBD utilizing the selectivity of electron-capture detectors by forming the dipentafluorobenzyl derivative of CBD.

EXPERIMENTAL

Reagents

All solvents were either chromatographic quality or redistilled in glass. Pentafluorobenzyl bromide (Pierce, Rockford, IL, U.S.A.) was used without further purification. Other reagents were analytical grade. Cannabidiol (CBD) was obtained from the National Institute on Drug Abuse (NIDA). Tetrahydrocannabidiol (THCBD) was prepared from CBD by hydrogenation over 10% palladium—charcoal catalyst and purified by column chromatography on Florisil (100—200 mesh).

Glassware

All glassware utilized in the recovery processes was previously silanized by soaking in a 10% solution of dichlorodimethylsilane in toluene for 4 h at room temperature.

Gas chromatograph

The analyses were performed on a Varian 1400 gas chromatograph with a ^{63}Ni electron-capture detector. A 1.8 m × 2 mm I.D. glass column packed with 5% OV-225 on Chromosorb W (100—120 mesh) was used at 230°C with nitrogen flow-rate of approximately 50 ml/min.

Preparation of CBD and THCBD derivatives for calibration

Ethanol solutions of both CBD and THCBD were prepared at concentrations of 2.0 μg/ml and stored at 5°C. Appropriate aliquots of the CBD solution were withdrawn to obtain 0.5, 1.0, 2.0, and 4.0 μg CBD and placed into separate 5-ml round-bottom flasks. To each flask were added 2.0 μg of THCBD (1.0 ml of the THCBD solution). The ethanol was evaporated under nitrogen at 60°C. Approximately 20 mg of anhydrous potassium carbonate were added to each flask, followed by the addition of approximately 3 ml of dry acetone (dried over anhydrous calcium chloride immediately prior to use). To this mixture, 50 μl of pentafluorobenzyl bromide were added and the reaction mixture re-

fluxed overnight with stirring. The reaction mixture was allowed to cool to room temperature then filtered. The flask was washed with three 3-ml portions of acetone and these washes passed through the filter paper. The filtrate, collected in a 15-ml conical centrifuge tube, was evaporated to dryness under nitrogen at 60°C. The residue was dissolved in 3.0 ml of hexane and aliquots of this injected into the chromatograph.

Analysis of plasma samples

Plasma samples (1.0 ml) were used throughout the standardization procedure. The basic extraction procedure is a modification of the protocol reported by Fenimore et al. [3] for Δ^9-THC. To a 50-ml conical centrifuge tube, 2.0 μg of THCBD (1.0 ml of ethanol solution of THCBD) were added. The ethanol was evaporated under nitrogen and 1.0 ml plasma added. The mixture was then vortexed and 10 ml of hexane—1.5% isoamyl alcohol were added, followed by vortexing for 30 sec and centrifuging at 400 *g* for 5 min. The hexane layer and most of the emulsified layer were transferred to a 15-ml conical centrifuge tube and the volume reduced under nitrogen by heating at 60°C being careful not to take to dryness. The plasma was reextracted with an additional 10-ml volume of hexane—1.5% isoamyl alcohol by vortexing and centrifuging as above. The volume of the combined hexane extracts was reduced to approximately 4 ml as before. The hexane extract was then washed successively with 2 ml of 0.1 *N* sodium hydroxide and 2 ml of 0.1 *N* hydrochloric acid and transferred to a 5-ml round-bottom flask, evaporated to dryness under nitrogen and derivatized as above.

The residue obtained after derivatization was dissolved in 0.3 ml hexane, then chromatographed with hexane on 1 g Florisil packed in a glass champagne column (Supelco, Bellefonte, PA, U.S.A.) with a 30-ml reservoir. The first 30 ml were collected, evaporated to dryness under nitrogen and the residue redissolved in 3.0 ml hexane. Aliquots of this sample were then injected into the chromatograph.

Spiked plasma samples for calibration purposes were treated in an identical manner with the addition of CBD and at the same time the internal standard (THCBD) was added to the sample.

Animal studies

Three male rhesus monkeys weighing 7—8 kg were utilized for the preliminary animal studies. The subjects were fasted overnight and anesthetized with 100 mg ketamine·HCl. CBD was administered orally via a nasal gastric tube in sesame oil or intravenously in ethanol. Blood samples were obtained via femoral puncture and collected in Vacutainer tubes containing disodium EDTA. The blood samples were centrifuged and plasma removed and stored in silanized containers at —20°C until analysis.

RESULTS AND DISCUSSION

The analysis of cannabinoids in biological fluids has drawn a lot of attention in the past decade because of the increased health problems associated with marihuana use as well as the increased number of clinical investigations

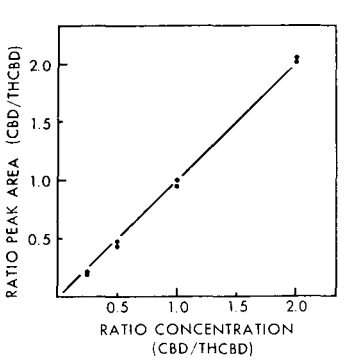
on certain cannabinoids. In this communication, we report a simple GC procedure for the analysis of CBD, a major cannabinoid which has received little attention in this respect. The selection of another cannabinoid to be used as an internal standard for the analysis of CBD was difficult since there are 61 cannabinoids known to exist naturally in *Cannabis* [20] and there are 22 known metabolites of CBD [21]. The use of THCBD as an internal standard was found to be satisfactory since the latter is not known to occur naturally or as a metabolite of CBD. THCBD was prepared by hydrogenation of CBD over palladium—charcoal catalyst. The product of the hydrogenation procedure was purified by column chromatography and then dissolved in ethanol. Quantitative hydrogenation was obtained as indicated by GC analysis with flame ionization detection [11]. The concentration of THCBD was calculated assuming the response factor to be the same as that utilized for CBD. The ethanol solution of THCBD became the stock solution of internal standard and was stored at 5°C. Appropriate dilutions were made to obtain the desired concentration of THCBD employed in the analyses.

Preliminary experiments to evaluate the procedure outlined above included monitoring of the derivatization and evaluating the extraction and recovery. Initially, the pentafluorobenzyl derivatives of CBD and THCBD were produced on milligram quantities of the materials and the reactions followed by thin-layer chromatography. No underivatized CBD or THCBD could be detected in the reaction mixture after 14 h and the conversion was quantitative. Thus, the overnight refluxing of the samples was selected out of convenience rather than necessity for the samples could be extracted one day, derivatized overnight and analyzed the next day.

Samples of the CBD and THCBD derivatives were analyzed by GC—MS to confirm the formation of the diether derivatives. The CBD and THCBD derivatives showed molecular ions of m/e 674 and 678 respectively indicating the formation of the desired products.

Detector response to CBD and THCBD derivatives was evaluated by adding varying amounts of CBD to 2.0 μg THCBD in the reaction vessel and derivatizing these samples. Fig. 1 illustrates the data obtained which demonstrate that the relative response factor for the CBD derivative and THCBD derivative remains constant over the range examined and is approximately 1.0.

The analysis of plasma samples spiked only with CBD at a level of 2 μg/ml and THCBD added after extraction (prior to derivatization) indicated the recovery of the CBD to be approximately 55%. The efficiency of this extraction did not remain constant over the concentration range examined; therefore, it was necessary to establish calibration data using plasma samples spiked with both CBD and THCBD. This variation in extraction and recovery efficiency is illustrated in Fig. 2. Even though these data exhibit non-linearity over the concentration range examined, a smooth curve can be drawn and the curve utilized to estimate the plasma concentration of CBD in the animal experiments. Fig. 3 illustrates a typical chromatogram of one of the samples from the animal studies illustrating the baseline resolution attainable. No peaks were seen in this region of the chromatograms from samples which contained no CBD or THCBD indicating the absence of interference by components extracted from the plasma.



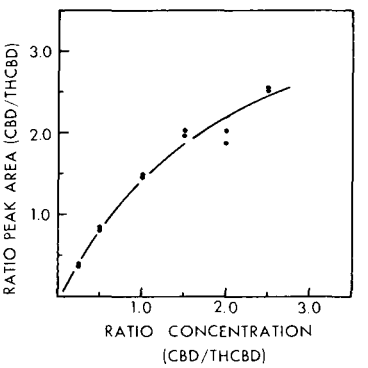


Fig. 1. Electron-capture detector response curve for the dipentafluorobenzyl derivatives of cannabidiol (CBD) and tetrahydrocannabidiol (THCBD).

Fig. 2. Calibration curve for cannabidiol (CBD) with tetrahydrocannabidiol (THCBD) added to plasma as an internal standard prior to extraction.

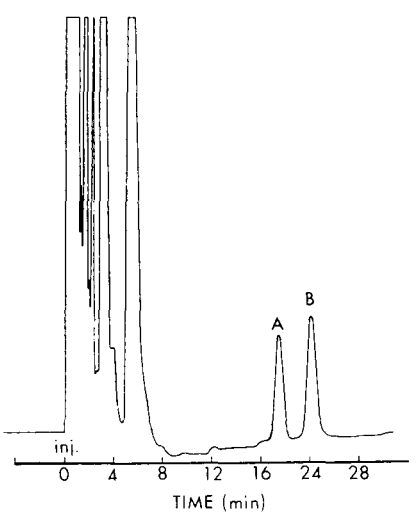


Fig. 3. Typical chromatogram from a plasma sample containing 2.0 μg internal standard (THCBD) and calculated to contain 1.5 μg cannabidiol (CBD). Peaks: A, THCBD derivative; B, CBD derivative.

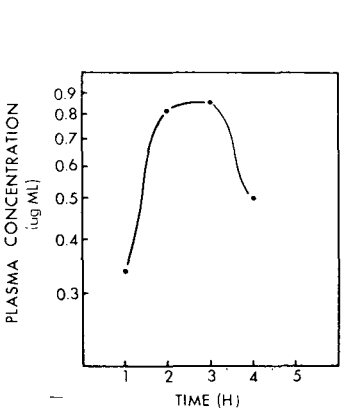
From the data utilized to generate Fig. 1, it is estimated that the absolute detection limit of the chromatographic system is approximately 80 pg. From the data utilized to generate Fig. 2, the detection limit of the total assay procedure as stated above is approximately 50 ng CBD per ml plasma.

Three monkeys were administered CBD either orally or intravenously and blood samples were withdrawn at selected times after administration. Fig. 4 illustrates the plasma concentrations of CBD detected after oral administration of 9.0 ml of a sesame oil solution of CBD (100 mg/ml) to a 7.9 kg male rhesus monkey (dose = 114 mg/kg). As is depicted in the figure, extremely low levels of CBD were detected over the time course of the experiment. The

two remaining animals were administered CBD intravenously in ethanol solution (10 mg/ml) at a 1.4 mg/kg dose. Fig. 5 illustrates the plasma concentrations of CBD detected in each of these experiments.

The actual fate of CBD in the body is not demonstrated in these preliminary animal experiments. However, it can be said that the free CBD rapidly disappears from the plasma with an apparent half-life in the order of 10 min. This is probably a distribution half-life rather than an elimination half-life for recently Siemens et al. [19] have shown the elimination half-life of CBD to be of the order of 11 h in rats. Their data indicated an initial rapid disappearance of CBD from the plasma and a multicompartmental system was necessary to describe the pharmacokinetics.

The results of these experiments indicate that the procedures presented can be successfully utilized for the quantitative detection of CBD in plasma samples by the formation of a high molecular weight fluorinated derivative. The procedure is applicable for the routine analysis of single or multiple samples with a standard gas chromatograph equipped with an electron-capture detector. The inclusion of the internal standard in the sample prior to extraction is a necessity.



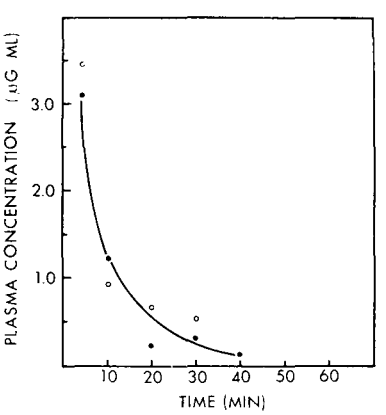


Fig. 4. Plasma concentration of free cannabidiol (CBD) following oral administration of the drug in sesame oil to a monkey. Dose = 114 mg/kg.

Fig. 5. Plasma concentration of free cannabidiol (CBD) following intravenous administration of the drug in ethanol to monkeys. Dose = 1.4 mg/kg; (○) experiment 1, (●) experiment 2.

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CHROMBIO. 1000

DETERMINATION OF ERGOT ALKALOIDS IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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SUMMARY

Liquid chromatographic methods for the determination of ergotamine and methylergometrine in plasma have been developed. The samples are extracted with an organic solvent at pH 9.0, cleaned by extractions and finally injected on an ODS-Hypersil reversed-phase column with acetonitrile—ammonium carbonate buffer as the mobile phase. The polarity of solvents used for extraction and the mobile phase are varied with the compounds of interest. Ergocristine is used as internal standard for ergotamine, and methysergide for the determination of methylergometrine. The stability of samples and standard solutions for calibration are discussed. Conditions for high selectivity and sensitivity of detection are given. Concentrations down to 100 pg/ml of plasma can be detected with a 3-ml sample.

INTRODUCTION

Ergotamine is still the mainstay in the treatment of migraine, and methylergometrine is used in obstetrics as a uterus-contracting agent. The therapeutic doses of these drugs are rather low (0.2—2 mg) and plasma concentrations after a single dose are in the range of ng/ml to pg/ml.

Determination of the total radioactivity after administration of tritium-labeled ergot alkaloids has been used in pharmacokinetic investigations [1, 2]. Several radioimmunoassay (RIA) methods for the determination of ergot alkaloids [3—6] and lysergic acid diethylamide (LSD) [7, 8] have been described. RIA methods using lysergic acid antisera for determination of ergopeptides are not selective or sensitive enough to be used in pharmacokinetic investigations. More selective and sensitive methods have been developed by Shran et al. [6] who used antisera elicited against conjugates of human serum albumin and ergopeptide linked via the indole nitrogen of the alkaloid.

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Fluorimetric [9] and thin-layer chromatographic methods [10] are not sensitive enough for analysis of ergot alkaloids in plasma samples. However, sensitive and selective methods for the determination of LSD by liquid chromatography with fluorescence detection have been used in forensic work [8, 11]. Several liquid chromatographic systems have been developed for separating ergot alkaloids [12—19] but no applications for plasma have been published to our knowledge. This report describes methods suitable for the determination of ergotamine and methylergotamine in plasma. The described methods can be used for other derivatives of lysergic acid after minor modifications.

EXPERIMENTAL

Ergotamine tartrate was obtained from WHO Collaborating Centre for Chemical Reference Substances (Solna, Sweden). Ergotaminine, ergocristine, methylergometrine hydrogen maleate, methysergide hydrogen maleate and dihydroergotamine methane sulphonate were kindly supplied by Sandoz (Basle, Switzerland). Ethyl acetate, cyclohexane, butanol and acetonitrile were of LiChrosolv® quality (Merck, Darmstadt, G.F.R.). Test tubes (50 ml) with glass stoppers were used for extraction and 10-ml conical tubes for evaporation of organic extracts. All test tubes were silanized with 2% Drifilm (Pierce, Rockford, IL, U.S.A.) in cyclohexane followed by three washes of methanol.

Preparation of calibration solutions

A stock solution of ergotamine tartrate and ergotaminine was prepared so that 40% of the total alkaloid content consisted of ergotaminine. Ethanol (96%) purged with nitrogen was used as solvent. The stock solution was further diluted with deoxygenated ethanol together with ergocristine to obtain seven solutions in the range of 0—75 ng/ml ergotamine and 100 ng/ml ergocristine.

Methylergometrine solutions with methysergide as internal standard were prepared in a similar manner; 0.1 ml of these solutions were used for calibration. The solutions were found to be stable for 60 days at −50°C.

Instruments

A liquid chromatographic pump (Waters M-6000 A) with a U6K injector was connected to a 250 × 4.6 mm I.D. stainless steel column packed with ODS-Hypersil, 5 μm particles (Chrompack, Middelburg, The Netherlands). Columns were packed with a slurry of particles in dichloromethane followed by methanol. The mobile phase consisted of acetonitrile—0.01 *M* ammonium carbonate in glass-distilled water. Thirty per cent acetonitrile was used for the separation of methylergometrine and methysergide; 50% acetonitrile was used to separate ergotamine, ergocristine and ergotaminine.

The outlet of the column was connected to a Schoeffel FS 970 fluorescence detector for liquid chromatography. The monochromator at the excitation side was set at 328 nm, combined with a band-pass filter of 320 nm to exclude higher orders of diffraction. A 389-nm cut-off filter was used at the emission side, and the time constant was set to 4 sec.

Extraction procedure

Plasma or serum (3 ml), 0.1 ml of internal standard solution and 3 ml of 1 *M* ammonia—hydrochloric acid buffer (pH 9.0) are extracted by 20 ml of organic solvent during 15 min, followed by centrifugation. The organic phase is transferred by pasteur pipette to a new tube. The organic phase is extracted with 2 ml of 0.05 *M* sulphuric acid during 10 min. The organic phase is aspirated off, and 2 ml of ammonia—hydrochloric acid buffer (pH 9.2) are added to the aqueous phase to yield pH 9.0. The aqueous phase is extracted by a fresh 10-ml portion of organic phase, transferred to a 10-ml tube and evaporated with a stream of nitrogen at 50°C. Then 0.2 ml of mobile phase from the liquid chromatograph is added, followed by mixing and agitation in an ultrasonic bath for 5 min. An aliquot of 50—150 μl is injected into the liquid chromatograph. The organic solvent used for extraction consisted of cyclohexane—1-butanol (9:1) for the extraction of ergotamine, and ethyl acetate for the extraction of methylergometrine. The concentrations of ergotamine, ergotaminine and methylergometrine are evaluated by using a calibration graph established from seven standard samples. The standard samples were prepared from standard solutions described above and 3 ml of blank Seronorm® (Nyegaard & Co., Oslo, Norway).

RESULTS AND DISCUSSION

Extraction

Ergotamine is an ampholytic compound with $pK_{a_1} = 6.25$ [20] and $pK_{a_2} \approx 11$. Kleimola [4] determined distribution ratios for ergotamine with different organic solvents and used benzene with 5% isoamyl alcohol for extraction in an RIA method for ergot alkaloids. Hooper et al. [9] also used benzene for extraction of biological samples in a fluorimetric assay of ergotamine. In general it is desirable to use a solvent with as low a polarity as possible to reduce co-extraction of the matrix components, but which still gives quantitative extraction. We preferred to use cyclohexane-1-butanol (9:1) prior to benzene due to the toxic properties of benzene. Cyclohexane—butanol gave pure extracts with a high absolute recovery. A more polar solvent was needed for extraction of methylergometrine. Ethyl acetate was found to give clean extracts and high absolute recovery (Table I). Re-extraction to acidic aqueous phase was used to increase the selectivity of the extraction and to concentrate the sample.

Chromatography

Separation of ergot alkaloids in fermentation media has been performed with LiChrosorb NH_2 [15]. Szepesy et al. [16] used silica columns for group separation and contaminants. Stereo- and structural isomers were separated on reversed-phase packings. Reversed-phase columns have been widely used for the separation of mixtures and isomers of ergot alkaloids [17, 19], and gradient systems are used for the determination of degradation products [13, 14].

Two modes of operation are possible for the chromatographic separation of bases on reversed-phase columns. The components can be chromatographed as free bases at alkaline pH or as cations in an ion-pair complex with a suitable counter-ion. The ion-pair mode of operation is advantageous with respect to

TABLE I

ABSOLUTE RECOVERY AND PRECISION

	n	Amount (ng) added to 3 ml of Seronorm	Absolute recovery (%)	Relative peak height versus internal standard (C.V. %)	Detection limit in 3-ml sample (pg/ml)
Methylergometrine	6	1.50	53 ± 4	10	100
Methysergide	6	10	50 ± 2		
Ergotamine	6	7.89	79 ± 7	2.5	100
	6	1.58	92 ± 6	3.2	
Ergotaminine	6	5.27	91 ± 7	6.6	200
	6	1.07	99 ± 6	7.7	
Ergocristine	12	10	84 ± 8		

column stability since silica is susceptible to hydrolysis at alkaline pH. However, a much better selectivity for the separation of epimers of the ergot alkaloids is obtained when the free bases are chromatographed (Table II). Large differences in capacity factors are obtained for lysergic acid—isolysergic acid epimers. Wurst et al. [15] suggested that this depends on a difference in the possibility of intramolecular hydrogen bonding. Epimers with an intramolecular hydrogen bond are much more lipophilic than epimers which lack such a bond. ODS-Hypersil columns with acetonitrile—aqueous ammonium carbonate mixtures as mobile phase provided very efficient and selective separation systems suitable for determination of ergot alkaloids in plasma.

We have used the same column for several months and hundreds of analyses without any sign of deterioration despite the high pH (9.0) used.

TABLE II

CAPACITY FACTORS FOR DIFFERENT MOBILE PHASES

	Mobile phase 1*	Mobile phase 2**
Ergotamine	6.4	2.3
Ergotaminine	7.7	8.5
Ergocristine	12.6	4.9

*Mobile phase 1 = 400 ml of acetonitrile, 1.9 g of sodium heptanesulphonate (0.05 *M*), 10 ml of glacial acetic acid and glass-distilled water to make one litre.

**Mobile phase 2 = 500 ml of acetonitrile diluted with 0.01 *M* ammonium carbonate to make one litre.

Detection

The combination of solvent extractions, high separation efficiency of the chromatographic system, intense fluorescence of the lysergic acid derivatives and selectivity of detection, makes it possible to measure concentrations down to 100 pg/ml with a 3-ml plasma sample. The detection limit in plasma samples

is restricted by the volume of sample available and the noise of the fluorimetric detector. Due to the efficiency of the chromatographic separation it is possible to optimize the conditions of fluorimetric detection for maximum sensitivity rather than selectivity. High sensitivity is gained by maximum ratio of output of photons to the photomultiplier, versus background fluorescence. Detectors equipped with filters give higher output of light than detectors with monochromators but the choice of wavelength is more restricted. The instrument used in this work was equipped with a diffraction-grating monochromator for excitation. A cut-off filter was used on the emission side to exclude scattered light and to minimize loss of light emitted. The intense fluorescence of the ergot alkaloids is diminished if the 9:10 double bond is hydrogenated. With the chromatographic system used for ergotamine the detection limit for dihydroergotamine was determined to be 1 ng at excitation wavelength 280 nm, without any filter on the emission side.

Stability of samples and standards

Ergotamine undergoes spontaneous epimerization at C-8 to ergotaminine, epimerization at C-2 in acidic solutions to form "aci-compounds". Addition of water to the 9:10 double bond can occur, catalyzed by UV light. Furthermore, the indole is susceptible to oxidation by oxygen. The stability of ergotamine tartrate in aqueous solution has been studied by Krielgård and Kisbye [21]. Heat treatment at pH 3.6 was used to obtain epimerization equilibrium at C-8 in solutions for injection. Hartman et al. [22] studied the stability of liquid hydrated ergot alkaloid preparations as a function of solvent polarity. Stable solutions were obtained with water—alcohol mixtures with a dielectric constant between 30 and 45. The information about the stability of solutions used for injections can not be extrapolated to the concentration range of standard solutions used for calibration. The rate of oxidation might be dependent on concentration, and solutions used for calibration differs by four to five orders of magnitude from solutions used for injection. The epimerization reactions will be without importance if the solutions are stored at a low enough temperature (−50°C), but oxidation might occur due to the low energy of activation for oxidation [22].

Another possibility to obtain solutions that are stable with respect to epimer composition is to prepare solutions with equilibrium concentrations of epimers.

We have used both approaches for the preparation of ergotamine/ergotaminine standard solutions. The ergotaminine concentration should be 40% of the total [21] to obtain equilibrium. Ergocristinine was not added since it is not readily available, and it would prolong the chromatography time due to its high elution volume.

Standard solutions prepared with nitrogen-purged ethanol can be stored at −50°C for at least 60 days without degradation. Serum standards were prepared from Seronorm and aqueous solutions of ergotamine and ergotaminine and stored at −50°C. The stability was followed by repeated analyses during the time of storage. It was found that the samples could be stored for at least 60 days without appreciable degradation (Table III). Prolonged storage of samples is not recommended until a thorough evaluation of long-term stability has

TABLE III

STABILITY OF SPIKED SERUM SAMPLES

Time of storage at −50°C (days)	*n*	Concentration found (ng/ml)		Calculated concentration (ng/ml)	
		Ergotamine	Ergotaminine	Ergotamine	Ergotaminine
0	1	38.8	24.4	39.6	26.2
24	1	31.2	20.7	39.6	26.2
44	1	31.4	20.5	39.6	26.2
129	1	38.8	27.5	39.6	26.2
144	1	40.5	28.2	39.6	26.2

been performed. A standard solution containing only ergotamine was extracted according to the "extraction procedure" to check if any ergotaminine was formed during work-up of the samples. No measurable concentration of ergotaminine was found. No ergocristinine was observed in chromatograms after work-up of samples if it was not present from the beginning.

The above-described method has been used in our laboratory for one year for the analysis of ergotamine, ergotaminine and methylergometrine in plasma with excellent results. Absolute recoveries and coefficients of variation are presented in Table I.

Plasma concentration profiles of ergot alkaloids after intravenous administra-

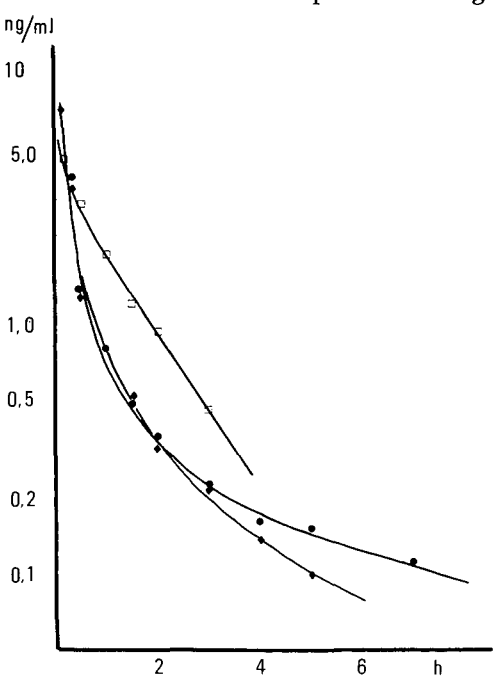

Fig. 1. Plasma concentration profiles from a patient after a 250-μg dose of ergotamine tartrate (containing 133 μg ergotamine base and 89 μg ergotaminine base); ergotamine (•), ergotaminine (♦). Plasma concentration profile from a patient after a 200-μg intravenous dose of methylergometrine maleate (□).

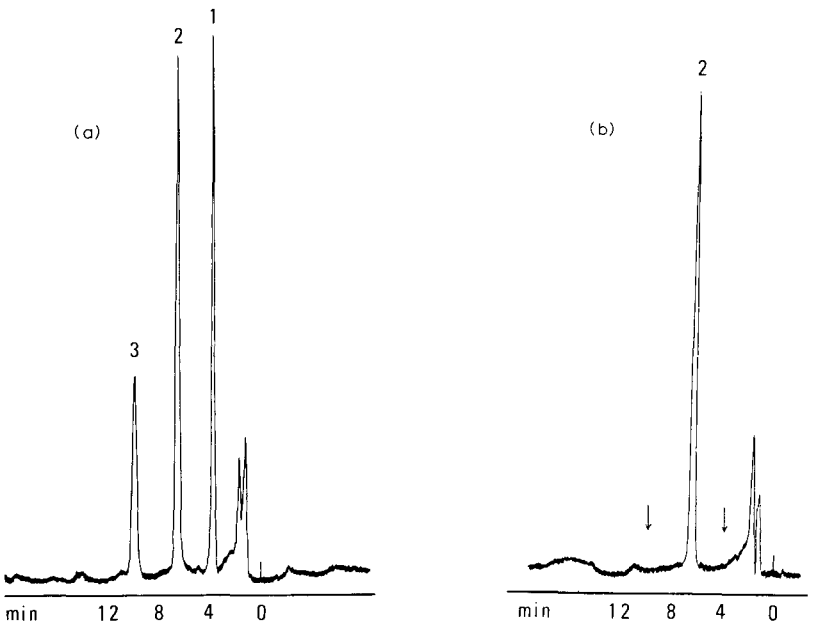

Fig. 2. (a) Chromatogram from a 3-ml plasma sample containing 3.2 ng of ergotamine (1), 2.1 ng of ergotaminine (3) and 10 ng of ergocristine (2). (b) The pertinent blank plasma containing 10 ng of ergocristine (2). Injection volume: 90 μl of a total 200 μl. Mobile phase 2; flow-rate = 1.5 ml/min.

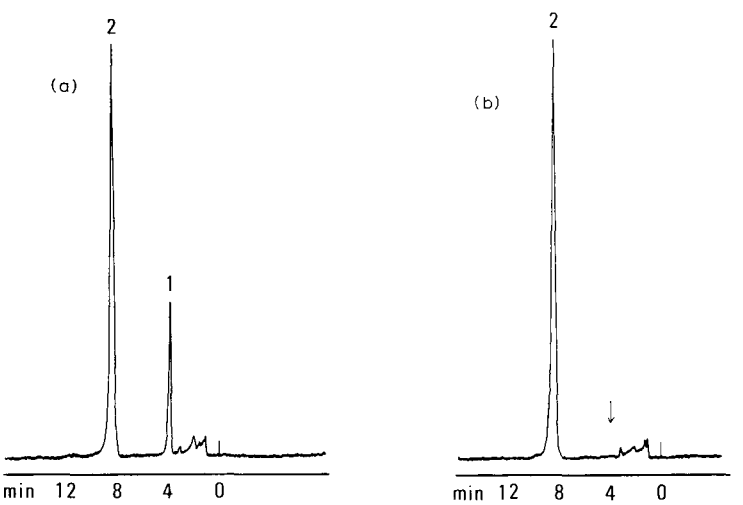

Fig. 3. (a) Chromatogram from a 3-ml plasma sample containing 2.3 ng of methylergometrine (1) and 9.0 ng of methysergide (2). (b) The pertinent blank plasma containing 9 ng of methysergide (2). Injection volume: 90 μl of a total 200 μl. Mobile phase was 300 ml of acetonitrile diluted with 0.01 *M* ammonium carbonate to one litre; flow-rate = 1.5 ml/min.

tion are shown in Fig. 1. Chromatograms obtained with 3-ml plasma samples are presented in Figs. 2 and 3 and with a 3-ml Seronorm blank in Fig. 4.

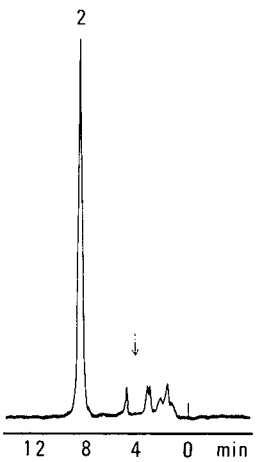

Fig. 4. Chromatogram obtained with a 3-ml Seronorm blank containing 9 ng of methysergide. Conditions as in Fig. 3.

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ANALYSIS OF BARBITURATES IN BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic (HPLC) assay for the identification and quantification of barbiturates in blood at therapeutic levels has been developed. An ODS-silica column is used with an eluent of 40% methanol at pH 8.5. The barbiturates are detected at 240 nm. The sample preparation procedure involves extraction of unfractionated blood (100 μl) with hexane—diethyl ether (50:50, v/v) and is very rapid. Talbutal is used as an internal standard.

The method has been applied to the determination of five barbiturates (amylobarbitone, butobarbitone, cyclobarbitone, pentobarbitone and quinalbarbitone) in blood after therapeutic doses of the drugs. An application of the HPLC assay to forensic casework is demonstrated.

INTRODUCTION

Our recent studies [1] have concluded that chromatographic systems using lipophilic stationary phases are the most effective for the separation of barbiturates, e.g. gas—liquid chromatography (GLC) using SE-30, high-performance liquid chromatography (HPLC) using ODS-silica. GLC is presently the most widely used method for the analysis of these drugs in biological fluids. Nevertheless, HPLC using ODS-silica can achieve several separations which are difficult by GLC [1] and could prove valuable for the identification of barbiturates in biological matrices. Furthermore, the GLC methods often involve lengthy extraction or derivatization steps while HPLC has the potential of requiring less complex sample preparation procedures which could save time in forensic and clinical analysis.

Some HPLC procedures for the analysis of barbiturates in biological fluids

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have been published [2—16]. These often deal with specific compounds and few procedures are suitable for the identification and quantification of a wide range of barbiturates. Furthermore the published procedures for blood analysis generally involve initial fractionation to either plasma or serum. This may be appropriate in clinical and pharmacokinetic laboratories where blood may be stored under ideal conditions from the time of collection but is of little value for haemolysed samples often encountered in forensic casework. A recent paper by Sprague and Poklis [17] gives a procedure for the analysis of barbiturates in post mortem blood by HPLC and, although whole blood is used, the method has limited sensitivity, particularly with decomposed specimens.

The analysis of barbiturates in biological fluids by HPLC requires a detection system capable of observing the compounds at low levels without interference from endogenous compounds. Derivatives of barbiturates suitable for enhancing both sensitivity and selectivity of detection in HPLC have been reported [18—20], however, these advantages are offset by the increase in sample preparation time. The purpose of the present work was to provide a rapid, simple, sensitive and high-resolution HPLC procedure without derivatization suitable for the identification and quantification of barbiturates in small volumes of blood including haemolysed samples.

EXPERIMENTAL

Materials

The extraction solvent was prepared from hexane (HPLC grade; Fisons, Loughborough, Great Britain) and diethyl ether (reagent grade, BDH, Poole, Great Britain), in the ratio 50:50, v/v. The ether was freshly distilled before use. All other chemicals used were of analytical grade.

Chromatography

The liquid chromatograph consisted of a pump (Waters M6000), an injection valve (Rheodyne 7120) fitted with a 1-ml loop and a variable-wavelength UV detector (Pye-Unicam LC-UV or Perkin-Elmer LC-75) operated at 240 nm. The column (10 cm × 5 mm I.D. stainless steel) was packed with 5 μm ODS-Hypersil (Shandon Southern Products, Runcorn, Great Britain) using isopropanol as the slurry medium and hexane as the pressurising solvent. The eluent consisted of aqueous sodium dihydrogen phosphate (0.1 *M*)—methanol (60:40, v/v) adjusted to pH 8.5 with concentrated sodium hydroxide solution and was degassed under vacuum before use. A flow-rate of 2 ml/min was used with an operating pressure of 1700 p.s.i. The column was washed with methanol—water (50:50, v/v) at the end of each working day.

Extraction procedure

Blood (100 μl) was transferred to a pyrex graduated test tube (10 ml, Exelo Permagold, Scientific Supplies, London, Great Britain) fitted with a polythene stopper (E-MIL, Scientific Supplies) using a safety pipetter (P-7000 Sampler, Oxford Laboratories, Athy, Ireland) and the internal standard solution (talbutal in ethanol 20 μl, 25.16 μg/ml) added with a syringe (SGE, London, Great Britain). The volumes of the blood and internal standard solution were mea-

sured accurately, whilst all further additions and transfers were made using the graduations on the test tubes. Phosphate buffer (0.1 *M*, pH 7.5) was then added to give a total volume of 1 ml. The extraction solvent (5 ml) was then added and the phases mixed by repeated inversion of the tube for approximately 1 min. A portion of the organic layer (4 ml) was decanted into a second tube and evaporated to dryness by a stream of nitrogen. The residue was dissolved in the HPLC eluent (500 μl) and 200 μl injected on to the column. The detector was operated at a range of 0.01—0.02 A full-scale.

Calibration solutions were prepared by dissolving the appropriate barbiturate and the internal standard (talbutal, 5-allyl-5-*sec.*-butylbarbituric acid) in aqueous methanol (40%, v/v) at known concentrations. The injection of each blood extract was followed by an injection of the appropriate calibration solution (200 μl). The concentration of barbiturate in the blood was calculated using peak height ratio measurements.

Oral ingestion of barbiturates

Experiments were carried out with laboratory staff with the permission of the Medical Ethics Committee, Chemical Defence Establishment, Porton Down. Five volunteers each took an oral dose of one barbiturate (see Table III) in the morning (approximately 09.00 h). Venous blood was collected before and about 2 h and about 8 h after ingestion of the drug. The blood was stored in plastic screw-top vials (2.5 ml) containing EDTA (Sterilin, Teddington, Great Britain) at 4°C.

RESULTS AND DISCUSSION

The HPLC eluent used in this study (40% methanol, pH 8.5) was chosen as that capable of separating the most commonly abused barbiturates: amylobarbitone, butobarbitone, cyclobarbitone, pentobarbitone and quinalbarbitone [1]. Analytical procedures for these five barbiturates have been examined in detail; however, a wide range of barbiturates can be analysed using this eluent. Capacity factors (k') for 30 barbiturates are given in Table I. Several separations can be achieved with the present eluent which are not possible under acidic or neutral conditions [1].

Most of the published procedures for the analysis of barbiturates in biological fluids by reversed-phase HPLC have used acidic (or neutral) eluents with detection at short wavelengths (< 220 nm) where the detection of barbiturates is not very specific. The drugs show no specific UV absorption under acidic conditions while at pH 10 they show an absorption maximum at 240 nm. Clark and Chan [21] have suggested the post-column mixing of the HPLC eluent with pH 10 buffer to enhance the detectability of barbiturates. Solutions of barbiturates in the present HPLC eluent do show an absorption maximum at 240 nm and experiments revealed that a change from pH 8.5 to 10 gave only a small increase (< 25%) in absorption. This increase would be further reduced by the dilution resulting from the post-column addition of buffer. Furthermore, this procedure would involve an increase in complexity of the HPLC equipment and consequently the present assay was developed using direct detection at 240 nm.

TABLE I

HPLC RETENTION DATA FOR 30 BARBITURATES ON ODS-SILICA

Eluent: 40% methanol at pH 8.5.

Barbiturate	Capacity factor (k')	Barbiturate	Capacity factor (k')
Barbitone	0.63	Methylphenobarbitone	3.84
Phenylmethylbarbituric acid	0.94	Talbutal	4.67
Phenobarbitone	1.23	Idobutal	4.77
Allobarbitone	1.33	Heptabarbitone	4.93
Probarbitone	1.57	Hexobarbitone	5.67
Brallobarbitone	1.72	Nealbarbitone	6.19
Metharbitone	1.99	Thialbarbitone	6.78
Aprobarbitone	2.22	Enallylpropymal	6.96
Vinbarbitone	2.32	Amylobarbitone	7.05
Ibomal	2.58	Pentobarbitone	8.07
Cyclobarbitone	2.61	Thiopentone	9.20
Secbutobarbitone	3.32	Quinalbarbitone	11.47
Butobarbitone	3.42	Sigmodal	12.37
Butalbital	3.48	Hexethal	20.39
Cyclopentobarbitone	3.84	Methohexitone	20.48

The assay was designed to employ the smallest volume of blood (100 μl) consistent with the need to detect barbiturates at therapeutic levels. Initial experiments were conducted using drug-free blood spiked with the appropriate barbiturates. Talbutal was chosen as an internal standard as it could be separated from the five commonly abused barbiturates (see Fig. 1). It can be seen that all these barbiturates can be analysed in less than 9 min. The use of an internal standard means that only the blood volume (100 μl) and the volume of internal standard solution (20 μl) need to be measured accurately so that the use of graduated test tubes for all further volume measurements makes the extraction procedure very rapid (see Experimental).

Barbiturates have pK_a values in the range 7.6—8.8 and their unionized forms are lipid soluble. They can be extracted with hexane—diethyl ether (50:50,

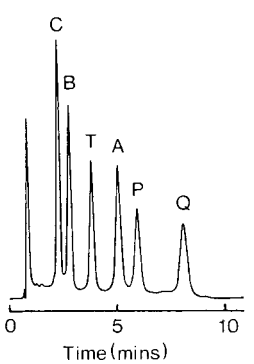

Fig. 1. HPLC of extract of spiked blood on ODS-silica. Peaks: C = cyclobarbitone; B = butobarbitone; T = talbutal; A = amylobarbitone; P = pentobarbitone; Q = quinalbarbitone (each about 5 μg/ml).

TABLE II

RECOVERY OF BARBITURATES ADDED TO BLOOD

Barbiturate	Blood concentration (μg/ml)	Recovery (%)
Cyclobarbitone	12.9	96.2
Butobarbitone	7.5	99.2
Talbutal	5.0	99.1
Amylobarbitone	8.4	99.2
Pentobarbitone	10.1	94.9
Quinalbarbitone	8.2	94.2

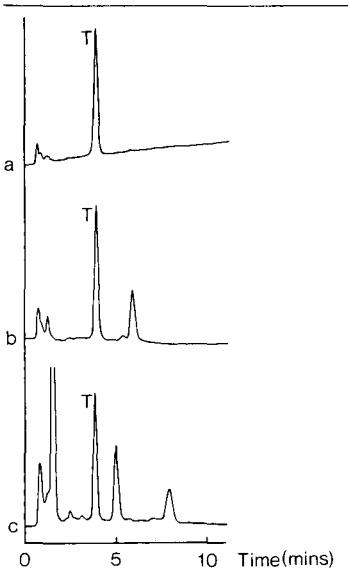

Fig. 2. Analysis of blood by HPLC: (a) drug-free blood spiked with internal standard (talbutal, T, 5 μg/ml); (b) sample from subject about 2 h after an oral dose of pentobarbitone (182 mg), drug peak at about 6 min; (c) sample from car driver suspected of taking barbiturates; peaks at about 5 and 8 min correspond to amylobarbitone and quinalbarbitone, respectively.

v/v) at pH 7.5 with recoveries of > 94% (Table II). Extraction at pH 7.5 gives little interference from endogenous materials over most of the chromatogram which allows the procedure to be applied to a wide range of barbiturates. Fig. 2a shows a typical chromatogram from a blank blood with an internal standard added at a level of 5 μg/ml. The amount of interfering material extracted with the barbiturates was increased by lowering the extraction pH or by raising the polarity of the extracting solvent. Low backgrounds were also obtained with blood from storage vials used for road traffic offences which contain preservatives and even with haemolysed samples. The detection limit for a barbiturate above the background of a blank blood was typically less than 1 μg/ml.

Quantification was performed using peak height ratio measurements with reductions of detector sensitivity for large peaks. The change in peak height ratio of quinalbarbitone to talbutal (internal standard, 5 μg/ml in blood) was

shown to be linear up to a blood concentration of 200 μg/ml. The high precision of the method was demonstrated by repeating the analysis of two blood samples containing quinalbarbitone. The samples gave mean values of 4.52 and 47.4 μg/ml with coefficients of variation of 2.9% (n = 6) and 1.5% (n = 6), respectively.

Most of the published procedures for the analysis of barbiturates involve adjustment of the pH of the body fluid followed by extraction with an organic solvent which is usually evaporated to dryness and the residue dissolved in the HPLC eluent for injection. However, in some assays complex back-extraction procedures have been employed to isolate further the barbiturates from interfering material (e.g. ref. 15). An alternative sample preparation procedure involves protein precipitation with acetonitrile or ethanol and then injection of the supernatant (e.g. refs. 4, 10, 13, 17). A further method involves the adsorption of the barbiturate from serum on to charcoal [3]. The most rapid procedures involve either a single-step solvent extraction or protein precipitation. Our initial experiments showed that protein precipitation with methanol did not remove enough endogenous material from whole blood to allow barbiturates to be detected at therapeutic levels and consequently the present assay was developed using a simple solvent extraction procedure.

Blood samples containing barbiturates after the oral ingestion of therapeutic doses of the drugs were also examined. The blank samples taken before ingestion of the drugs showed no interfering peaks on the chromatograms. Fig. 2b shows the chromatogram for a blood sample from one subject about 2 h after taking pentobarbitone. The results of all assays are given in Table III and plotted in Fig. 3. It can be seen that the levels of amylobarbitone, butobarbitone, pentobarbitone and quinalbarbitone have started to decrease by the end of the 8-h period while the level of cyclobarbitone has not reached a maximum, reflecting slow absorption of the drug by the subject.

The HPLC assay has also been applied to a forensic case where barbiturate abuse was involved in a driving accident. Fig. 2c shows the chromatogram obtained for the analysis of the blood sample by the present HPLC procedure. The two unknown peaks were found to correspond to amylobarbitone and

TABLE III

BARBITURATE BLOOD LEVELS FOR FIVE VOLUNTEERS AFTER ORAL DOSES OF THE DRUGS

Approx. time (h) after oral dose	Barbiturate blood level (μg/ml)				
	Subject 1, cyclobarbitone 185 mg*	Subject 2, butobarbitone 200 mg	Subject 3, amylobarbitone 182 mg**	Subject 4, pentobarbitone 182 mg**	Subject 5, quinalbarbitone 183 mg**
2	0.86	3.32	2.29	3.15	1.99
8	2.95	2.66	1.75	2.26	1.57

*Given as the calcium salt.
**Given as the sodium salt.

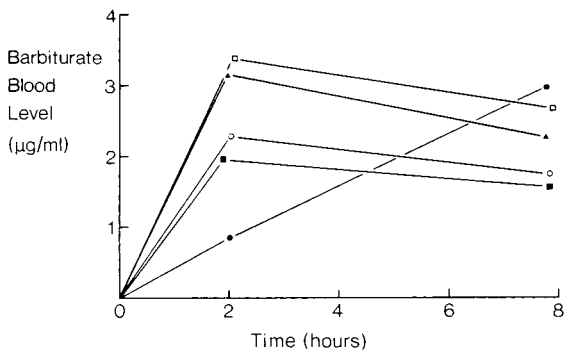

Fig. 3. Changes in barbiturate blood levels determined by HPLC over an 8-h period for five subjects each taking an oral dose of one barbiturate (see Table III). (•) Cyclobarbitone; (□) butobarbitone; (▲) pentobarbitone; (○) amylobarbitone; (■) quinalbarbitone.

quinalbarbitone (5.8 and 3.9 μg/ml, respectively) by co-injection of authentic samples.

In conclusion, the present HPLC assay provides a rapid method for the identification and/or quantification of therapeutic levels of barbiturates in blood which should prove useful in forensic and clinical analysis.

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SENSITIVE AND RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF METHOTREXATE AND ITS METABOLITES IN PLASMA, SALIVA AND URINE

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SUMMARY

A simple and sensitive high-performance liquid chromatographic assay of methotrexate (MTX) and its two active metabolites, 7-hydroxymethotrexate (7-OH-MTX) and 2,4-diamino-N^{10}-methylpteroic acid (APA) in plasma, saliva and urine was developed. The method involved deproteinization with acetonitrile followed by addition of isoamyl alcohol and ethyl acetate. After extraction the sample was chromatographed on a cation-exchange column and monitored at 313 nm. The retention times were 5, 7 and 9 min and detection limits 20, 10 and 5 ng/ml for 7-OH-MTX, MTX and APA, respectively. For concentrations greater than 100 ng/ml one-step deproteinization of 0.1 ml sample with 0.25 ml acetonitrile was satisfactory for sample preparation. The method has been evaluated in samples from patients and rabbits receiving MTX.

INTRODUCTION

Methotrexate (MTX; 4-amino-N^{10}-methylpteroylglutamic acid), a potent antifolate, has been widely used for the treatment of various malignant diseases as well as non-neoplastic disorders [1]. With the advent of high-dose therapy followed by leucovorin rescue, plasma monitoring of MTX levels has been regarded as being mandatory to allow early detection of patients at high risk of toxicity [1—4].

Many assay methods have been developed. They include, for example, fluorometry [5], competitive protein binding [6, 7], enzyme inhibition assay [8], radioimmunoassay [9], radioassay [10], enzyme immunoassay [11] and high-performance liquid chromatography (HPLC) [12—21]. It has been reported that many non-HPLC methods lack specificity due to the potential interference of active metabolites, such as 7-hydroxymethotrexate

(7-OH-MTX) and 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA). Furthermore, the non-HPLC methods are not capable of quantitating these two metabolites [11, 14, 20]. A major drawback of many previous pharmacokinetic studies is the use of these non-specific assay methods for the determination of MTX concentrations in biological fluids. A need for the re-evaluation of the past pharmacokinetic studies has been recently advocated [20].

In reviewing published HPLC methods, it appears that they have one or more of the following limitations or drawbacks. For example, 1 ml [12, 14, 16—18] to 3 ml [15, 21] of plasma or serum samples are needed. The sample preparations involving extraction, evaporation and reconstitution are relatively complex and may take more than 20 min [14, 16, 17]. Applicability for the determination of 7-OH-MTX [12] and APA [14—16, 19] in plasma was not studied. Retention times up to 20 or 30 min together with relatively low sensitivity for APA were reported [17, 18, 21]. In addition, only a few HPLC methods reported their application for urine analysis [13, 18, 21]. Furthermore, none of the published methods have shown their feasibility for saliva analysis. The purpose of this paper is to describe a simple, sensitive and micro HPLC assay for the simultaneous determination of MTX, 7-OH-MTX and APA in plasma, saliva and urine.

EXPERIMENTAL

Reagents and standards

All reagents were of analytical grade. MTX and APA were kindly supplied by Dr. Ven L. Narayanan from the National Institute of Health (Bethesda, MD, U.S.A.), and Dr. Maharaj K. Raina from the Lederle Labs. (Pearl River, NY, U.S.A.). The purified 7-OH-MTX was generously supplied by Dr. David Johns from the National Cancer Institute, and Dr. Kenneth K. Chan from the University of Southern California (Los Angeles, CA, U.S.A.). Additional samples used for routine standard curve study were isolated from rabbit liver homogenates on a DEAE-cellulose column according to the procedure of Watson et al. [14]. Ammonium phosphate, phosphoric acid and glass-distilled acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Isoamyl alcohol was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Ethyl acetate was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Most drugs tested for potential interferences of the assay were donated by the Hospital Pharmacy, University of Illinois Medical Center (Chicago, IL, U.S.A.).

Standard solutions (1 μg/ml to 10 mg/ml) of MTX and APA were prepared in distilled water. 7-OH-MTX purified from DEAE-cellulose column and dissolved in 10 m*M* Tris—HCl buffer (pH 7.5) was used for spiking directly. Its concentration was determined by comparing the HPLC peak height with those from authentic samples. All standard solutions were stored at 4°C in a refrigerator.

Sample preparations

Plasma, serum or saliva (0.2 ml) from normal subjects or patients was pipetted into 13 × 100 mm screw-capped culture tubes. The deproteinization was

carried out by adding 0.5 ml of acetonitrile, followed by vortexing for 10 sec and centrifugation at 800 *g* for 2 min. The entire supernatant was poured into a 5-ml glass tube which had a tapered base. After addition of 100 μl of isoamyl alcohol and 1 ml of ethyl acetate, the tube was vortexed for 10 sec and then centrifuged at 800 *g* for 4 min. The lower aqueous portion, 10—30 μl, was directly collected into the syringe and injected onto the column.

Urine samples were prepared by the same deproteinization procedure as described above. Since concentrations in urine are usually much higher, the deproteinized supernatant (20—50 μl) was injected directly onto the column. Peak height measurements with the assistance of a micrometer (Vernier Caliper from Fisher Scientific, Chicago, IL, U.S.A.) were used for quantitation [22]. Standard curves were constructed by supplementing blank human plasma, saliva and urine with known concentrations of MTX, 7-OH-MTX and APA.

HPLC instrumentation

The liquid chromatographic system consisted of a solvent delivery pump (Model M6000A), a fixed-wavelength detector with 313-nm filter (Model 440) obtained from Waters Assoc. (Milford, MA, U.S.A.), a syringe loading sample injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) and an ion-exchange column (Partisil PXS 10/25 SCX, 25 cm × 4.6 mm I.D., particle size 10 μm, available from Whatman, Clifton, NJ, U.S.A.). The output from the detector was connected to a 10-mV potentiometric 25.4-cm recorder (Linear Instruments, Irvine, CA, U.S.A.).

The mobile phase was prepared by mixing 10 parts of acetonitrile with 90 parts of 0.02 *M* monobasic ammonium phosphate solution acidified with phosphoric acid (0.2%). This was pumped through the HPLC system at a flow-rate of 2 ml/min, and the resulting pressure was approximately 136 bar. The recorder chart speed was 10 cm/h [23]. All experiments were carried out at ambient temperature. The optimal mobile phase used may vary with the column.

Reproducibility study

Reproducibility studies were carried out at two concentrations for each of MTX, 7-OH-MTX and APA. Six replicate analyses of plasma samples spiked with stock solutions of the three compounds to give final concentrations of 0.1 and 10 μg/ml were carried out as described earlier.

Drug interference study

Many anticancer drugs and therapy-related compounds were tested to determine if they would interfere: 5-fluorouracil, 6-mercaptopurine, adriamycin, bleomycin sulfate, cisplatin, cyclophosphamide, vincristine, vinblastine, carmustine, folic acid, folinic acid (leucovorin), 5-methyltetrahydrofolic acid, acetazolamide, hydralazine and trimethoprim. Aliquots of stock solutions of each compound were injected directly onto the column and monitored at 313 nm.

RESULTS AND DISCUSSION

Chromatograms from blank human plasma, saliva, urine and those spiked with known concentrations of MTX, 7-OH-MTX and APA, together with plasma from a patient on MTX therapy are shown in Figs. 1 and 2. The peak shape from MTX, 7-OH-MTX or APA was all symmetrical with no interferences from endogenous substances. Although there was an endogenous peak between 7-OH-MTX and MTX, it did not affect the present assay. The retention times for MTX, 7-OH-MTX and APA were 7, 5 and 9 min, respectively.

The uniqueness of this method is that a considerably shorter retention of APA (less than 10 min) was accomplished by the use of the cation-exchange column. In all the previous assays, reversed-phase [12, 13, 15—21] or anion-exchange [13—15] columns were used. It appears that reversed-phase columns could not elute APA in a short period of time presumably due to the marked difference in polarity between MTX and APA. The apparent drawback of the anion-exchange column is that a higher pH (about 7.0) of the mobile phase [13, 14] is required, which tends to deteriorate the column more rapidly.

A higher sensitivity was obtained with salivary samples than plasma as shown in Fig. 2. This might be in part due to the lower content of electrolytes in saliva which had resulted in the reduction of the final aqueous volume after the extraction. Standard curves were linear over the concentration range

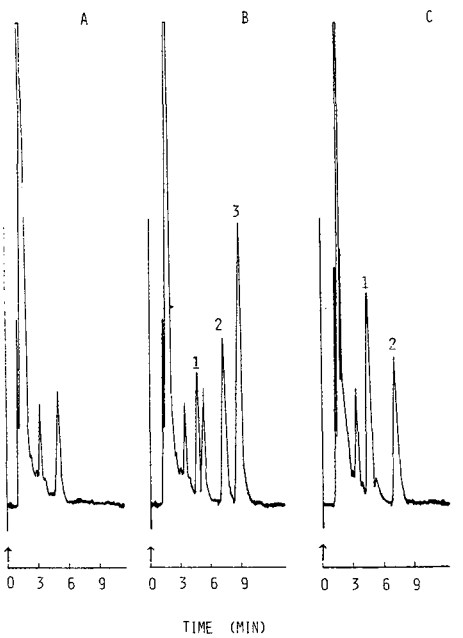

Fig. 1. Chromatograms of extracts from (A) blank human plasma; (B) plasma spiked with 0.5 μg/ml of MTX, 7-OH-MTX and APA; (C) patient plasma collected at 8 h after the end of intravenous infusion for 25 h on a dose of 750 mg/m² MTX. Peaks: 1 = 7-OH-MTX, 2 = MTX and 3 = APA. The arrow marks the point of injection. Detector sensitivity was 0.005 a.u.f.s. and recorder chart speed was 20 cm/h.

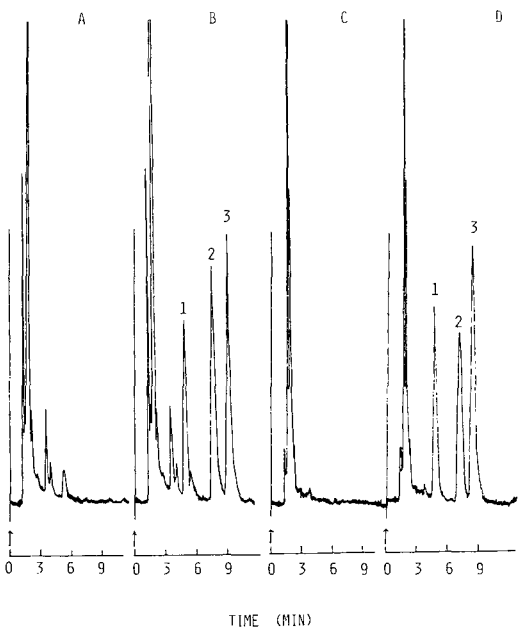

Fig. 2. Chromatograms from (A) blank human saliva extract; (B) extract of saliva spiked with 0.5 μg/ml of MTX, 7-OH-MTX and APA; (C) deproteinized blank human urine; (D) deproteinized human urine spiked with 5 μg/ml of MTX, 7-OH-MTX or APA. Peaks: 1 = 7-OH-MTX, 2 = MTX and 3 = APA. The arrow marks the point of injection. Detector sensitivity setting was 0.005 a.u.f.s. and recorder chart speed was 20 cm/h.

(0.1—10 μg/ml) studied for the three compounds (Tables I—III) as indicated by the constancy of the response factors (peak height divided by concentration).

The detection limits with a 20-μl injection volume for MTX, 7-OH-MTX

TABLE I

RESPONSE FACTORS FOR MTX, 7-OH-MTX AND APA IN HUMAN PLASMA

Spiked plasma concentration (μg/ml)	Response factor (cm/μg/ml)*		
	MTX	7-OH-MTX	APA
0.1	11.35	8.48	18.13
0.5	11.28	8.17	18.88
1	11.04	8.53	18.20
5	10.84	8.94	18.30
10	11.25	9.17	18.16
Mean ± S.D.**	11.15 ± 0.209	8.65 ± 0.396	18.33 ± 0.312
C.V.*** (%)	1.88	4.58	1.70

*Response factor = peak height/concentration; peak heights (cm) were based on the 20-μl injection and normalized sensitivity setting of 0.005 a.u.f.s.
**S.D. = standard deviation.
***C.V. = coefficient of variation.

TABLE II

RESPONSE FACTORS FROM MTX, 7-OH-MTX AND APA IN SALIVA

Spiked saliva concentration (μg/ml)	Response factor (cm/μg/ml)		
	MTX	7-OH-MTX	APA
0.1	15.35	11.30	17.25
0.5	15.22	11.32	17.12
1	15.60	11.65	17.22
5	15.72	11.45	17.44
10	16.07	11.58	17.66
Mean ± S.D.	15.59 ± 0.332	11.46 ± 0.155	17.33 ± 0.214
C.V. (%)	2.13	1.35	1.23

and APA in plasma are 15, 25 and 10 ng/ml, respectively. Higher sensitivity could be obtained when 0.5 ml rather than 1 ml of ethyl acetate was used for extraction. This was primarily attributed to the lower volume of the final aqueous solution obtained after extraction. The above modification could result in detection limits down to 10 ng/ml (approximately $2 \cdot 10^{-8}$ *M*) for MTX, 20 ng/ml for 7-OH-MTX and 5 ng/ml for APA.

The intra-day coefficients of variation for three compounds were between 1.23 and 5.28%. Under the condition described, three replicate analyses in three days gave inter-day coefficients of variation of 4.6% for MTX, 7.9% for 7-OH-MTX and 3.8% for APA.

Although there is no internal standard used in the present assay, excellent reproducibility was obtained as shown in Table IV. The extraction efficiencies for plasma samples were 70, 50 and 77% for MTX, 7-OH-MTX and APA, respectively. Recoveries for saliva samples were higher, being 98, 61 and 79% for these three compounds, respectively. Compared with many other HPLC methods, the present assay offers higher recovery, lower limit of sensitivity as well as lower coefficients of variation for MTX and its two metabolites in plasma [14—18, 20].

TABLE III

RESPONSE FACTORS FOR MTX, 7-OH-MTX AND APA IN URINE

Spiked urine concentration (μg/ml)	Response factor (cm/μg/ml)		
	MTX	7-OH-MTX	APA
1	1.20	1.0	1.75
2.5	1.07	0.98	1.77
5	1.04	1.09	1.71
7.5	1.09	1.11	1.68
10	1.10	1.07	1.73
Mean ± S.D.	1.10 ± 0.058	1.05 ± 0.055	1.72 ± 0.034
C.V. (%)	5.28	5.23	1.94

TABLE IV

REPRODUCIBILITY DATA FOR MTX, 7-OH-MTX AND APA IN HUMAN PLASMA

Compound	0.1 μg/ml (n = 6)			10 μg/ml (n = 6)		
	Mean peak height* (cm)	S.D.	C.V. (%)	Mean peak height** (cm)	S.D.	C.V. (%)
MTX	1.135	0.079	7.0	5.625	0.150	2.67
7-OH-MTX	0.848	0.038	4.4	4.587	0.103	2.25
APA	1.813	0.136	7.5	9.082	0.353	3.89

*Based on 20-μl injection at a sensitivity setting of 0.005 a.u.f.s.
**Based on 20-μl injection at a sensitivity setting of 0.1 a.u.f.s.

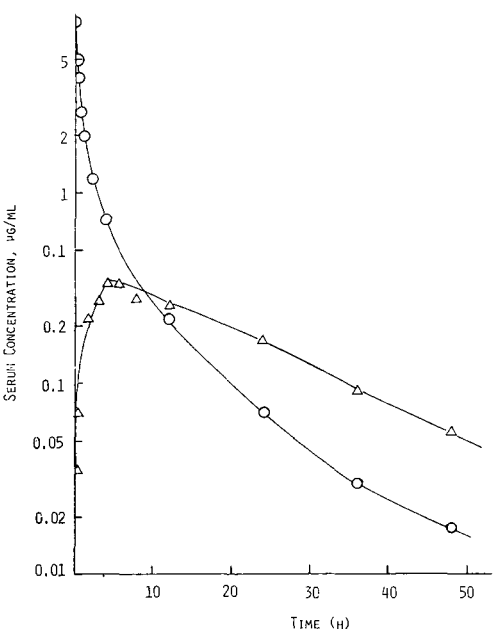

Fig. 3. Serum concentration profiles of MTX (○——○) and 7-OH-MTX (△——△) in a patient following intravenous administration of 40 mg/m² of MTX.

The results of interference studies showed that none of the drugs tested interfered with the analysis of MTX and its two metabolites.

By using the simple deproteinization method, MTX concentrations in urine can be readily detected down to 0.1 μg/ml (about $2 \cdot 10^{-7}$ M). This method is also applicable to plasma with MTX levels above 0.1 μg/ml. Therefore, analysis of drug levels in plasma or serum by the one-step deproteinization procedure might be adequate for routine monitoring in high-dose therapy [1].

Typical plasma level profiles from a patient receiving 40 mg/m² of MTX by intravenous push and from a rabbit after 15 mg/kg intravenous bolus injection are shown in Figs. 3 and 4, respectively. No APA was found in the patient's plasma, saliva and urine collected up to 72 h. This was probably due to the much lower concentrations of APA present in biological fluids or to

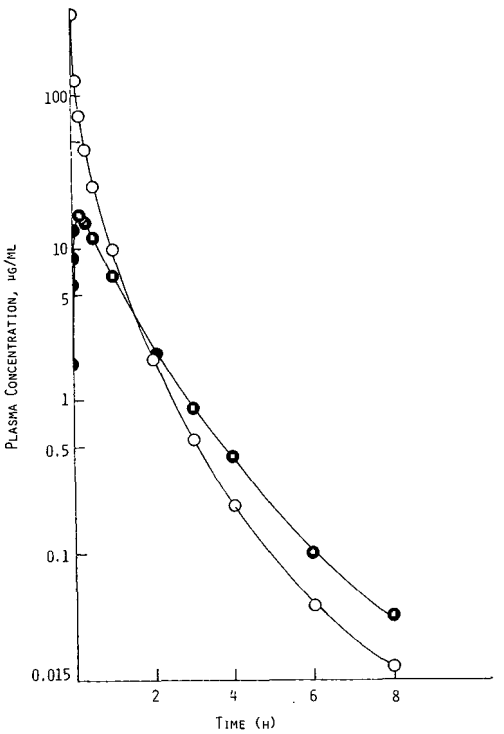

Fig. 4. Plasma concentration profiles of MTX (o——o) and 7-OH-MTX (•——•) from a rabbit following intravenous administration of 15 mg/kg of MTX.

the failure of the production of APA in this patient. In this case, APA can be used as an internal standard for the assay. Our data indicate that MTX is eliminated from the rabbit by multi-exponential decay with the terminal half-life much longer than 25 min which was reported previously [18].

Acetonitrile appears to be an ideal deproteinizing agent. The volume ratio of 2.5 between acetonitrile and plasma, saliva or urine was satisfactory to assure virtual completeness of the deproteinization process. Such a simple deproteinization method has been successfully used in the assay of creatinine [24], gentamicin [25], procainamide [26], tolbutamide [27], furosemide [28], and other drugs developed from this laboratory.

In attempting to increase the sensitivity of the assay, efforts have been made by acidification or alkalization during the extraction procedure. Surprisingly, the peak heights were all decreased, indicating the reduced extraction efficiency in both cases. Addition of isoamyl alcohol in the present assay was found to enhance the sensitivity by 1.5- to 2-fold for the three compounds. Due to the high viscosity of this reagent, the use of a syringe (Hamilton, Reno, NV, U.S.A.) rather than a micro pipettor is suggested to assure complete delivery. The final volume of aqueous phase would be constant if an accurate amount of isoamyl alcohol was introduced. Careful sample preparation prior to injection onto the column is essential in reducing analytical errors. The use of a micrometer also increases the accuracy of the measurement of peak heights.

The method described here permits a rapid, simultaneous determination of MTX and its two active metabolites in biological fluids. The sample preparation prior to chromatography is easy and no evaporation or reconstitution steps are needed. In view of the simplicity, specificity and sensitivity, the method may be of great use in pharmacokinetic studies in humans and animals. It may also be suitable for routine monitoring of serum levels of MTX as well as its two metabolites, 7-OH-MTX and APA, which have received wide attention recently due to their implication in nephrotoxicity during MTX therapy [1, 29—31].

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DETERMINATION OF THE ANXIOLYTIC AGENT 8-CHLORO-6-(2-CHLOROPHENYL)-4H-IMIDAZO-[1,5-*a*] [1,4]-BENZODIAZEPINE-3-CARBOXAMIDE IN WHOLE BLOOD, PLASMA OR URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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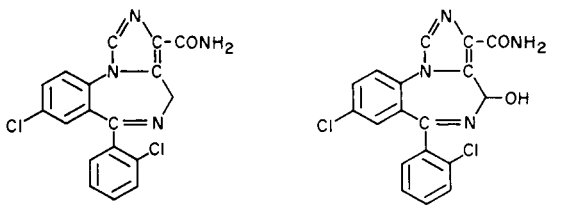
SUMMARY

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of 8-chloro-6-(2-chlorophenyl)-4H-imidazo-[1,5-*a*]-[1,4]-benzodiazepine-3-carboxamide [I] and its 4-hydroxy metabolite, 8-chloro-6-(2-chlorophenyl)-4-hydroxy-4H-imidazo-[1,5-*a*][1,4]-benzodiazepine-3-carboxamide [II] in whole blood, plasma or urine. The assay for both compounds involves extraction into diethyl ether—methylene chloride (70:30) from blood, plasma, or urine buffered to pH 9.0. The overall recoveries of [I] and [II] are 92.0 ± 5.4% (S.D.) and 90.3 ± 4.9% (S.D.), respectively. The sensitivity limit of detection is 50 ng/ml of blood, plasma, or urine using a UV detector at 254 nm. The HPLC assay was used to monitor the blood concentration—time fall-off profiles, and urinary excretion profiles in the dog following single 1 mg/kg intravenous and 5 mg/kg oral doses, and following multiple oral doses of 100 mg/kg/day of compound [I].

INTRODUCTION

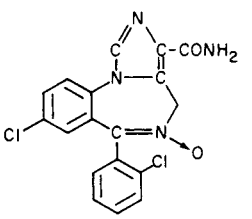
The compound 8-chloro-6-(2-chlorophenyl)-4H-imidazo-[1,5-*a*] [1,4]-benzodiazepine-3-carboxamide, [I] (Fig. 1), synthesized by Walser and co-workers [1,2], is a member of the imidazo-1,4-benzodiazepine class of compounds undergoing evaluation as anxiolytic agents [3]. It is structurally analogous to midazolam; a water soluble imidazo-1,4-benzodiazepine in clinical evaluation as a preoperative anesthesia inducing agent [4,5].

Studies on the biotransformation of ^{14}C-labeled [I] by the dog [6], indicated that the compound was metabolized by hydroxylation producing the 4-hydroxy compound, 8-chloro-6-(2-chlorophenyl)-4-hydroxy-4H-imidazo-[1,5-*a*] [1,4]-benzodiazepine-3-carboxamide, [II], as the major plasma metabolite



I

II



III (Ref. Std.)

Fig. 1. Chemical structures of compounds [I], [II], and [III].

(Fig. 1). Electron-capture gas–liquid chromatography (EC–GLC) used in the determination of midazolam [7] was not applicable to the analysis of [I] at low concentration due to poor precision and reproducibility. The presence of the carboxamide group in the molecule adversely influences the chromatographic behavior of the compound by EC–GLC.

High-performance liquid chromatography (HPLC) was investigated and resulted in the development of a rapid, sensitive and specific assay for the determination of compounds [I] and [II] in blood, plasma or urine. The method presented herein determines compounds [I] and [II] by normal-phase HPLC using their ultraviolet (UV) absorbance at 254 nm for quantitation. The analogous compound, 8-chloro-6-(2-chlorophenyl)-4H-imidazo-[1,5-*a*][1,4]-benzodiazepine-3-carboxamide-5-oxide, [III] (Fig. 1) is used as the internal standard. The HPLC assay was used to monitor the blood concentration–time fall-off profiles, and urinary excretion profiles in the dog following single 1 mg/kg intravenous and 5 mg/kg oral doses, and following multiple oral doses of 100 mg/kg/day of compound [I].

EXPERIMENTAL

HPLC analysis of compounds [I] and [II] in blood or plasma

Column. The column used was a 0.25 m × 4.6 mm I.D. stainless-steel column containing 10-μm Partisil silica gel, generating 32,200 plates/m (Whatman, Clifton, NJ, U.S.A.).

Instrumental parameters. A Waters Model ALC/GPC-204 high-performance liquid chromatograph equipped with a Model 440 absorbance detector, operated at 254 nm, a Model M6000A solvent delivery system and a U6K injector was used. The isocratic mobile phase used was methylene chloride–methanol–ammonium hydroxide (96:3.85:0.15) at a pressure of 6.2 MPa and a constant

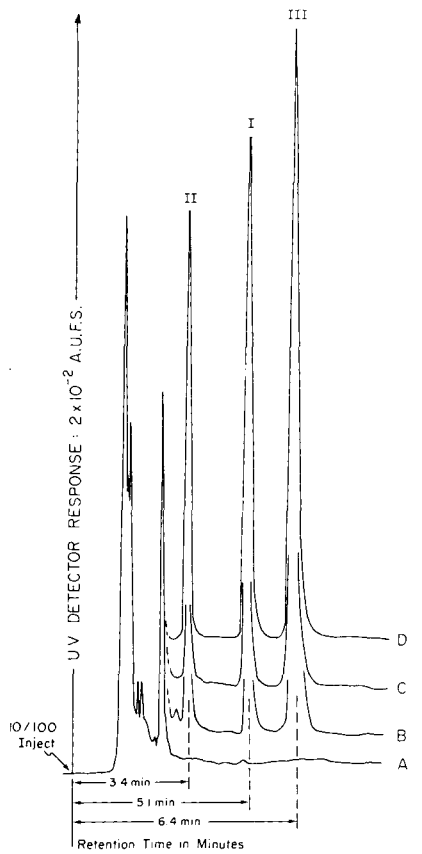


Fig. 2. Chromatograms of HPLC analysis of diethyl ether—methylene chloride extracts of (A) control dog blood, (B) dog blood following chronic oral dosing of a 100 mg/kg/day, (C) authentic standards recovered from control dog blood, and (D) authentic standards added to the residue of control dog blood.

flow-rate of 2.1 ml/min. Under these conditions, the retention times of compounds [I], [II] and [III] were 5.1, 3.4 and 6.4 min, respectively, with capacity factors (k') of 2.10, 1.05 and 2.85 for compounds [I], [II], and [III], respectively (Fig. 2). The UV detector sensitivity was $2 \cdot 10^{-2}$ a.u.f.s. and the chart speed on the 10-mV Hewlett-Packard recorder (Model No. 7132A) was 1.25 cm/min. Under these conditions 100 ng of [I], 60 ng of [II] and 140 ng of [III] per 10 μl injected gave nearly full scale pen response. The minimum detectable amount of [I] or [II] is 50 ng/ml of blood, plasma or urine.

Analytical standards. Compound [I] ($C_{18}H_{12}Cl_2N_4O$, M.W. = 371.23, m.p. = 300°C), compound [II] ($C_{18}H_{12}Cl_2N_4O_2$, M.W. = 387.24, m.p. = 304—307°C), and compound [III] ($C_{18}H_{12}Cl_2N_4O_2$, M.W. = 387.22, m.p. = 286—288°C) of pharmaceutical grade purity (> 99%) are used as analytical standards.

Prepare stock solutions of compounds [I], [II] and [III] in separate 10-ml volumetric flasks by dissolving 10 mg of each compound in 1 ml of methanol followed by 1 ml of mobile phase (see Reagents). Sonicate if necessary for 15—30 min for complete solubilization and dilute to volume with mobile phase.

These stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions by suitable dilutions in the mobile phase to contain the following amounts as indicated below:

Standard solution	Concentration (ng per 100 μl)		
	[I]	[II]	[III] (internal standard)
A	50	50	1400
B	100	100	1400
C	400	200	1400
D	800	400	1400
E	1000	600	1400
F	—	—	1400

Aliquots (10 μl) of solutions A—E are injected to establish the HPLC parameters using the UV detector at 254 nm. Aliquots (100 μl) of the same solutions are evaporated to dryness, the residue dissolved in 0.1 ml of methanol and reconstituted in 1 ml of control blood, plasma or urine as the processed standard calibration curve for the determination of the concentration in the unknowns. The determination of percent recovery requires that the processed standards be compared to authentic standards (100 μl of standards A—E) which have been added to the residue of extracted control blood, plasma or urine, to constitute the external standard curve. This is necessary due to a chromatographic enhancement effect which compounds [I], [II] and [III] exhibit; i.e. the peak height response is 10—20% greater when the compounds are chromatographed in the residue of biological extracts, than when chromatographed as pure standards.

A separate stock solution of the internal standard [III] containing 1 mg/ml of methanol is diluted in methanol to yield a working solution F containing 14 μg/ml, 100 μl of which are added only to the unknown blood or plasma samples. It is not added to the unknown urine samples due to an interfering peak whose retention time is close to that of compound [III]. Therefore, calculations in urine are carried out using the direct calibration technique which is discussed under Calculations.

Calibration of compounds [I], [II] and [III] by HPLC. Calibration (external standard) curves of the peak height ratio of [I] to [III], and [II] to [III] vs. concentration of [I] and [II] per 100 μl of mobile phase are constructed. Fresh calibration curves of the external standards and of the processed recovered standards are prepared for each day of analysis to establish the reproducibility of the HPLC system.

Reagents. All reagents must be of analytical reagent grade (> 99% purity). Potassium phosphate buffer, 1.0 *M*, pH 9.0, is prepared by dissolving 174.18 g of K_2HPO_4 in 1 l of distilled deionized water and titrating to pH 9.0 with 1.0 *N* hydrochloric acid. Mix well and check final pH with a pH meter. Diethyl ether—methylene chloride (70:30) is the extraction solvent and a mixture of methylene chloride—methanol—ammonium hydroxide (96:3.85:0.15) is used as both the mobile phase for HPLC analysis and the solvent for preparing

calibration standard solutions of compounds [I], [II] and [III]. Diethyl ether (absolute), opened for no more than five days was purchased from Mallinckrodt (St. Louis, MO, U.S.A.); methylene chloride and methanol from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); hydrochloric acid and ammonium hydroxide from J.T. Baker (Phillipsburg, NJ, U.S.A.). A 1% aqueous solution of Prosil-28 (P.C.R. Research Chemicals, Gainesville, FL, U.S.A.) is used for siliconizing all the glassware used in the assay.

Analysis of blood or plasma

The flow diagram of the extraction procedure is shown in Fig. 3.

Into a 15-ml siliconized conical centrifuge tube (PTFE No. 13 stoppered), add 1400 ng of compound [III] (100 μl of solution F) as the internal standard, 0.2 ml of distilled deionized water, 1.0 ml oxalated whole blood or plasma, 2.5 ml of 1 *M* phosphate buffer, pH 9.0 (mix well), and extract with 8 ml of diethyl ether—methylene chloride (70:30) by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80—100 strokes/min. Along with the samples, run six 1.0-ml specimens of control blood or plasma (one used as a control blank and five to be used for the preparation of external standards) and five 1.0-ml specimens of control blood or plasma containing 0.1 ml of standard solutions A—E (equivalent to 50, 100, 400, 800, and 1000 ng of [I], 50, 100, 200, 400 and 600 ng of [II], and 1400 ng of [III] (internal standard) per 1.0 ml of blood or plasma). Centrifuge the samples at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, rotor No. 253, Damon/IEC, Needham, MA, U.S.A.) at 5°C for 10 min and transfer a 7.0-ml aliquot of the upper organic layer into another siliconized 15-ml conical centrifuge tube. Evaporate the organic layer to dryness at 45°C in a N-EVAP evapo-

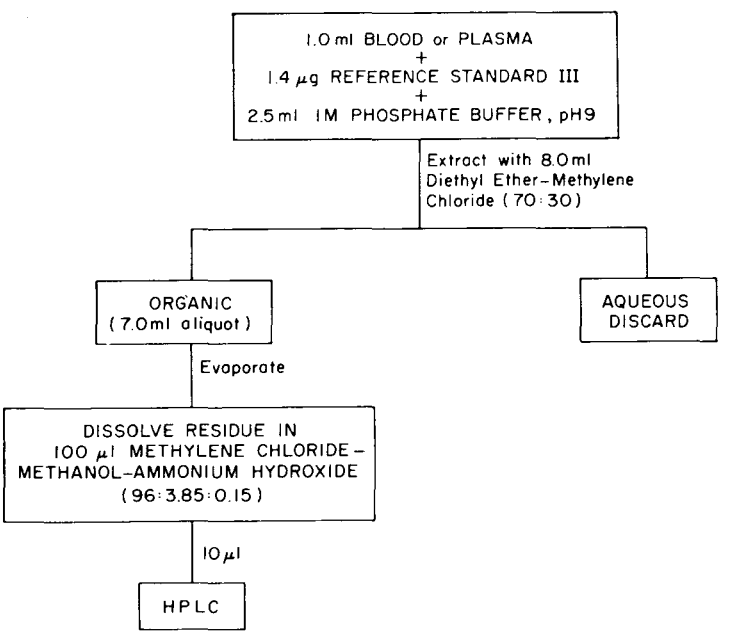


Fig. 3. Flow diagram of the extraction procedure for compounds [I], [II], and [III] from blood or plasma.

rator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residue in 100 μl of mobile phase [methylene chloride—methanol—ammonium hydroxide (96:3.85:0.15)] and inject a 10-μl aliquot into the liquid chromatograph. Typical chromatograms of blood extracts are shown in Fig. 2. The analysis of urine is identical to that for blood or plasma except that the addition of the internal standard [III] to the unknowns is omitted.

Calculations

The peak height ratio of [I] or [II] to [III] of the respective processed standards recovered from blood or plasma is determined and plotted graphically vs. concentration (ng/ml of specimen) to establish the calibration curve. Similarly, the peak height ratio of [I] or [II] to [III] in the aliquots of the respective blood or plasma unknowns injected is also determined. The concentrations of [I] and [II] in the unknowns represented by their peak height ratios are interpolated directly from their respective standard curves, because the recovery factors, and therefore the peak height ratio of [I] or [II] to [III] remains constant, irrespective of the sample aliquot injected. Thus, concentration (ng) in the unknowns interpolated from the processed standard curve = ng [I] or [II] per 1.0 ml of blood or plasma.

If, however, the peak due to the internal standard [III] is either diluted out, due to high concentration (> 10 μg) of [I] or [II] in blood or plasma, or in urine where it is omitted, then the direct calibration technique must be used whereby a calibration curve of peak height of the recovered standards of [I] or [II] vs. concentration (ng/ml of specimen) is plotted and used for the quantitation of the unknowns. The amount of [I] or [II] per aliquot of the unknown sample injected has to be corrected for the total volume of the sample and the recovery factor for each compound.

RESULTS AND DISCUSSION

A sensitive and specific HPLC assay was developed for the determination of compound [I] and [II] from 1 ml of blood, plasma or urine using UV detection at 254 nm for quantitation. This method enabled the rapid and accurate quantitation of compounds [I] and [II] for routine analysis of the large number of samples obtained during pre-clinical pharmacokinetic and toxicological studies. The major UV absorption bands of compounds [I], [II], and [III] occur at 215—220 nm and 255—260 nm, and are shown in Fig. 4. The Waters Model 440 absorbance detector used in conjunction with a 254-nm wavelength kit and a medium-pressure mercury lamp, allowed for quantitation of [I], [II], and [III] in the nanogram range.

Normal-phase (adsorption) HPLC analysis is the method of choice, since it is a simple three-step operation which involves selective extraction, sample concentration, and direct analysis by HPLC which ensures optimum resolution, peak symmetry and sensitivity of compounds [I], [II] and [III]. The shorter retention time of compound [II] with respect to that of compound [I] is probably due to intramolecular hydrogen bonding between the amide and hydroxyl group in [II] making it apparently less polar than [I]. Reversed-phase

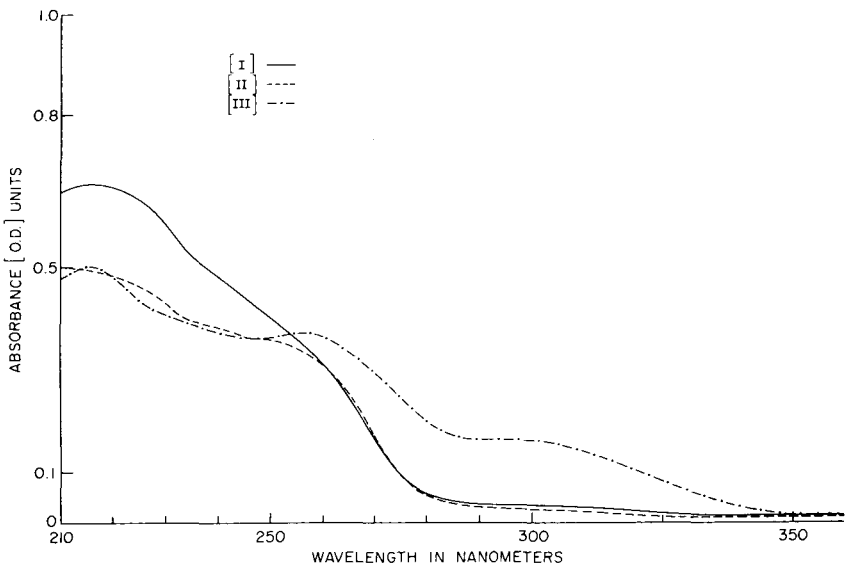


Fig. 4. UV absorption spectra of 5 μg/ml solutions of compounds [I], [II], and [III] in methanol.

HPLC was not practicable due to poor resolution between [I] and [II], and poor peak shape which adversely affected the sensitivity and quantitation of both compounds.

Compound [III] which is the N-oxide of [I], was chosen as the internal standard in the assay, due to its similar extraction and chromatographic behavior as compounds [I] and [II]. Compound [III] has not been identified as a metabolite of [I].

Recovery, sensitivity limits and statistical validation of the HPLC assay

The overall recoveries of compounds [I] and [II] from blood, plasma or urine are of the order of 92.0 ± 5.4% (S.D.) and 90.3 ± 4.9% (S.D.), respectively. The sensitivity limit of detection of [I] is 50 ± 5.3 ng (S.D.) and for [II] is 50 ± 1.7 ng (S.D.) per ml of blood, plasma or urine, using UV detection at 254 nm.

The intra-assay variability of [I] over the concentration range of 100—1000 ng/ml of blood showed a mean coefficient of variation of 1.5% while that for [II] over the concentration range of 100—600 ng/ml of blood was 2.3% (Table IA). The inter-assay variabilities of [I] and [II] showed mean coefficients of variation of 2.6 and 2.3%, respectively (Table IB).

Application of the method to biological specimens

The HPLC method was used to determine the blood concentration—time fall-off profiles and urinary excretion profiles in the dog following single 1 mg/kg intravenous and 5 mg/kg oral doses of compound [I]. The blood concentration of compound [I] following the intravenous administration ranged from 0.99 μg/ml at 1 min to 0.11 μg/ml at 30 h (Table II). Following oral administration (5 mg/kg) a peak concentration of 0.37 μg [I] per ml was observed at

TABLE I

STATISTICAL EVALUATION OF THE HPLC ASSAY

For A, $n = 4$; for B, $n = 3$.

Compound	Amount added (ng)	Amount found (ng ± S.D.)	Coefficient of variation (%)
A. Intra-assay variability			
[I]	100	94.6 ± 2.8	2.9
	400	403.3 ± 4.0	0.9
	800	814.5 ± 7.1	0.9
	1000	987.6 ± 11.1	1.1
			Average = 1.5
[II]	100	96.8 ± 3.0	3.1
	200	197.7 ± 2.0	1.0
	400	412.6 ± 14.1	3.4
	600	592.9 ± 10.1	1.7
			Average = 2.3
B. Inter-assay variability			
[I]	100	101.8 ± 6.4	6.2
	400	393.4 ± 10.4	2.6
	800	811.7 ± 8.1	0.9
	1000	993.1 ± 5.7	0.6
			Average = 2.6
[II]	100	104.2 ± 4.5	4.3
	200	191.7 ± 6.7	3.5
	400	402.7 ± 4.2	1.0
	600	600.0 ± 1.4	0.2
			Average = 2.3

0.5 h (Table II), with drug concentrations measurable through 10 h. Compound [II] was non-measurable following either single-dose administration of compound [I].

The urine concentration of directly extractable (unconjugated) [I] was non-measurable in the two dogs following the 1 mg/kg intravenous administration (0—48 h), whereas following the 5 mg/kg oral dose, urinary excretion of directly extractable parent drug [I] accounted for 16.3% of the dose in one dog, and 2.1% of the dose in the second dog (Table III). The metabolite [II] was non-measurable in all cases. Attempts at measuring the conjugated fraction of [I] and [II] were hampered by extracted endogenous impurities.

The assay was also used to monitor the blood concentrations of compounds [I] and [II] following multiple oral dosing in the dog (Table IV). Blood specimens were obtained from six dogs at 1, 3 and 6 h on day 15, following consecutive daily doses of 100 mg/kg of compound [I]. The maximum concentrations of compound [I] occurred at 1 h and ranged between 0.10 and 0.25 μg/ml of blood. Compound [II] was measurable at every time point with maximum concentrations occurring at 3 h, except for dog A, whose maxima occurred at 1 h. Maximum concentrations of compound [II] were consistently

TABLE II

CONCENTRATIONS OF COMPOUND [I] IN DOG BLOOD FOLLOWING THE ADMINISTRATION OF INTRAVENOUS AND ORAL DOSES OF COMPOUND [I]

Time	Concn. (μg [I] per ml of blood)	
	Intravenous Dose 1 mg/kg	Oral Dose 5 mg/kg
1 min	0.99	N.S.T.*
2.5 min	0.87	N.S.T.
5 min	0.84	N.S.T.
10 min	0.83	0.13
15 min	0.74	N.S.T.
20 min	0.69	0.31
30 min	0.73	0.37
45 min	0.59	0.35
1 h	0.47	0.33
1.5 h	0.57	0.32
2 h	0.58	0.31
3 h	0.55	0.26
4 h	0.50	0.24
6 h	0.32	0.16
8 h	0.31	0.12
10 h	0.27	0.10
24 h	0.15	N.M.**
30 h	0.11	N.M.
48 h	N.M.	N.M.

*N.S.T. = No sample taken.
**N.M. = Non-measurable (< 50 ng/ml).

TABLE III

URINARY EXCRETION OF DIRECTLY EXTRACTABLE (UNCONJUGATED) [I] IN TWO DOGS FOLLOWING INTRAVENOUS AND ORAL DOSES OF COMPOUND [I]

Excretion period (h)	Percentage of dose excreted as directly extractable [I]			
	Dog A		Dog B	
	Intravenous Dose 1 mg/kg	Oral Dose 5 mg/kg	Intravenous Dose 1 mg/kg	Oral Dose 5 mg/kg
0—24	N.M.*	16.11	N.M.	2.05
24—48	N.M.	0.16	N.M.	N.M.
Total	N.M.	16.27	N.M.	2.05

*N.M. = Non-measurable (< 50 ng/ml).

higher than those of compound [I], and ranged between 0.12 and 0.35 μg/ml of blood.

Gas chromatographic behavior of [I]

Although the sensitive and specific gas chromatographic analysis of the 1,4-

TABLE IV

CONCENTRATIONS OF COMPOUNDS [I] AND [II] IN DOG BLOOD FOLLOWING MULTIPLE ORAL DOSING FOR 15 DAYS

Dose: 100 mg [I]/kg/day.

Dog	Time (h)	Concn. (μg/ml of blood)	
		[I]	[II]
A	1	0.15	0.23
	3	0.07	0.22
	6	N.M.*	0.13
B	1	0.10	0.08
	3	0.07	0.12
	6	0.05	0.10
C	1	0.13	0.16
	3	0.07	0.18
	6	N.M.	0.11
D	1	0.13	0.11
	3	0.08	0.16
	6	N.M.	0.08
E	1	0.25	0.27
	3	0.18	0.35
	6	0.05	0.27
F	1	0.12	0.10
	3	0.06	0.15
	6	N.M.	0.08

*N.M. = Non-measurable (<50 ng/ml).

benzodiazepines using electron-capture detection is well documented [8], it was not applicable per se to the determination of [I] at low concentrations. The EC—GLC assay developed for midazolam [7] was initially applied to the determination of [I] which showed tailing on both OV-1 and OV-17 liquid phases, albeit with intrinsic high sensitivity to electron-capture detection. The chromatograms also indicated ghosting or memory effects on GLC analysis probably due to adsorption in the system resulting in poor precision and reproducibility especially at low concentration. The compound also showed adsorption losses on glassware which was circumvented by treatment with Prosil-28®, a siliconizing agent. It was apparent that both derivatization of the compound and clean up were necessary to improve its chromatographic behavior. Since back extraction of benzodiazepines into acid from a biological extract is an effective means of sample clean up, this step was investigated. It was noted that back washing the compound with dilute acid (0.1 *N* hydrochloric acid) resulted in a sharp symmetrical Gaussian shaped peak with enhanced electron-capture detector sensitivity (500 pg for full scale response at $0.5 \cdot 10^{-9}$ A), on a 1.22 m × 4 mm I.D., 3% OV-17 column at 245°C, indicating some form of rearrangement to a less polar moiety (Fig. 5). The authentic compound gave a tailing peak with a 4.9-min retention time (Fig. 5, trace A) whereas the acid-washed compound showed a sharper (Gaussian shaped) peak with a shorter

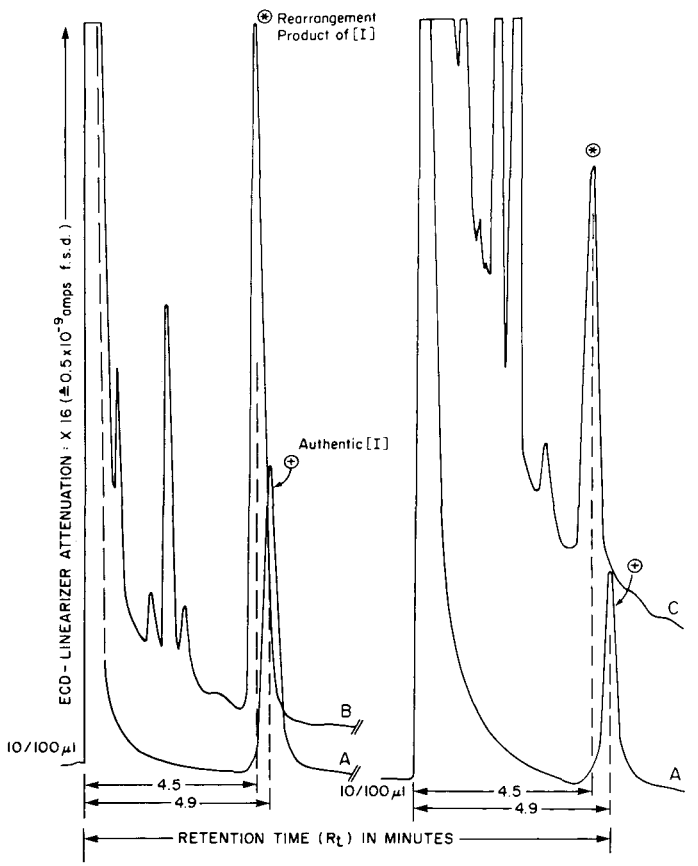


Fig. 5. Chromatograms of the EC—GLC analysis of (A) authentic [I]; (B) authentic [I] after treatment with 0.1 *N* hydrochloric acid indicating a rearrangement product with a shorter retention time; (C) authentic [I] extracted from blood, after dilute acid treatment, indicating a similar rearrangement product.

retention time of 4.5 min (Fig. 5, trace B), suggesting a rearrangement product. Similar behavior was observed for the compound recovered from blood after back extraction into acid (Fig. 5, trace C).

The partial ring opening of 1,4-benzodiazepines in dilute acids (e.g. 0.1 *N* hydrochloric acid) due to hydrolysis of the 4,5-azomethine group to yield the open ketone was demonstrated by differential pulse polarographic (DPP) analysis [7,9]. Similarly the DPP analysis of [I] indicated that 50% hydrolysis of [I] in 0.1 *N* hydrochloric acid was achieved in approximately 30 min, based on the polarographic reduction of the 4,5-azomethine group [10]. At equilibrium approximately 70% of [I] is in the open ketone form. Although this reaction is reversible upon alkalination for most benzodiazepines [7,9], compound [I], however, after standing in acid, and then alkalinized (pH 7.0 or 13.0) (which should undergo cyclization back to the expected parent compound), apparently undergoes a rearrangement to a new product, as indicated by a greatly diminished polarographic reduction peak for the 4,5-azomethine group.

These leads are under investigation for the development of a more sensitive EC—GLC assay (potential sensitivity of 250—300 pg/ml) for future clinical pharmacokinetic evaluation.

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DETERMINATION OF 2-HYDROXYDESIPRAMINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple reversed-phase high-performance liquid chromatographic assay of 2-hydroxydesipramine (2-OH-DES) in plasma is described, using 2-hydroxyimipramine (2-OH-IMI) as the internal standard. Extraction of the plasma samples by methylene chloride—isoamyl alcohol was followed by back-extraction of 2-OH-DES into acidic phosphate buffer. Precautions include silanizing test tubes and rinsing pipettes to minimize adsorptive loss, and washing with extraction solution to eliminate chromatographic interference peaks. Analyses were carried out by using a high carbon load C-18 column (15%) with phosphate buffer—acetonitrile as the mobile phase at 43°C. Detection at 254 nm was monitored at extended attenuation of 0.001 or 0.002 a.u.f.s. Peak height ratios of 2-OH-DES/2-OH-IMI were linearly correlated with 2-OH-DES concentration between 10 and 100 ng/ml of plasma. Detection limit was 3 ng. Coefficients of variation for within-run and day-to-day studies were 2.2% and 5.0%, respectively. A significant amount of 2-OH-DES was identified from the plasma extract of a psychiatric patient taking a daily dose of desipramine. This assay may be used for monitoring of 2-OH-DES in evaluating clinical side effects and for pharmacokinetics studies.

INTRODUCTION

Plasma tricyclic antidepressant (TCA) levels have proven to be useful for correlation with clinical response in patients [1]. Tricyclics are metabolized in the liver to both active and inactive metabolites prior to elimination [2]. Hydroxylated metabolites of desipramine and imipramine are examples of such active metabolites. 2-Hydroxydesipramine (2-OH-DES) has been shown to produce cardiotoxicity in dogs [3]. Thus, either clinical responses or side

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effects may depend on levels of parent TCA and such active metabolite as 2-OH-DES.

Hydroxylated metabolites of desipramine and imipramine were quantitated by several methods. Stiller et al. [4] utilized alkaline flame-ionization gas chromatography to monitor plasma levels of imipramine and desipramine and their hydroxylated metabolites. Sutfin and Jusko [5] reported a normal-phase high-performance liquid chromatographic (HPLC) assay of these same drugs and metabolites. A fluorescence detector was used to achieve a sensitivity of 1 ng for each drug in 1 ml of plasma. More recently, Suckow and Cooper [6] used reversed-phase paired-ion liquid chromatography and a highly sensitive electrochemical detector to monitor these drugs with a sensitivity of less than 1 ng/ml of plasma. Godbillon and Gauron [7] analyzed clomipramine, imipramine and their metabolites using a silica gel column with ethanol—hexane—dichloromethane—diethylamine as the eluent and a 254-nm detector. Detection limits ranged from 5 to 10 ng. Fekete et al. [8] used a reversed-phase column with water—ethanol—decylamine at pH 9.5 or pH 11 as the mobile phase for the measurement of chlorpromazine, imipramine and their metabolites. Detection limits ranged from 1 to 3 ng.

The present study is a continuation of efforts within our laboratory to develop methodologies for tricyclics level determination [9, 10]. Since attempts to assay both desipramine and 2-OH-DES using the previously published procedure [9] were not successful as explained in the discussion later on, a reversed-phase HPLC assay for 2-OH-DES has been developed using a high carbon load C-18 column and a 254-nm UV detector.

EXPERIMENTAL

Reagents

Acetonitrile, methanol and methylene chloride were ultraviolet grade, distilled in glass, obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was double distilled in glass. 2-OH-DES and 2-hydroxyimipramine (2-OH-IMI) were gifts from Dr. Albert A. Maniam, National Institute of U.S.A.). Water was double distilled in glass. 2-OH-DES and 2-hydroxyimipramine (2-OH-IMI) were gifts from Dr. Albert A. Maniam, National Institute of Mental Health (NIMH) (Rockville, MD, U.S.A.). Sodium carbonate was reagent grade from Mallinckrodt (St. Louis, MO, U.S.A.). Methylene chloride—isoamyl alcohol (98:2) was washed with 0.05 *M* phosphate buffer, pH 2.5. Sodium carbonate buffer, (1 *M*, pH 11.0) was washed with the above methylene chloride—isoamyl alcohol.

Mobile phase

To 2 l of distilled water, potassium dihydrogen phosphate (13.68 g) was added and the pH adjusted to 4.7 with diluted potassium hydroxide. The solution was filtered and kept at 4°C. Prior to the analysis, the phosphate solution was mixed with acetonitrile (75:25), followed by degassing.

Standards

A primary stock solution of 2-OH-DES (0.1 mg/ml free base) was prepared

by dissolving the powder in distilled water in a silanized 10-ml volumetric flask and stored at 4°C. For the preparation of calibration standards, a working aqueous stock solution of 1 μg/ml was prepared. A stock solution of the internal standard, 2-OH-IMI, was prepared by dissolution in methanol in a silanized volumetric flask. This solution was kept at −20°C. For spiking purposes, a working standard solution of 1 μg/ml was prepared.

High-performance liquid chromatography

The liquid chromatograph consisted of a Model 6000A pump, a Waters guard column, 2.3 cm × 3.9 mm, packed with Bondapak/Porasil particles, a U6K injector and a Model 440 254-nm detector (Waters Assoc., Milford, MA, U.S.A.). The column was a Partisil ODS-2 reversed-phase column, 250 mm × 4.6 mm (Whatman, Clifton, NJ, U.S.A.). The column packing consisted of 10-μm silica gel with 15% of the surface hydroxyls bonded with octadecyl groups. Detector attenuation was set at either 0.01 or 0.02 a.u.f.s. with the recorder input voltage set at 1 mV. In effect, the attenuation was extended to 0.001 or 0.002 a.u.f.s. The analysis was carried out by using the previously prepared mobile phase at a flow-rate of 2.7 ml/min and at an elevated temperature of 43°C.

Sample extraction

To a series of silanized test tubes, each containing 2 ml of plasma, 0, 10, 20, 40, 80, and 100 ng of 2-OH-DES were added per ml of plasma. In addition, a psychiatric patient plasma and five previously spiked plasma samples (20 ng/ml) were also included for checking the precision of this assay. To these samples, 75 ng/ml of 2-OH-IMI were added, followed by 2 ml of carbonate buffer (1 *M*, pH 11) and 10 ml of methylene chloride—isoamyl alcohol (98:2). The sample tubes were capped, shaken for 15 min and spun for 10 min. The upper, aqueous phase was discarded, and the lower organic phase transferred into another silanized test tube, containing 400 μl of phosphate buffer (0.05 *M*, pH 2.5, previously washed with hexane). The extraction was completed by shaking for 15 min and centrifuging for 5 min. The lower phase was transferred by rinsed pipettes [methylene chloride—isoamyl alcohol (98:2)] to another set of silanized test tubes, and 350 μl were injected for HPLC analysis.

Quantitation

Peak height ratios of 2-OH-DES to 2-OH-IMI were plotted against concentrations of 2-OH-DES. The concentrations of 2-OH-DES of precision studies and patient's sample were estimated from these plots.

Recovery

Percentage recoveries of 2-OH-DES were established for the concentrations of 20 and 50 ng/ml. At both concentrations, five determinations were made. These samples were spiked with internal standard, followed by extraction as described previously. Then, the peak height of extracted 2-OH-DES was compared to that of a known amount for recovery percentage estimation.

Interferences

Interferences were checked by comparing the capacity factor, k', of some commonly used drugs with those of 2-OH-DES and 2-OH-IMI.

RESULTS

Measurements of 2-OH-DES concentration in plasma were achieved by using a rapid three-step extraction, followed by a reversed-phase HPLC analysis. Fig. 1A shows the chromatogram of a blank (unspiked) plasma sample spiked with the internal standard, 2-OH-IMI. Fig. 1B shows the chromatogram of a plasma sample spiked with 20 ng of 2-OH-DES per ml of plasma, and the internal standard. The two peaks were well resolved, and the capacity factors of 2-OH-DES and 2-OH-IMI were 6.20 and 8.90, respectively. The sensitivity for a signal-to-noise ratio of 5:1 was 3 ng. Blank plasma sample (Fig. 1A) did not show any endogenous interference peaks with 2-OH-DES. Each analysis was completed within 13 min.

Calibration studies showed that the peak height ratios of 2-OH-DES/2-OH-

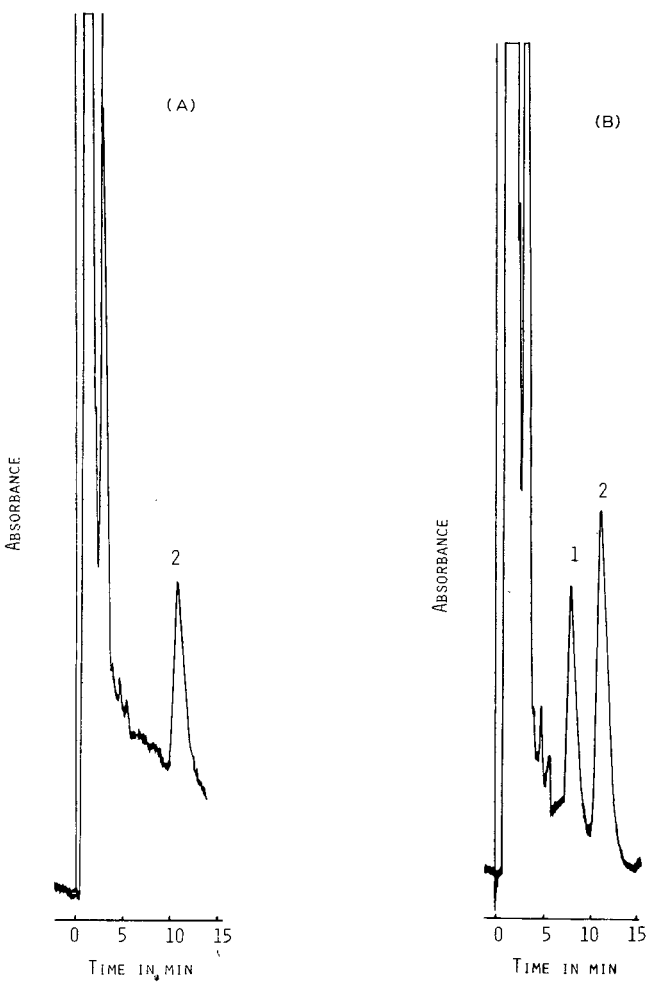

Fig. 1. Chromatograms of (A) extract of drug-free plasma spiked with internal standard, 2-OH-IMI, and (B) extract of plasma spiked with 20 ng of 2-OH-DES per ml of plasma. Peaks: 1 = 2-OH-DES, 2 = 2-OH-IMI.

TABLE I

CAPACITY FACTOR, k', OF COMMON DRUGS CHECKED FOR INTERFERENCE

Drug	k'	Drug	k'
Acetaminophen	0.00	Doxepin	a
Codeine	0.65	Cimetidine	a
Phenobarbital	2.16	Desipramine	a
2-Hydroxydesipramine	6.20	Nortriptyline	a
Phenytoin	6.85	Propoxypene	a
Meperidine	7.03	Imipramine	a
Pentobarbital	7.05	Amitriptyline	a
2-Hydroxyimipramine	8.90	Diazepam	a
Secobarbital	10.20	Chlorpromazine	a
Oxazepam	12.90	Clomipramine	a
Lorazepam	a*	Perphenazine	a
Flurazepam	a	Thioridazine	a
Chlordiazepoxide	a	Trifluoperazine	a
		Prochlorpherazine	a

*a, Capacity factor greater than 12.90.

IMI were linearly correlated with concentrations between 10—100 ng of 2-OH-DES per ml of plasma. The correlation coefficient was 0.9994 with a slope of 0.0321 and an intercept of 0.0868. Within-run precision was estimated by the determination of five 2-ml plasma samples containing 20 ng of 2-OH-DES per ml of plasma. Coefficient of variation was 2.2%. This experiment was repeated five times over a period of a month in order to evaluate the day-to-day variability, and the coefficient of variation was 5.0%.

Recovery of 2-OH-DES was estimated by using five 20 ng/ml and five 50 ng/ml samples. Peak height of the 2-OH-DES standard, either 20 or 50 ng, was compared to that of the extracted 2-OH-DES. The percentage recoveries were 66% and 68% for the 20 ng/ml and 50 ng/ml samples, respectively.

Table I lists the capacity factors, k', of about 20 common drugs as analyzed by our chromatographic system. None of these drugs interfere with our assay of 2-OH-DES or 2-OH-IMI. As a result of the high carbon load of this Partisil-10 ODS-2 column, the other TCA, such as desipramine and imipramine were well retained by the column with a k' greater than 10.

To evaluate the applicability of this assay to clinical and pharmacokinetics studies, a plasma sample from a psychiatric patient taking a daily dose of 150 mg of desipramine was measured by the procedure. Fig. 2 shows the chromatogram of plasma extract from this patient. Concentration of 2-OH-DES was estimated to be 51 ng/ml. The desipramine concentration, determined by our previously published procedure [9], was 80 ng/ml.

DISCUSSION

Preliminary studies of the simultaneous assay of 2-OH-DES and desipramine using the published procedure [9] were not successful as explained later on in this section. The approach of development of a new 2-OH-DES assay was chosen, resulting in a systematic search for optimization of extraction proce-

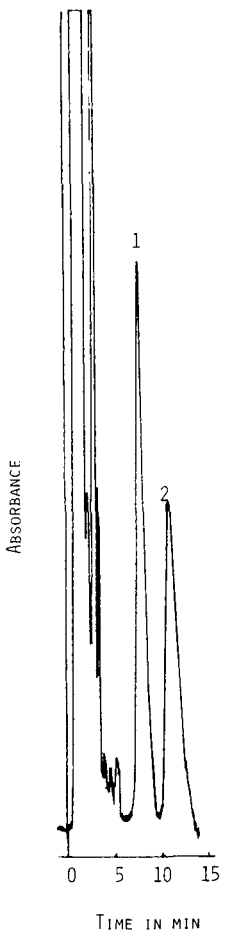

Fig. 2. Chromatogram of a patient plasma extract, showing 2-OH-DES at a concentration of 51 ng/ml. Plasma sample was taken 8 h post-ingestion.

dures and chromatographic conditions. Based upon our previous experience, the following precautions were taken in order to avoid adsorptive losses and interference peaks from extraction reagents. To minimize adsorptive losses, the test tubes, volumetric flasks and transfer pipettes were either silanized or rinsed with the extraction solution [methylene chloride—isoamyl alcohol (98 : 2)]. To avoid interference peaks from the extraction reagents, the washing of sodium carbonate buffer and methylene chloride—isoamyl alcohol solutions was carried out according to the procedures of the Experimental section. That these steps ensured precisions in replicate determinations is evident from the small coefficient of variation obtained.

Another important aspect of the assay development was the chromatography. Due to the obvious advantages of reversed-phased HPLC for biological assays, the initial effort was concentrated on using a μBondapak C_{18} column with a 254-nm detector as described in the published assay [9]. However, attempts at retaining the polar 2-OH-DES with k' between 2 and 10 and achiev-

ing resolution from endogenous interference were not successful. Thus, it became necessary to use a reversed-phase column with a high carbon load in order to obtain the desired retention characteristics and resolution. The column of choice was a Partisil-10 ODS-2 with a 15% carbon load. Because of the higher percentage of C-18, the 2-OH-DES and 2-OH-IMI molecules experienced more interaction with the column packing than that with a 10% carbon load μBondapak C_{18} column. Due to increased interaction, peak broadening occurred. This was overcome by carrying out the separation at an elevated column temperature of 43°C. In contrast to several previous attempts by other workers, the detection mode utilized a readily available 254-nm UV detector. An extended attenuation of either 0.001 or 0.002 a.u.f.s. was used, and the response was linear for 2-OH-DES concentrations of 10—100 ng/ml of plasma. The coefficients of variation for within-run and day-to-day studies were equal or less than 5%. The present assay identified the presence of a significant amount of 2-OH-DES in the plasma of a psychiatric patient.

Since the present procedure involved extraction of 2-OH-DES and 2-OH-IMI from plasma samples, the possibility of simultaneous assay of these two metabolites by modifying the present method was investigated. The approach was the external standard method. Modifications included: firstly, the preparation of plasma calibration standards for both 2-OH-DES and 2-OH-IMI; secondly, quantitative transfer of methylene chloride—isoamyl alcohol; and finally, quantitative injection of the phosphate buffer extract. Preliminary studies show that the calibration curves for both 2-OH-DES and 2-OH-IMI concentrations versus the respective peak heights were linear. This modified method may be used for assaying both concentrations of plasma samples from patients taking imipramine.

Thus, the present study shows that measurement of 2-OH-DES in plasma can be achieved by using a rapid three-step extraction process, and a reversed-phase column. By following precautions to avoid adsorptive loss and extractant interference, this procedure may be used for both pharmacokinetics studies and patient monitoring of 2-OH-DES levels, which may be correlated with clinical side effects.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF LORAZEPAM IN MONKEY PLASMA

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SUMMARY

A simple, sensitive and selective method for the determination of lorazepam in monkey plasma has been developed using high-performance liquid chromatography in a reversed-phase mode. The limit of detection for lorazepam in plasma is about 2 ng/ml. The method has been applied to plasma samples obtained from cynomolgus monkeys after oral doses of 0.15 mg/kg and intravenous doses of 0.05 mg/kg of lorazepam. In this species, mean peak plasma concentrations of 12 ng/ml occurred at 2 h after oral dosing and declined with a half-life of 2.5 h; the mean terminal half-life after intravenous dosing was 1.4 h.

INTRODUCTION

Lorazepam[7-chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one] (Fig. 1) is a member of the benzodiazepine group of anxiolytic drugs. It is currently used as a premedicant in anaesthesia [1, 2] and for the relief of anxiety states in man [3, 4]. Lorazepam is extensively metabolised in the rat, but in man and other investigated species there is only one major metabolite, lorazepam glucuronide [5, 6], which has no psychopharmacological activity [7].

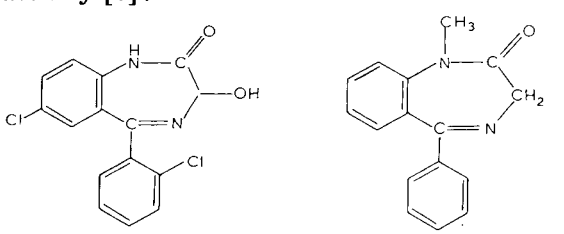

Fig. 1. Structures of lorazepam (left) and internal standard (diazepam) (right).

For accurate pharmacokinetic studies of drugs in biological fluid, the analytical techniques employed should discriminate between the parent drug and its metabolites and should quantify them at levels normally encountered during drug therapy. Early gas chromatographic (GC) methods employed for the analysis of lorazepam either lacked specificity as a result of utilising acid hydrolysis to the benzophenone, or sensitivity due to on-column adsorption phenomena [8]. More recent GC methods require minimal clean-up procedures, require no derivatisation and are specific for lorazepam [9, 10].

This paper describes a rapid, sensitive and selective assay for lorazepam in monkey plasma using high-performance liquid chromatography (HPLC) in a reversed-phase mode. The system is non-destructive and uses an internal standardisation technique employing the benzodiazepine, diazepam, as the internal standard. In contrast to a recent review [11] which considered HPLC to be insufficiently sensitive as a technique for the quantitation of therapeutic concentrations of lorazepam, this paper demonstrates that HPLC can be applied to pharmacokinetic studies in monkeys at dose levels which are within the human therapeutic range.

EXPERIMENTAL

Materials

Acetonitrile was HPLC (far UV) grade. All other reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Diethyl ether was redistilled prior to use. Borate buffer (1 *M*) containing potassium chloride (1 *M*) was adjusted to pH 9.0 using sodium carbonate solution (1 *M*). Standard solutions of lorazepam were prepared in methanol at concentrations of 1 μg/ml and 10 μg/ml; a stock solution of the internal standard, diazepam, was prepared at a concentration of 10 μg/ml.

Extraction

Plasma samples (1 ml) were spiked with internal standard (100 ng) and mixed with borate buffer (1 ml) to adjust the pH to 9.0. The samples were extracted by vortex mixing with diethyl ether (5 ml) for 30 sec. After centrifugation, the ether phase was transferred to a 10-ml pointed centrifuge tube and evaporated to dryness under a stream of dry nitrogen at 37°C. The residue was washed to the bottom of the tube with a small volume of diethyl ether, which was again evaporated to dryness. The residue was redissolved in 100 μl of acetonitrile—water (50:50, v/v) and half of the sample (50 μl) was injected into the liquid chromatograph.

High-performance liquid chromatography

The chromatograph consisted of an M6000A pump (Waters Assoc., Cheshire, Great Britain) fitted to a Pye LC3 variable-wavelength UV absorption detector (Pye Unicam, Cambridge, Great Britain) operated at 230 nm (a λ_{max} for lorazepam in methanol). Injection was via an automatic sampler WISP™ 710A (Waters Assoc.).

The column was constructed of stainless steel (30 cm × 0.4 cm I.D.) prepacked with μBondapak C_{18} (mean particle diameter 10 μm) (Waters Assoc.). A

pre-column (7 cm × 0.2 cm I.D.) constructed of stainless steel and dry-packed with pellicular Co:Pell® ODS (particle diameter 27—37 μm, Whatman, Kent, Great Britain) was installed in front of the main analytical column.

Chromatography was performed in a reversed-phase mode using a solvent system of 40% (v/v) acetonitrile in aqueous 0.1% (w/v) sodium dihydrogen orthophosphate, the final pH of the mobile phase was adjusted to 3.0 with phosphoric acid. The mobile phase flow-rate was 2 ml/min.

Chromatograms were recorded using either a Trilab computing integrator (Trivector Systems, Sandy, Great Britain) or a 3380A computing integrator (Hewlett-Packard, High Wycombe, Great Britain). Peak height measurements were used in preference to peak area measurements since these gave greater precision of measurement.

Under the conditions described, lorazepam and the internal standard (diazepam) eluted from the column with retention times of 4.3 and 8.8 min, respectively (Fig. 2).

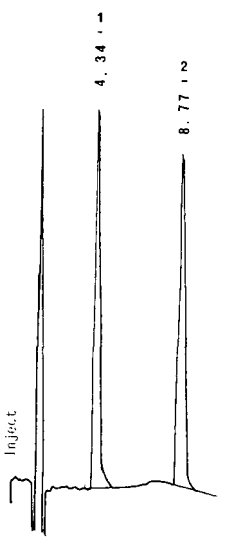

Fig. 2. Chromatogram of a standard mixture containing lorazepam (1) and internal standard, diazepam (2). Column: 30 × 0.4 cm I.D. prepacked with μBondapak C_{18}; flow-rate, 2 ml/min; solvent system, 40% (v/v) acetonitrile—aqueous 0.1% (w/v) sodium dihydrogen orthophosphate; detector, UV, at 230 nm; integrator attenuation, 16.

Calibration procedure

Calibration lines of peak height ratio measurements of lorazepam to internal standard against concentrations of lorazepam (ng/ml) were constructed over the concentration range 0—100 ng/ml. Samples of blank (drug-free) plasma (1 ml), and plasma spiked with lorazepam at concentrations of 5, 10, 30, 50, 70 and 100 ng/ml and with internal standard at a fixed concentration of 100 ng/ml, were taken through the extraction procedure described previously. Five replicate extractions were made at each concentration over the calibration range.

Gas chromatography—mass spectrometry

Gas chromatography—mass spectrometry (GC—MS) was carried out using a Pye 104 gas chromatograph (Pye-Unicam) linked via a single-stage, glass jet separator to a Micromass 16F mass spectrometer (V.G. Analytical, Cheshire, Great Britain). The mass spectrometer was operated in the electron impact mode of ionisation with an electron energy of 70 eV, a trap current of 100 μA and a source temperature of 200°C. Mass spectra were obtained at 10-sec intervals and the data stored using a Display Digispec data system (V.G. Analytical) on floppy diskettes.

The gas chromatograph oven was fitted with a glass column (15 m × 0.2 cm I.D.) packed with 3% OV-17 on Diatomite CC1 (100—120 mesh) and was operated at 260°C. Helium was used as the carrier gas at a flow-rate of 20 ml/min. The temperature of the GC—MS interface was 250°C.

Lorazepam samples were derivatised with N,O-bis(trimethylsilyl)acetamide in pyridine [Trisil/BSA Formula 'P'; Pierce and Warriner (U.K.), Cheshire, Great Britain]. Sample residues (ca. 1—10 μg) were heated with the derivatising agent (10—20 μl) for 15 min at 40°C; aliquots containing 0.5—1 μg of lorazepam were injected for GC—MS. Under the above conditions the bis(trimethylsilyl) derivative of lorazepam was formed and gave a retention time of 5 min.

Studies in the cynomolgus monkey (Macaca fascicularis)

Five adult male cynomolgus monkeys were each given single oral doses of 0.15 mg lorazepam per kg bodyweight and 0.05 mg/kg intravenous doses. The oral doses were administered in 10 ml water via a stomach tube and were washed in with an equal volume of water. The animals were fasted for 12 h preceding drug administration and for 6 h following drug administration.

Blood samples (3—5 ml) were withdrawn from the femoral veins of the animals into heparinised tubes, at 0 h (predose) and at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, 7 and 24 h after dosing; an additional sample was taken at 2 min after intravenous dosing. Blood cells were removed by centrifugation and the separated plasma was analysed immediately.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration were repeated on five occasions. The precision of the method for the measurement of lorazepam in plasma as indicated by the coefficient of variation of peak height ratio measurements was ± 8% at 5 ng/ml, ± 2% at 30 ng/ml and ± 2% at 100 ng/ml (Table I).

Accuracy

A calibration line for the measurement of lorazepam in plasma, constructed over the concentration range 0—100 ng/ml was linear ($y = -0.014 + 0.0126x$, correlation coefficient 0.9958) where y is the peak height ratio and x the concentration of lorazepam in plasma (ng/ml). However, two overlapping calibration lines were constructed for the ranges 0—50 ng/ml ($y = -0.004 + 0.0118x$, correlation coefficient 0.9943) and 30—100 ng/ml ($y = -0.048 + 0.0131x$, correlation coefficient 0.9891). The use of two calibration lines improved the

TABLE I

PRECISION OF THE METHOD AND RECOVERIES OF LORAZEPAM FROM PLASMA

Values corrected for 100% recovery of internal standard.

Concentration added to plasma (ng/ml)	Coefficient of variation (%)	Recovery (%)
5	8	66
10	9	71
30	2	69
50	6	71
70	2	75
100	2	78

accuracy of measurement in the lower regions of the calibration range; clinical doses of lorazepam produce plasma concentrations of the drug of this order of magnitude. The standard error of the calibration line as a measure of the concentration of lorazepam in plasma was 2.25 ng/ml.

Recovery

The recovery of internal standard from plasma (100 ng/ml) was 77 ± 6% S.D. (n = 6). The mean recovery of lorazepam over the concentration range 5—100 ng/ml was determined by comparison of non-extracted standards to those of extracted standards corrected for 100% recovery of internal standard, and was 72 ± 4% S.D. (Table I).

Limit of detection

No interfering peaks with the same retention time as lorazepam were present in the predose (blank) plasma samples taken from the cynomolgus monkeys. The limit of accurate measurement of the method based on a signal-to-noise ratio of 2:1 was set at 5 ng/ml (Fig. 3). Since, however, the extraction method described could also be used for the extraction of 2 ml of plasma without deviation in peak height ratios from the calibration line based on 1 ml of plasma, it was also possible to measure 5 ng of lorazepam in 2 ml of plasma giving a limit of detection of the order of 2—3 ng/ml. This limit of detection is of the same order of magnitude as that of the reported GC methods [9, 10].

Selectivity of the analytical method

The selectivity of the analytical method was determined by GC—MS. The mass spectra for the bis(trimethylsilyl) derivatives of the authentic drug, and lorazepam separated by HPLC from the plasma of treated cynomolgus monkeys, were virtually identical (Fig. 4). The spectrum of the derivatised compound had a molecular ion at m/e 464 and characteristic fragments at m/e 449 (M − CH_3) and m/e 429 (M − Cl) all showing the expected isotope pattern of Cl-containing ions.

Concentrations of lorazepam in monkey plasma

After single oral doses of lorazepam to cynomolgus monkeys, a peak of mean concentrations of 12 ng/ml was reached at 2 h after dosing (Table IIa).

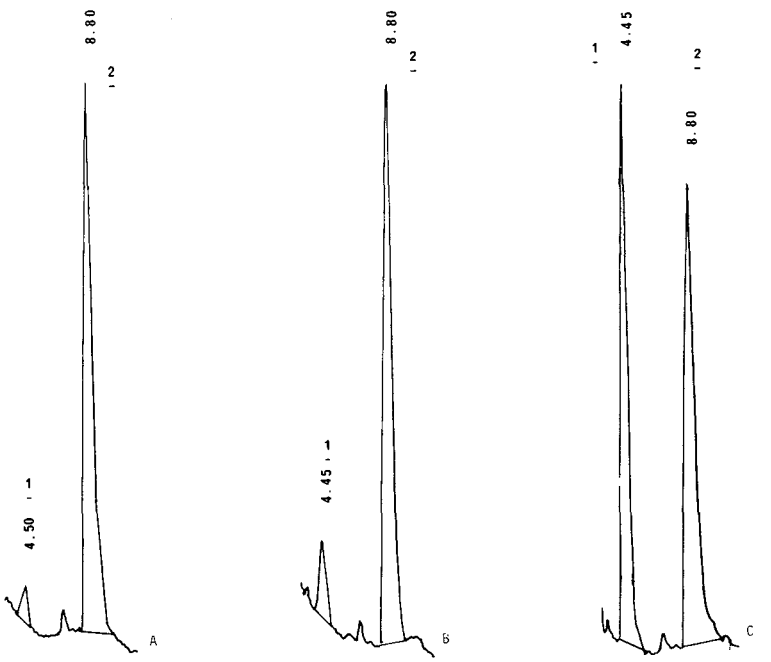

Fig. 3. Chromatograms of plasma standards containing (A) 5 ng/ml, (B) 10 ng/ml and (C) 100 ng/ml lorazepam. Conditions as for Fig. 2. Peaks: 1 = lorazepam, 2 = internal standard.

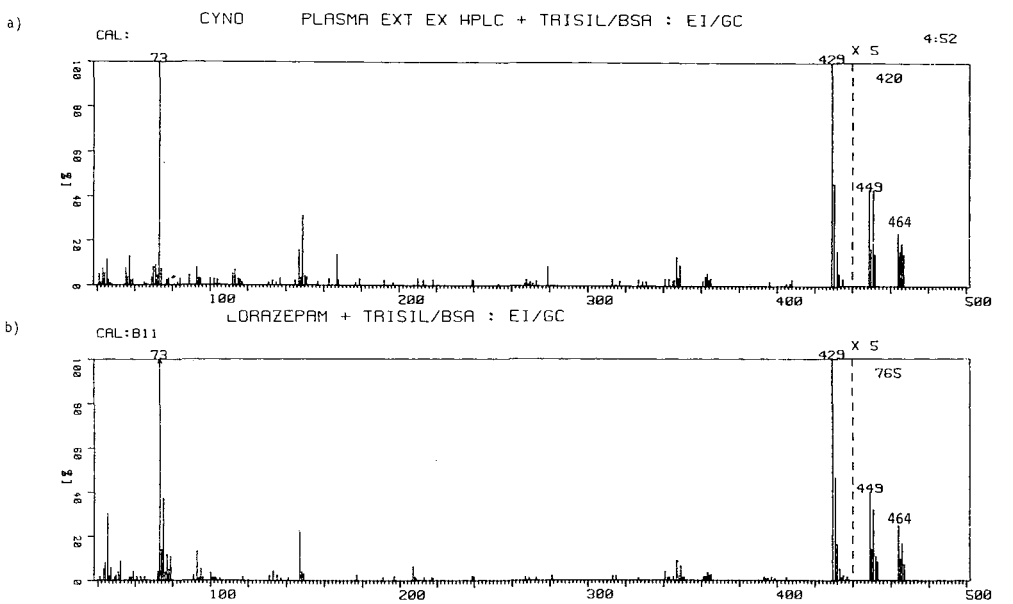

Fig. 4. Mass spectra of the trimethylsilyl derivatives of (a) lorazepam separated by HPLC from the plasma of a treated cynomolgus monkey and (b) authentic drug.

TABLE II

PLASMA CONCENTRATIONS OF LORAZEPAM (ng/ml) IN CYNOMOLGUS MONKEYS

Time (h)	Monkey number					Mean ± S.D.
	1	2	3	4	5	
(a) After single oral doses of 0.15 mg/kg						
0.25	4	6	6	4	6	5 ± 1
0.50	8	8	7	10	11	9 ± 2
0.75	8	7	10	8	16	10 ± 4
1.00	11	11	9	9	15	11 ± 2
1.50	8	7	9	15	17	11 ± 4
2.00	7	15	8	13	17	12 ± 4
3.00	4	12	8	9	11	9 ± 3
4.00	3	8	7	8	8	7 ± 2
5.00	<2	5	5	7	7	5 ± 3
7.00	2	5	<2	5	3	3 ± 2
(b) After single intravenous doses of 0.05 mg/kg						
0.03	46	84	24	78	74	61 ± 25
0.25	34	34	27	73	64	46 ± 21
0.50	22	28	23	56	48	35 ± 16
0.75	18	20	17	42	39	27 ± 12
1.00	12	16	14	37	31	22 ± 11
1.50	9	13	10	22	25	16 ± 7
2.00	8	10	9	20	18	13 ± 6
3.00	4	7	6	13	11	8 ± 4
4.00	<2	6	4	5	8	5 ± 3
5.00	<2	8	3	4	4	4 ± 3
7.00	<2	2	<2	<2	<2	<2

Thereafter mean concentrations declined to 3 ng/ml at 7 h after dosing. The mean half-life of lorazepam in the plasma of cynomolgus monkeys after 0.15 mg/kg oral doses was 2.5 h. This is markedly shorter than the half-life of the drug in human plasma (12—14 h) [12, 13] after single oral doses. The dose applied to the cynomolgus monkeys was three times the human clinical dose but rapid elimination of lorazepam by the cynmolgus monkey precluded accurate assessment of pharmacokinetic parameters at lower dose levels by this route of administration.

Plasma concentrations of lorazepam in the same monkeys after 0.05 mg/kg intravenous doses of the drug are shown in Table IIb. Mean plasma concentrations of lorazepam declined with a terminal half-life of 1.4 h. Representative plots of lorazepam concentrations in the plasmas of three cynomolgus monkeys after oral and intravenous doses are shown in Fig. 5. Plasma concentrations during the terminal β-phase after intravenous doses of lorazepam to monkeys are of the same order of magnitude as would be encountered after single therapeutic doses of lorazepam to humans.

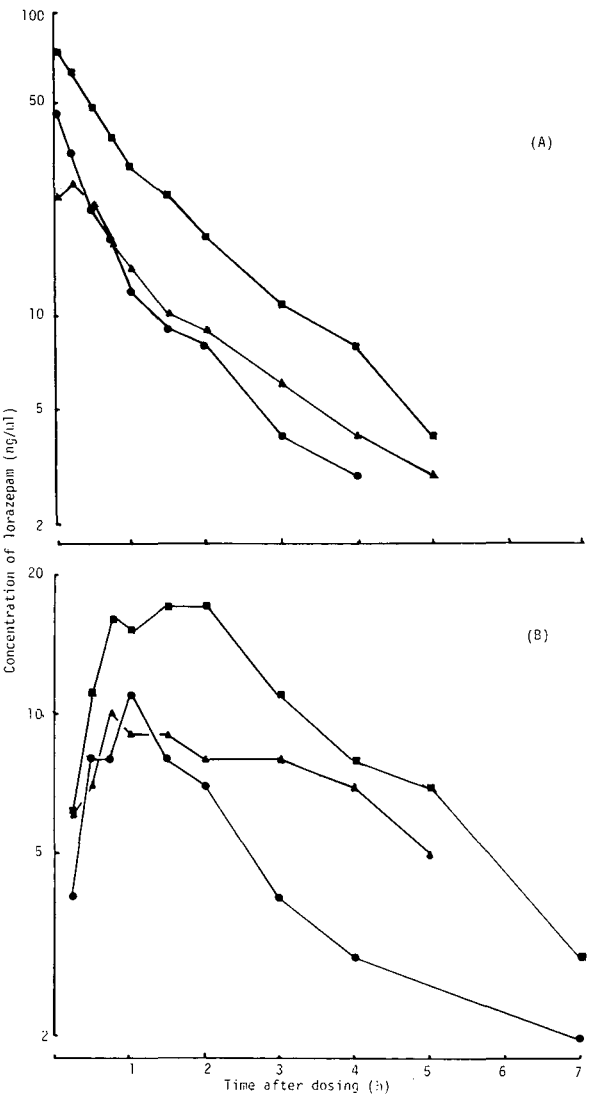

Fig. 5. Representative plots of concentrations of lorazepam in the plasma of cynomolgus monkeys after (A) an intravenous dose of 0.05 mg/kg and (B) an oral dose of 0.15 mg/kg. Semi-logarithmic scale. Symbols: ● = animal No. 1; ▲ = animal No. 3; ■ = animal No. 5.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION, ISOLATION AND IDENTIFICATION OF 1,2,3-THIADIAZOLE-5-CARBOXALDOXIME GLUCURONIDE IN RABBIT URINE

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SUMMARY

Reversed-phase high-performance liquid chromatography was used to separate and isolate the glucuronic acid conjugate of 1,2,3-thiadiazole-5-carboxaldoxime from urine of rabbits after intravenous injection of the oxime. The conjugate was identified by gas chromatography—mass spectrometry as its trimethylsilylated methyl ester and by nuclear magnetic resonance spectrometry. Additional information was obtained from thin-layer chromatography and high-voltage paper electrophoresis.

INTRODUCTION

Among other oximes 1,2,3-thiadiazole-5-carboxaldoxime (TDA) was tested as an antidote in organophosphate poisoning. To that end its physicochemical and biological properties were studied [1]. During this investigation high-performance liquid chromatography (HPLC) of the urine of rabbits that had been given TDA intravenously revealed that only a negligible amount of unaltered TDA was excreted, whereas at the same time a new UV-absorbing product was found. This paper deals with the separation, isolation and identification of the metabolised TDA.

EXPERIMENTAL

High-performance liquid chromatography

The HPLC equipment was assembled from two pumps (Waters Assoc. Model 6000A), a solvent programmer (Waters Assoc. Model 660), a Valco six-way sampling valve (Model CV-6-UHPa-N60) provided with a 100-μl sample loop

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for analytical purposes or with a 1-ml sample loop for preparative separations, and a variable-wavelength UV detector (Tracor Model 970). In all cases home-made HPLC columns were used. They were packed with LiChrosorb RP-18 according to a modified procedure of Lindner et al. [2]. Before application the performance of the columns was tested. The analytical columns (250 × 5 mm, 5-μm particles) showed 19,000 plates for cumene as test substance (k' = 1.7) in the mobile phase methanol—water (5:1, v/v) at a flow-rate of 1 ml/min. The semi-preparative column (250 × 10 mm, 10-μm particles) gave 6000 plates for cumene (k' = 1.7) in the same mobile phase at a flow-rate of 2 ml/min.

The following other mobile phases were used. (A) In the case of conjugate analysis the mobile phase was composed of water—methanol—acetic acid (98:2:0.5, v/v). During a chromatographic run a linear gradient was applied to change the mobile phase from 2 to 100% methanol in 30 min. (B) In the case of TDA the mobile phase consisted of water—methanol (4:1, v/v).

Thin-layer chromatography

Silica-gel F254 plates (Merck, No. 11798) were used. The plates were developed with ethyl acetate—water—acetic acid (5:2:2, v/v). The development chamber (20 × 20 × 10 cm) was saturated with the mobile phase. The compounds were detected using UV irradiation (254 nm) on thiadiazole nuclei and *p*-anisidine phthalate (PAP) spray reagent [3] on polyvalent alcohols.

High-voltage paper electrophoresis

The electrophoretic migration was carried out on Whatman No. 1 paper placed in a CAMAG-6100 high-voltage paper-electrophoresis (HVPE) system at pH 3.2 (sodium citrate + hydrochloric acid, 0.1 *M* buffer), 2000 V and 0.03 A.

Mass spectrometry

Electron-impact (EI) and chemical-ionisation (CI; isobutane as reaction gas) spectra were recorded on a VG Micromass 70-70F mass spectrometer using 60 eV electron energy and a source temperature of 200°C.

Gas chromatography

Gas chromatography (GC) combined with mass spectrometry (MS) was carried out on a Varian 1400 gas chromatograph. A glass capillary column (60 m × 0.7 mm I.D.) was used coated with SE-30 as stationary phase. The total ion current served as a detector.

Nuclear magnetic resonance

A Varian XL-100 NMR FT spectrometer was used. The samples were repeatedly dissolved in ^2H_2O and evaporated to dryness in order to remove free hydroxyl protons and were finally run in ^2H_2O solution using sample tubes with an internal diameter of 5 mm. In proton experiments a pulse delay was chosen so as to minimise the solvent peak.

Derivatization reagents

TDA conjugate was derivatized in two sequential steps as described by Compernolle et al. [4]. First the carboxyl group was esterified with diazo-

methane [5], followed by silylation of the hydroxyl groups using trimethylchlorosilane and hexamethyldisilazane [6]. In the case of TDA and the hydrolysed conjugate only the aforementioned silylation reagent was used.

Hydrolysis

The hydrolyses of the conjugate were carried out according to the following methods. (A) Acid hydrolysis: 4 *N* HCl was added to urine (in the ratio 1:1, v/v) and the mixture was set aside at room temperature for 30 min. (B) Enzymatic hydrolysis: a mixture of 2 ml of urine and 3 ml of 0.1 *M* sodium acetate buffer (pH 5.2) was incubated with 0.2 ml of a solution of β-glucuronidase + arylsulfatase (Boehringer, Mannheim, G.F.R.) at 37°C for 3 h.

RESULTS AND DISCUSSION

Urine from rabbits given an intravenous dose of 125 mg of TDA per kg was collected for 24 h. HPLC analysis (solvent system B, see Experimental) of the urine revealed that less than 0.2% of TDA was excreted unchanged [1]. Analysis of the urine using solvent system A (see Experimental) showed the appearance of a new UV-absorbing compound with $k' = 2.5$ as compared with the urine of untreated rabbits (Fig. 1).

The new compound eluted from the HPLC column with a retention time different from that of TDA ($k' = 7$). The addition of acetic acid to the mobile phase proved to be essential to obtain a better separation between the TDA

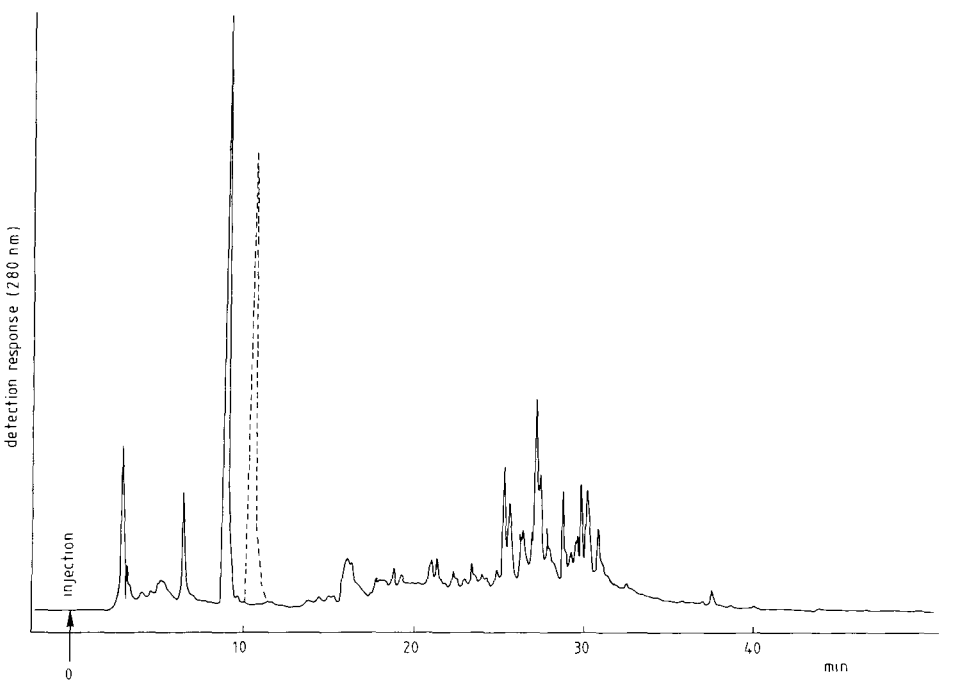

Fig. 1. Typical chromatogram of urine samples from rabbits. — — , urine from untreated rabbits; — — —, TDA conjugate found after intravenous administration of TDA.

TABLE I

THIN-LAYER CHROMATOGRAPHY* OF TDA CONJUGATE AND ITS HYDROLYSATE

Sample	R_F	Characterized as	Detection
TDA conjugate	0.46	—	UV + PAP
Hydrolysed TDA conjugate	0.18	Glucuronolactone	PAP
	0.64	Glucuronic acid	PAP
	0.99**	TDA	UV

*For conditions see Experimental.
**Using ethyl acetate as the mobile phase R_F of TDA is 0.70.

conjugate and the front peaks with k' about zero. On addition of acetic acid the retention time of the TDA conjugate increased considerably. This effect may be explained by assuming the presence of a carboxyl group in the conjugate the dissociation of which decreases on addition of acetic acid. As a result the compound will become more lipophilic and consequently will adhere more strongly to the stationary phase. This assumption of a carboxyl group was sustained by the results of an HVPE experiment in which the conjugate migrated to the anode whereas TDA stayed at the origin.

The HPLC fraction containing the conjugate was compared with a hydrolysed sample (hydrolysis method A, see Experimental) using thin-layer chromatography. The results are presented in Table I. The R_F values and detection results obtained from the hydrolysed conjugate correspond with those of the glucuronolactone, glucuronic acid and TDA, which could be confirmed by the application of GC—MS after silylation of the hydrolysed TDA conjugate. The gas chromatogram is presented in Fig. 2.

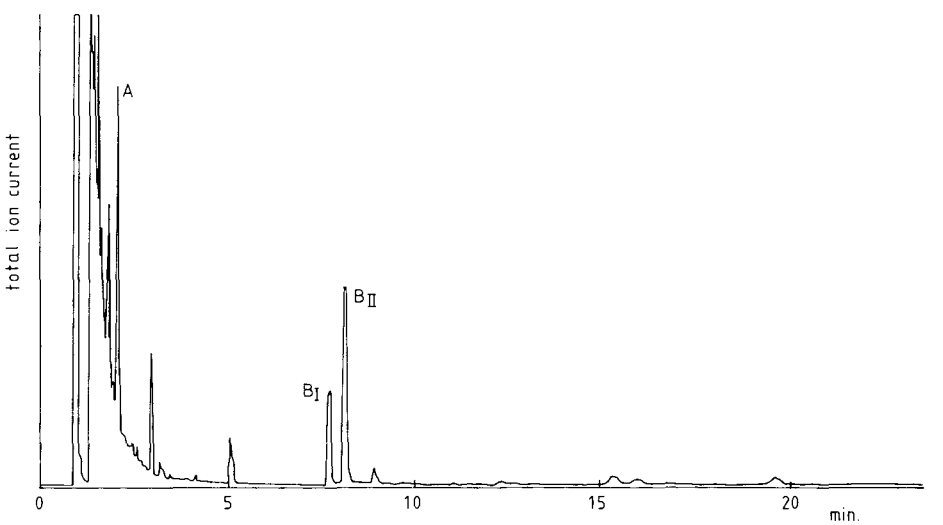

Fig. 2. Gas chromatogram of hydrolysed TDA conjugate after silylation. Column temperature = 200°C. Peaks: A = TMS-TDA; B_I = TMS-α-glucuronolactone; B_{II} = TMS-β-glucuronolactone (TMS = trimethylsilyl).

Table II gives the mass spectra taken from the GC peaks A, B_I and B_{II}. These spectra proved to be identical with those of trimethylsilylated TDA and trimethylsilylated α- and β-glucuronolactone [7], respectively, as was concluded from the use of reference compounds. The presence of the mixture of α- and β-glucuronolactones may be explained by isomerisation of glucuronic acid during the hydrolysis under acid conditions. From these experiments it was concluded that the TDA conjugate is most likely a conjugate of TDA and glucuronic acid.

TABLE II

THE MASS SPECTRA* (EI) OF GAS CHROMATOGRAPHIC PEAKS A, B_I AND B_{II} AS PRESENTED IN FIG. 2

A, B_I and B_{II} were identified as TMS-TDA, TMS-α-glucuronolactone and TMS-β-glucurolactone, respectively (TMS = trimethylsilyl).

Peak A		Peak B_I		Peak B_{II}	
m/e	Rel. Int. (%)	*m/e*	Rel. Int. (%)	*m/e*	Rel. Int. (%)
43	5	45	8	45	8
45	12	73	100	73	100
47	5	74	8	74	9
57	6	75	25	75	18
59	10	103	6	103	7
73	100	133	5	129	6
74	10	147	28	147	20
75	19	217	10	189	5
103	6	230	67	217	15
131	8	231	14	230	73
201	12	232	5	231	18
		259	5	232	6
		287	11	243	7
				245	8
				287	6
				377	7

*Relative intensities $\geq$ 5%.

Without any derivatization both MS (EI, direct inlet) and GC—MS analysis of the isolated conjugate showed only one substance that could be identified as 5-cyano-1,2,3-thiadiazole. Neither the mass spectrum (EI, direct inlet) nor the gas chromatogram showed any sign of glucuronic acid. This fact indicates that extreme care must be taken in interpreting a cyano derivative as an oxime metabolite [8—10]. Due to thermal instability an oxime conjugate may decompose into a cyano derivative during isolation and analysis.

A stable and volatile derivative of the TDA conjugate was only obtained after methylation followed by silylation, otherwise the conjugate decomposed at high temperatures. In Fig. 3 the gas chromatogram of this derivatized TDA conjugate is presented. The CI-mass spectrum is given in Table III. Besides the protonated molecular ion (*m/e* = 536) the mass spectrum showed the ions *m/e* = 407, 317, 275 and 217, which are characteristic for the glucuronic acid part

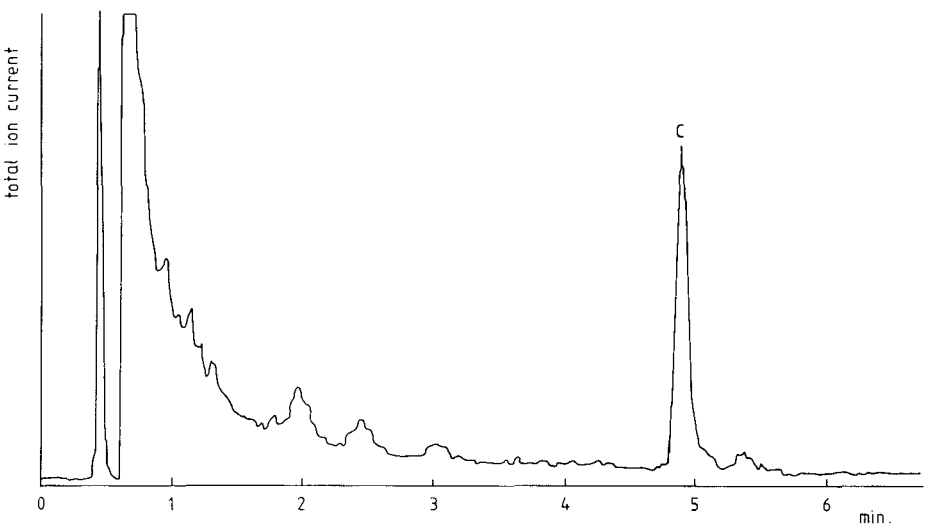

Fig. 3. Gas chromatogram of the methylated and silylated TDA conjugate. Column temperature = 260°C. For the CI-MS data of peak C see Table III.

TABLE III

MASS SPECTRUM* (CI) OF PEAK C IN FIG. 3

m/e	Rel. Int. (%)	*m/e*	Rel. Int. (%)	*m/e*	Rel. Int. (%)	*m/e*	Rel. Int. (%)
73	26	119	6	187	6	275	10
75	16	130	11	202	6	317	100
89	6	131	5	204	7	318	26
91	43	155	5	215	5	319	13
112	16	159	6	217	19	335	5
114	19	172	6	227	9	407	14
117	5	175	6	245	12	408	6
						536	12

* Relative intensities ⩾ 5%.

of the conjugate derivatized as the trimethylsilyl and methyl ester [11]. The TDA part is represented by the ions *m/e* = 130, 114 and 112, which correspond with protonated TDA, TDA ($M^+ - O$) and TDA ($M^+ - H_2O$), respectively.

To carry out ^1H- and ^{13}C-NMR experiments milligram amounts of the TDA conjugate were isolated using the semi-preparative HPLC column (mobile phase A, see Experimental). Volumes of up to 0.5 ml of the urine samples could be injected, giving only a small loss in separation efficiency in comparison with the analytical column. Fig. 4 shows the 100 MHz proton spectrum of the isolated TDA conjugate in ^2H_2O. The numbering of the peaks refers to the corresponding atoms in the formula. The peaks at δ = 9.2 and 8.3 ppm belong to the protons of the thiadiazole ring, which has been verified by means of a reference spectrum of TDA giving the same δ values. The doublet at δ = 5.2 ppm is characteristic for the glucosidic proton 1'. The high value for the coupling

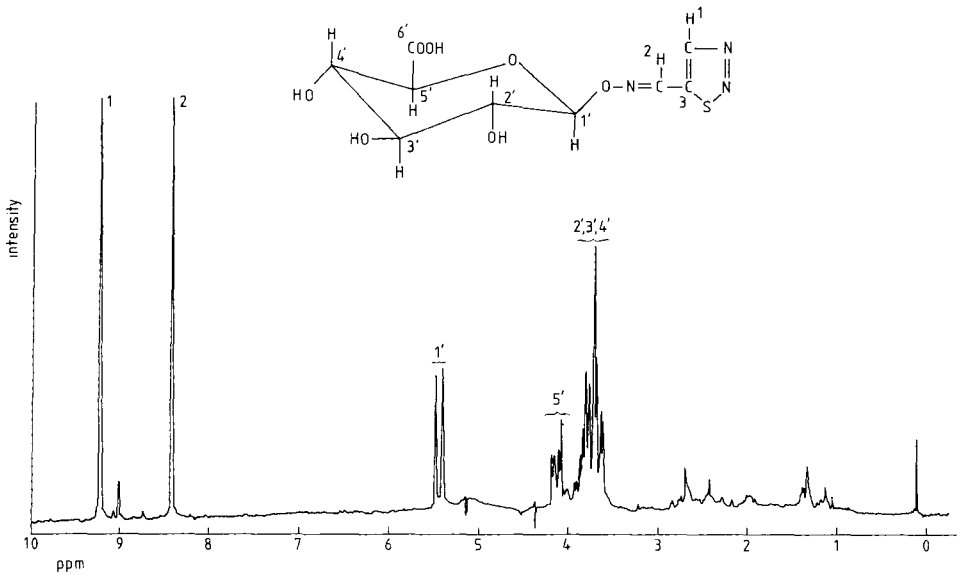

Fig. 4. 100 MHz ^{1}H-NMR spectrum of the isolated TDA conjugate together with the proposed chemical structure. Reference: sodium salt of 3-(trimethylsilyl)propanesulphonic acid.

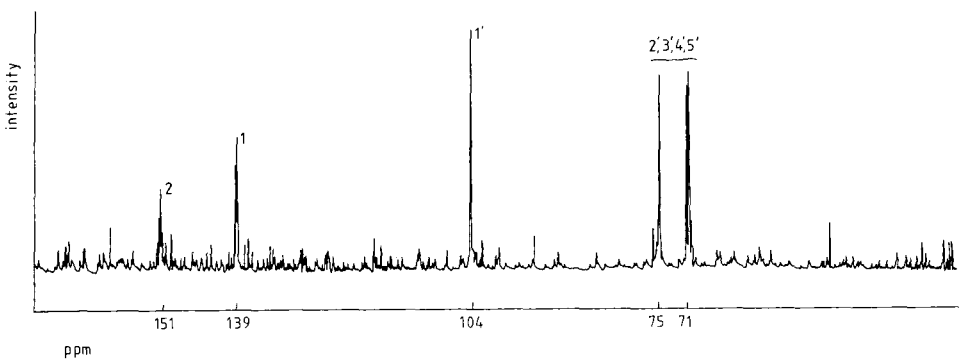

Fig. 5. ^{13}C-NMR spectrum of the isolated TDA conjugate. Numbering is according to chemical structure given in Fig. 4.

constant (J = 7.8 Hz) indicates a *trans* position to the next ring proton 2′ or an axial orientation [12], and points to a β-configuration of the glucosidic linkage [6]. From de-coupling experiments it could be demonstrated that the peak δ = 4 ppm may be ascribed to the proton at site 5′ of the chemical structure. The multiplet at δ = 3.6 ppm belongs to the protons 2′, 3′ and 4′.

Fig. 5 represents the ^{13}C-NMR spectrum of the isolated TDA conjugate. The numbering of the peaks refers to that of the atoms of the chemical structure presented in Fig. 4. The signals at δ = 139 and 151 ppm have been derived from the correspondingly coded carbon atoms (1 and 2) in the thiadiazole part of the conjugate as could be found from reference experiments with TDA. When comparing signals from carbon atoms (1′) in the conjugate and in glucuronic

acid (mixture of α and β form) it was found that the peaks at δ = 92—96 ppm had shifted to δ = 104 ppm in the conjugate linkage. The peaks at δ = 71—75 ppm are comparable with those of glucuronic acid derived from the carbon atoms coded as 2′, 3′, 4′ and 5′. The signals from the other carbon atoms (3 and 6′) were too faint to distinguish them from the noise level because of the relative insensitivity for this type of carbon atom. The ^{13}C-NMR spectrum of (α + β)-glucuronic acid is presented in Fig. 6. From the aforementioned NMR experiments it can be concluded that the conjugate consists of TDA and glucuronic acid linked together by a β-linkage. The presence of a β-glucosidic linkage was further confirmed when TDA was liberated from the isolated TDA conjugate on incubation with β-glucosidase.

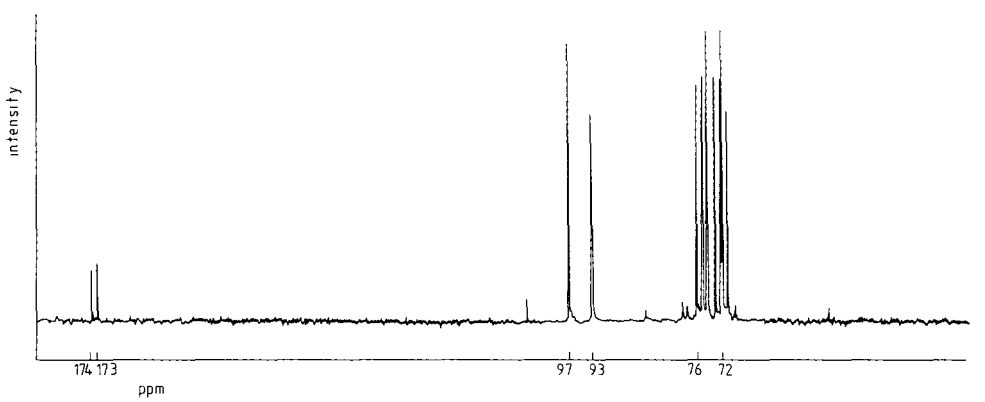

Fig. 6. ^{13}C-NMR spectrum of (α + β)-glucuronic acid.

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CHROMBIO. 992

DETERMINATION OF THERAPEUTIC PLASMA CONCENTRATIONS OF TETRABENAZINE AND AN ACTIVE METABOLITE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic method for the determination of tetrabenazine and a hydroxy metabolite in plasma is described. Tetrabenazine and the hydroxy metabolite are quantified as their dehydro derivatives using fluorescence detection. This method has been applied to the analysis of plasma samples from patients with Huntington's chorea and has been found to be sensitive, reliable and specific for tetrabenazine and the hydroxy metabolite. The plasma concentrations of tetrabenazine found in patients were lower than could be detected using previously published methods.

INTRODUCTION

Tetrabenazine (TB) is currently used to treat the abnormal movements of Huntington's chorea and other disorders [1]. Although its urinary metabolites have been described [2] little is known of its pharmacokinetics in man.

In order to study relationships between dose, plasma levels and therapeutic effect of TB, a reliable and sensitive method to estimate the plasma concentrations of TB and its metabolites is necessary. Quinn et al. [3] have described a spectrofluorometric method for the determination of TB in plasma and tissues. This method detects levels of 2—5 μg in plasma and relies on the extraction procedure for specificity. The sensitivity of the method was improved by a derivatization step [4], the limit of sensitivity being between 30 and 200 ng per g of biological material. This method does not appear to have excluded interference by metabolites. In the application of these methods to quantifying the distribution of TB in animal tissues [3, 5], no differentiation between the concentrations of TB and any of its metabolites in these tissues was attempted.

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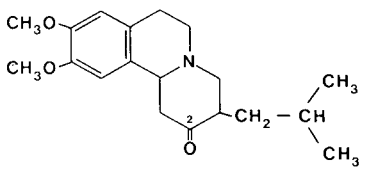
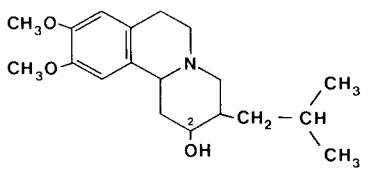


TETRABENAZINE



HYDROXYTETRABENAZINE

Fig. 1. Structures of tetrabenazine and its metabolite, hydroxytetrabenazine.

Nine metabolites of TB have been detected in urine after its administration to man [2].

This paper describes a reversed-phase, high-performance liquid chromatographic (HPLC) method for the determination of TB and a hydroxy metabolite (HTB, Fig. 1) in plasma. This method has been applied to the analysis of plasma samples from patients with Huntington's chorea and has been found to be sensitive, reliable and specific for TB and HTB. Plasma concentrations of TB found in patients were well below levels detectable by previous methods [3, 4].

EXPERIMENTAL

Standards and reagents

Tetrabenazine (1,3,4,6,7,11*b*-hexahydro-3-isobutyl-9,10-dimethoxy-2H-benzo[*a*]quinolizin-2-one) and the *cis* and *trans* isomers of the 2-hydroxy derivative of tetrabenazine (HTB) were kindly supplied by Roche Products (Sydney, Australia).

The mercuric acetate reagent used for the derivatization of TB and HTB to their dehydro derivatives [2] consisted of 2.0% mercuric acetate in pH 4 acetate buffer [96 ml glacial acetic acid, 32 ml sodium hydroxide (40%) and 4 ml distilled water] [4].

Acetonitrile, methanol (specially purified for HPLC) and PIC B-5 (pentanesulphonic acid) were supplied by Waters Assoc. (Milford, MA, U.S.A.). All other chemicals were reagent grade.

Instrumentation

Reversed-phase HPLC was performed using a Waters Model M45 solvent delivery system, a U6K universal injector, a Phenyl μBondapak column and a C_{18} μBondapak column (each 30 × 3.9 mm I.D., 10 μm average particle size). A guard column packed with Bondapak C_{18}/Porasil B was used in all studies. The absorbance of the eluent was determined using a Waters Model 450 variable-wavelength UV absorption detector. The fluorescence of the eluent was monitored using a Schoeffel PS970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.).

Liquid chromatography

The influence of the following parameters on the chromatography of TB and its dehydro derivative were examined: pH, buffer composition, acetonitrile concentration, methanol concentration, addition of PIC reagents to mobile phase and support type.

Thin-layer chromatography

Since authentic samples of most TB metabolites were not available, thin-layer chromatography (TLC) of urine extracts from patients on TB therapy was carried out to identify and isolate individual metabolites. Urine (50 ml) was concentrated to 10 ml with a rotary evaporator (Büchi, Flawil, Switzerland) made to pH 5.2 with 1 ml 1 *M* acetate buffer and incubated at 37°C overnight with 0.5 ml ext. Helix pomatia (Boehringer, Mannheim, G.F.R.) to hydrolyze glucuronide conjugates. This solution was then adjusted to pH 9.6 with 5 *M* sodium hydroxide and TB and its metabolites extracted into 25 ml ethyl acetate by vortexing for 30 sec. After centrifuging at 1500 g for 10 min, the ethyl acetate layer was removed and evaporated to dryness. The residue was redissolved in 2 ml dichloromethane and spotted on a 0.25 mm thick silica gel plate (Kieselgel G, Merck, Darmstadt, G.F.R.). The plate was developed with a chloroform—*n*-butanol—2.5% ammonia solution (80:20:0.6) system and spots were visualized by converting TB metabolites to their dehydro compounds with mercuric acetate reagent [2]. Spots were scraped off and extracted in 2 ml methanol. The methanol was evaporated to dryness under nitrogen and the residue dissolved in 1 ml 0.25 *M* sulphuric acid prior to HPLC.

Plasma assay

Procedure. Plasma samples were processed by transferring 2 ml into a glass centrifuge tube containing 1 ml 0.1 *M* carbonate buffer (pH 9.6), and 2.5 ml diethyl ether were added. After the sample was vortexed for 20 sec it was centrifuged for 10 min at 1500 *g*. The lower aqueous phase was snap-frozen with acetone—dry ice and the ether decanted into a calibrated 3-ml glass centrifuge tube. The ether was evaporated to about 1 ml in a 40°C water bath prior to the addition of 100 µl 0.5 *M* sulphuric acid. This mixture was then vortexed for 15 sec and centrifuged for 5 min at 1500 *g*. The lower aqueous phase was snap-frozen with acetone—dry ice and the upper ethereal layer discarded. The aqueous phase was mixed with 100 µl mercuric acetate reagent, heated in a boiling water bath in an all-glass stoppered centrifuge tube for 30 min and cooled. The tubes had been tared and were made to weight with distilled water after heating. An aliquot (20 µl) of this solution was then injected onto the liquid chromatographic system consisting of a C_{18} µBondapak column and fluorescence detector. The mobile phase was acetonitrile—1% acetate buffer, pH 4.5 (50:50) at a flow-rate of 1 ml/min. The fluorescence of the eluent was quantified using an excitation wavelength of 265 nm and an emission filter (KV418).

Quantitation. The procedure was standardized by analyzing drug-free plasma samples spiked with known amounts of the analytes. Calibration curves were established by plotting absolute peak heights of analytes for a given injection

volume against plasma analyte concentration. Standards were run each day to control changes in detector response.

Recovery. Recoveries from plasma were estimated by a comparison of peak heights obtained from the injection of known quantities of the analytes in aqueous solutions treated with mercuric acetate reagent and known quantities added to plasma before extraction and derivatization.

Reproducibility. Within-day precision was determined by performing replicate analyses of spiked plasma samples.

Specificity. The specificity of the plasma assay for TB and HTB was verified by injection of other TB metabolites which had been separated by TLC and by processing plasma samples from patients on various medications.

RESULTS AND DISCUSSION

Chromatographic behaviour and fluorescence of tetrabenazine

Preliminary studies on the chromatographic behaviour of TB were carried out with a Phenyl μBondapak column and UV absorbance detection. TB at a concentration of 3 μg/ml gave a good symmetrical peak at 282 nm after 7 min with a methanol—0.01 *M* ammonium hydrogen phosphate, pH 6.5 (60:40) system. However, no detectable concentrations of TB were found in plasma extracts from patients on steady-state TB therapy for Huntington's chorea when the eluent was monitored for absorbance. With this mobile phase, TB showed no fluorescence. Fluorescence of TB was only significant in an acidic mobile phase. Unfortunately, with the various acidic mobile phases tried, tetra-

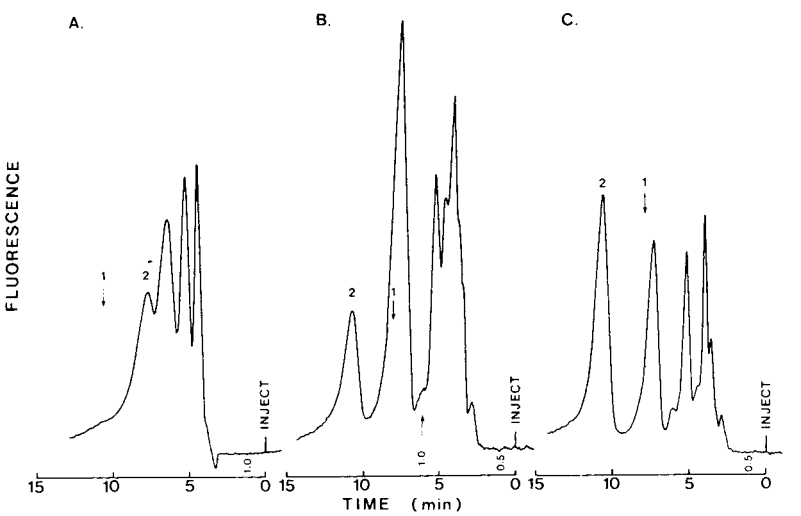


Fig. 2. Chromatograms of urine extracts from a patient being treated with TB. Urine was extracted as described for thin-layer chromatography, but heated with mercuric acetate reagent before HPLC. Mobile phase and λ_{ex}: (A) methanol—1% acetate buffer, pH 4.0 (60:40), 310 nm; (B) acetonitrile—1% acetate buffer, pH 4.5 (50:50), 315 nm; (C) acetonitrile—1% acetate buffer, pH 4.5 (50:50), 265 nm. (1) shows the retention of DTB in each system (TB was not found in urine) and (2) shows the position of DHTB. Fluorescence range is shown in μA, and the arrow marks a change.

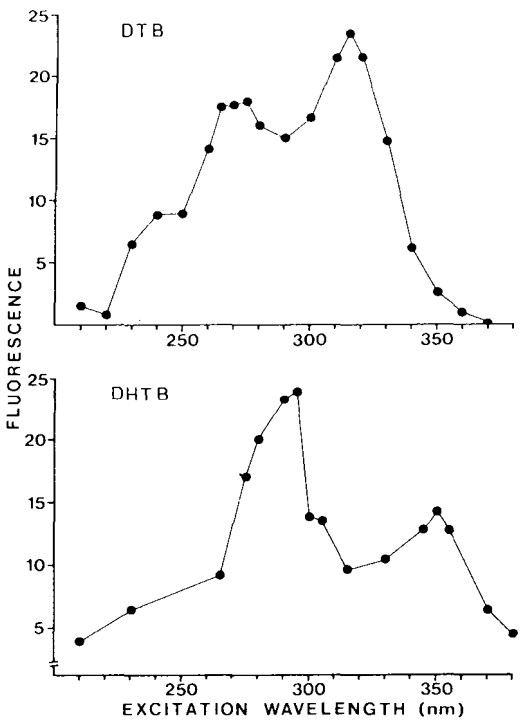


Fig. 3. Excitation spectra of DTB and DHTB. Each point was obtained by injecting a 2-ng sample onto the HPLC system.

benazine did not resolve sufficiently from the solvent front for quantitative studies.

Satisfactory fluorescence and column retention could only be achieved after derivatization of tetrabenazine. TB and HTB form highly fluorescent dehydro derivatives (DTB and DHTB) when heated in the presence of mercuric acetate [4]. Maximum fluorescence was observed after heating with mercuric acetate in a boiling water bath for 30 min. Heating of HTB for 40 min (or longer) resulted in a diminished fluorescence of DHTB and an extraneous peak on the chromatogram.

The composition of the mobile phase was found to be critical for the separation of DTB and DHTB (Fig. 2A, B). The lower excitation maximum for DTB (265 nm, Fig. 3) was used to reduce the potential interference from a urinary metabolite of TB (Fig. 2C), which was observed at 310 nm (Fig. 2B). In all HPLC systems used, we were unable to resolve the two stereoisomers of DHTB. They also had the same fluorescence intensity over all excitation wavelengths (Fig. 3). Maximum fluorescence intensity for DHTB was found at 305 and 350 nm, but satisfactory sensitivity was present at 265 nm.

Plasma assay

Fig. 4 shows chromatograms of blank plasma and plasma from a patient on TB therapy. The retention times for DTB and DHTB were 7.0 and 9.7 min, respectively. Approximately 0.1 ng/ml of TB and 1 ng/ml of HTB in plasma

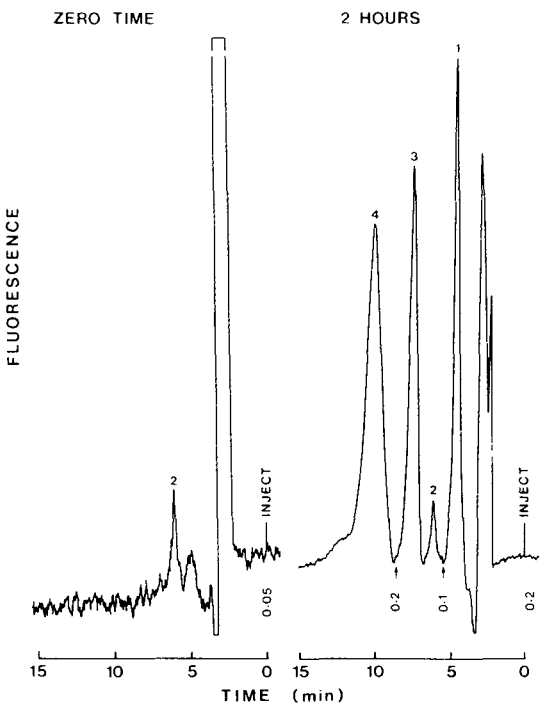


Fig. 4. Chromatograms of extracts of plasma taken before (zero time) and 2 h after a single oral dose of TB (50 mg). Fluorescence range is shown in μA, and changes are marked with an arrow. Peaks: (1) metabolite(s) of TB; (2) plasma; (3) DTB; (4) DHTB.

could be detected. Other metabolites of TB found on thin-layer plates eluted together (Fig. 4) and could not be quantified.

A number of potential internal standards were evaluated for the plasma assay. Unfortunately, a substance having both a desirable retention time and an acceptable fluorescence emission was not found. Plasma from patients taking a variety of medications (Table I) were found not to contain any substances which interfered with the assay.

Standard curves of the fluorescence plotted against plasma concentrations for DTB and DHTB gave correlation coefficients of 0.998 and 0.997, respec-

TABLE I

DRUGS TESTED FOR INTERFERENCE

Amiloride	Hydrochlorothiazide
Amoxycillin	Methyldopa
Bendrofluazide	Nitrazepam
Chlorothiazide	Potassium chloride
Digoxin	Prednisolone
Ferrous sulphate	Propranolol
Folic acid	Pyridoxine
Furosemide	Salicylate
Glyceryl trinitrate	Vancomycin
Heparin sulphate	

tively. The good linearity of response was found to be consistently reproducible for each set of standards prepared. The precision of the assay is given in Table II. For both TB and HTB the coefficient of variation was less than 5%. The recoveries of TB and HTB from plasma after extraction were 74% and 65%, respectively.

TABLE II

REPRODUCIBILITY OF ASSAY

	Concentration (ng/ml)	Mean	*n*	S.D.	C.V. (%)
Tetrabenazine	2.5	4.85	8	0.20	4.1
	10.0	18.98	10	0.51	2.7
Hydroxytetrabenazine	10.0	26.34	10	0.64	2.4

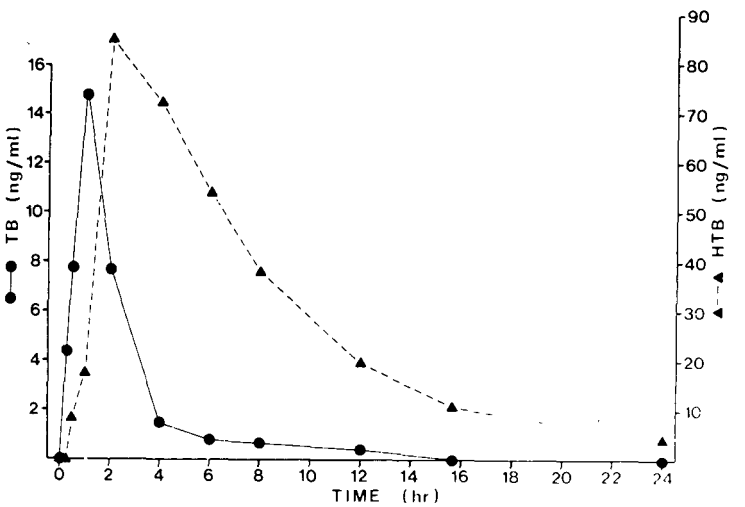


Fig. 5. Plasma concentrations of TB and HTB following a single 50-mg oral dose administered to a fasting patient.

The application of the present method to the analysis of TB and HTB in the plasma of a patient given a single 50-mg dose of TB is shown in Fig. 5. It is observed that the plasma concentrations of TB are lower and present for a shorter period of time after dosing than those of HTB. These high levels of HTB may be clinically significant because HTB is known to have brain amine depleting activity similar to that of TB [6]. These high concentrations of HTB also demonstrate the need for an assay for TB and HTB which is not only sensitive but also specific.

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CHROMBIO. 1003

DETERMINATION OF IBUPROFEN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND APPLICATION TO IBUPROFEN DISPOSITION

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SUMMARY

A high-performance liquid chromatographic method for quantitation of ibuprofen from serum and application of this method to ibuprofen disposition in the dog is described. The drug was extracted from acidified plasma with dichloromethane. The internal standard used was a methanolic solution of 4-*n*-butylphenylacetic acid. A μBondapak C_{18} column was used for analysis; the mobile phase was methanol—water—glacial acetic acid (pH 3.4) (75 : 24 : 1, v/v). A wavelength of 272 nm was used to monitor ibuprofen and the internal standard.

Method sensitivity was 0.5 μg/ml serum using either 0.5 or 1.0 ml of sample, and no interference was found from endogenous compounds or other commonly used anti-inflammatory agents. The coefficients of variation of the method were 4.2% and 6.0% for samples containing 50.0 and 6.25 μg/ml of ibuprofen, respectively, and the calibration curve was linear for the range of 0.5 to 100 μg/ml. This method was demonstrated to be suitable for pharmacokinetic and/or biopharmaceutical studies of ibuprofen in man and the dog.

INTRODUCTION

Ibuprofen [DL-2-(*p*-isobutylphenyl)propionic acid, IBU] is a 2-phenylalkylcarboxylic acid derivative possessing potent anti-inflammatory, antipyretic and analgesic properties [1—3]. It is used extensively for the treatment of adult and juvenile rheumatoid arthritis [3—5], and additionally in the treatment of pain associated with dysmenorrhea [6] and for antipyresis [7].

IBU and its metabolites have been assayed in plasma by a variety of techniques including gas—liquid chromatography (GLC) with derivatization [8], colorimetric determination of a copper complex [9], GLC with electron-capture detection [10], and a combined gas chromatography—mass spectrometry

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method [11]. Several GLC methods have obviated the need for derivatization [12, 13] and hence have simplified the analysis of IBU. These methods, however, require 1.0—2.0 ml of plasma, and thereby render a pharmacokinetic or bioavailability study of IBU in children difficult.

Pitrè and Grandi [14] measured IBU in 1.0-ml samples of spiked canine plasma by utilizing high-performance liquid chromatography (HPLC). They did not, however, demonstrate appropriate utility or evaluate their method in a biological system. We developed a rapid, specific, and sensitive HPLC method for IBU in serum volumes of 0.5—1.0 ml. Application of this method to human serum and to IBU disposition in the dog is described. Suitability of the method for therapeutic drug monitoring is discussed.

MATERIALS AND METHODS

Standards

IBU and 4-*n*-butylphenylacetic acid, the internal standard (IS), were donated by Boots Pharmaceutical (Nottingham, Great Britain).

Preparation of sample

Ten micrograms of the IS were added to a serum sample (0.5 or 1.0 ml) contained in a 12.0-ml conical glass-stoppered tube. Hydrochloric acid (5 *M*, 0.2 ml) was added to precipitate serum proteins. Dichloromethane (3.0 ml) was added and the extraction was performed on a shaking board mixer for 10 min. The sample was centrifuged for 5 min at 1500 *g*, the clear upper aqueous layer removed by aspiration and the lower dichloromethane layer decanted into a 3.0-ml conical vial. The organic layer was evaporated to dryness under a gentle stream of nitrogen in a 37°C water bath. Evaporated samples were reconstituted with 40 μl of the HPLC eluent, mixed thoroughly and recentrifuged at 1500 *g* for 10 min. The clear supernatant fraction was transferred to a sample vial and capped in preparation for automated analysis.

Conditions of analysis

A Waters Assoc. (Milford, MA, U.S.A.) HPLC apparatus with the following instrumentation was used: M6000A Solvent Delivery System, Waters Intelligent Sample Processor (WISP), Data Module and M450 variable-wavelength UV detector. A μBondapak C_{18} column, particle size 10 μm (reversed-phase) preceeded by a guard frit and a guard column packed with C_{18}/Corasil bulk packing were also used (Waters Assoc.). The eluent (pH 3.4) was a mixture of 75% methanol (Waters Assoc.), 24% deionized water and 1% glacial acetic acid. All solutions were prefiltered with an FH-type filter (Millipore, Bedford, MA, U.S.A.), prewet with methanol.

The flow-rate was 1.5 ml/min at room temperature (20—22°C). The WISP and Data Module were programmed to inject 5.0—20.0 μl of the extracted samples. Both IBU and the internal standard were eluted for a period of 6 min. Absorbance detection at a wavelength of 272 nm and an instrument range setting of 0.01 were used to monitor both compounds.

RESULTS

Chromatogram and elution characteristics

A typical chromatographic tracing is seen in Fig. 1. Variable injection volumes (5—20 μl) were used to ensure appropriate resolution characteristics for peak height determination. With a constant flow-rate (1.5 ml/min) and chart recording speed of 1 cm/min, IBU and the internal standard eluted at retention times of 4.4 and 3.97 min, respectively. Both compounds consistently produced symmetrical peaks with minimal tailing. Adequate separation was obtained during the elution of both compounds (α-value of 1.22 for IBU).

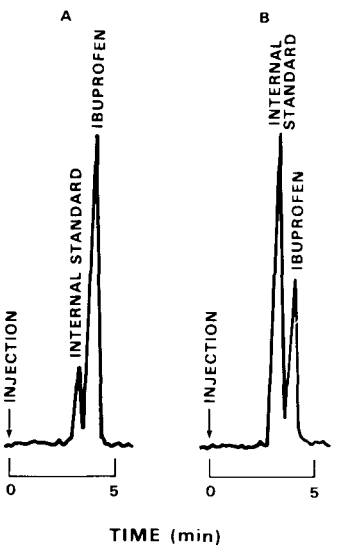

Fig. 1. Representative chromatographic tracings of 1.0-ml human serum, spiked with (A) 50 μg/ml and (B) 6.25 μg/ml ibuprofen. Detector sensitivity was changed in the recording of the tracings for qualitative purposes.

Standard curve and method sensitivity

Three standard curves were prepared using 1.0 ml of canine serum, and 0.5 and 1.0 ml of human serum. Correlation analysis was performed on each set of data obtained from the standard curves and the following tests were conducted: (1) a *t*-test; the null hypothesis (H_0) being that the slope of the regression line equals zero; (2) a correlation coefficient (r), H_0 being that x (IBU serum concentration in μg/ml) and y (the ratio of the peak heights calculated by dividing the IBU peak height by that of the internal standard) are independent; and (3) an analysis of variance, H_0 being that the regression of y on x is not linear. The p values for the t, r and analysis of variance were less than 0.005 in all cases, substantiating linearity of each standard curve to an IBU concentration of 100 μg/ml. Table I summarizes the results obtained from the linear regression analysis for each of the standard curves. The detection limit of 0.5 μg/ml and linearity through 100 μg/ml render the method suitable for monitoring IBU concentrations commonly found in man after therapeutic administration of the drug [2].

TABLE I

IBUPROFEN STANDARD CURVES IN CANINE AND HUMAN SERUM

Sample	$n^{\star}$	Equation	$r^{\star\star}$	$F^{\star\star\star}$
1.0 ml human serum	7	$y = 0.089 + 0.061x$	0.999§	2268.95§
1.0 ml canine serum	5	$y = 0.093 + 0.068x$	0.999§	4163.67§
0.5 ml human serum	1	$y = 0.120 + 0.07x$	0.999§	1955.82§

$^{\star}n$ = Number of replicate samples analyzed at each of six concentration points in the construction of the standard curves.
$^{\star\star}r$ = Correlation coefficient.
$^{\star\star\star}F$ = Analysis of variance for linearity.
§$p < 0.005$.

Comparison of standard curves using a paired *t*-test revealed no statistically significant differences in the slopes or intercepts for dog vs. human serum, or for 1.0 vs. 0.5 ml of human serum. Rearrangement of the equation from the linear regression analysis of a standard curve permits calculation of IBU plasma concentrations from a corresponding peak height ratio determined from a given serum sample

Variability and stability

Serum samples containing 6.25 and 50.0 μg/ml of IBU, respectively, were extracted and prepared for analysis as described. Variability was assessed by analysis of seven extracted serum samples of both concentrations. The coefficients of variation for replication of the extraction were 4.2% and 6.0% for serum samples containing 50.0 and 6.25 μg/ml of IBU, respectively.

Sample stability in the automated sample processor (WISP) was assessed by analyzing extracted serum samples (n = 7), corresponding to IBU concentrations of 6.25 and 50.0 μg/ml, for a 10-h period. The average variation was 5.45% (range 47.5—58.2 μg/ml) for the 50.0 μg/ml standard and 6.27% (range 5.21—5.92 μg/ml) for the 6.25 μg/ml IBU standard. There was no trend indicative of compound instability while samples resided for 10 h in the WISP.

Variability in the automated sampling process was assessed by determining the reproducibility of absolute peak heights from ten 10-μl injections of the internal standard (which corresponded to a concentration of 100 μg/ml). Variability of automated sampling was minimal as revealed by a coefficient of variation of 3.3%.

The stability of frozen aliquots of human serum containing 50.0 and 6.25 μg/ml of IBU was examined by analysis of extracted samples (n = 6) of each concentration for ten days. The variations ($\bar{x}$ ± S.E.) for the samples were 5.46 ± 1.24% and 10.22 ± 2.35%, respectively. There was no trend indicative of compound instability as a result of freezing and thawing for at least ten days.

Selectivity

Table II contains the resolution characteristics for IBU, structurally and non-structurally related analgesic compounds, and substances which could be found concomitantly with IBU in plasma. The only compound evaluated which

TABLE II

RESOLUTION CHARACTERISTICS OF IBUPROFEN, OTHER ANALGESIC COMPOUNDS, AND POTENTIAL INTERFERING SUBSTANCES

Each substance was prepared as a 20% methanol in water solution and injected without prior extraction. Detection was evaluated at 272 nm during a 10-min elution period. The separation factor, α, was calculated with regard to the internal standard (4-*n*-butylphenylacetic acid) according to $\alpha = (V_2 - V_0)/(V_1 - V_0)$, where V_0 is the void volume, V_1 is the internal standard elution volume, and V_2 is the elution volume of the compound of interest. Retention times (in minutes) were used instead of volumes for the calculation of α. The concentration of the drug solutions is not intended to represent those found at steady state upon therapeutic administration of the respective agents.

Drug	Concentration (μg/ml)	Retention time (min)	α
4-*n*-Butylphenylacetic acid	100	4.09	—
Ibuprofen	100	4.52	1.22
Indomethacin	125	4.31	1.11
Phenylbutazone	125	3.55	0.72
Tolmetin Sodium	250	3.12	0.49
Fenoprofen Calcium	250	3.68	0.78
Sulindac	200	3.29	0.58
Salicylic acid	250	Not detectable	
Salicylamide	250	2.49	0.16
Salicyluric acid	250	2.42	0.13
Gentisic acid	250	6.02	2.01
Acetaminophen	25	2.36	0.09
Phenacetin	250	2.68	0.26
Codeine sulfate	250	2.75	0.30
Caffeine	250	2.60	0.22
Penicillin G	250	Not detectable	
Sodium Phenobarbital	250	2.51	0.17

demonstrated a potential for interfering with IBU analysis was indomethacin. Since these compounds were analyzed in a non-extracted solution of methanol and water, analyses of serum samples containing these compounds may yield somewhat different values, and each should be evaluated if the IBU method described herein is adapted to routine clinical use.

Method selectivity was assessed by HPLC recycling of the IBU peak from an extracted serum sample containing 100 μg/ml of IBU. A 1-h recycling period (equivalent to six cycles) revealed that the peak could not be split into two or more components, and this confirmed the presence of the single compound, IBU. Comparison of IBU plasma concentrations assayed by a standard GLC technique in dogs [15] revealed that our method was sufficiently specific, from both a biochemical and biological perspective.

Extraction efficiency

Known amounts of IBU in methanol (corresponding to representative serum concentrations of 6.25 and 50.0 μg/ml), and similar tubes evaporated to dryness and reconstituted with 40 μl of the eluent were compared to serum samples containing identical concentrations of IBU. Five serum samples of each

concentration were extracted, and aliquots of each were analyzed as previously described. Since IBU and the internal standard behaved in a similar fashion quantitatively (as determined by peak height comparison), the ratios of IBU: internal standard peak heights were used for comparison of extraction efficiency. The extraction efficiencies from 1.0 ml of serum were 82.5% and 100.0% for IBU concentrations corresponding to 50.0 μg/ml and 6.25 μg/ml, respectively. Evaporation did not account for a significant loss of compound as the mean recoveries from evaporated samples corresponding to IBU concentrations of 50.0 μg/ml and 6.25 μg/ml were 100.8% and 96.7%, respectively.

Application — serum levels of IBU in the dog

To demonstrate the utility of the analytical method, a male mongrel dog was administered 400 mg (13.8 mg/kg) of sodium IBU* as a single intravenous injection over a 5-min period. A peak serum concentration of 111.47 μg/ml was observed 10.9 min after termination of the injection. ESTRIP [16] analysis of the plasma concentration versus time points (Fig. 2) revealed that a bi-exponential equation best described IBU disposition in this animal. IBU disposition was modeled using an open, two-compartment pharmacokinetic model. Pharmacokinetic parameters calculated from the data analysis are described in Table III. The serum elimination half-life of IBU determined in the application of our method correlates closely to that extrapolated from the study of IBU plasma levels in dogs utilizing a colorimetric method for analysis [9], as well as that from a study of IBU bioavailability in dogs employing a GLC analysis method [15].

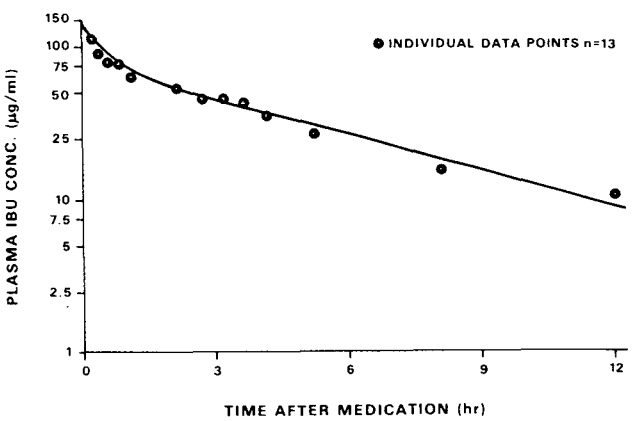

Fig. 2. Plasma concentrations of IBU versus time in the dog following a 400-mg (13.8 mg/kg) intravenous injection of sodium IBU. Biexponential curve fit is from estimates generated by ESTRIP [16] analysis of the data.

*Sodium IBU for intravenous use was kindly supplied by the Upjohn Company, Kalamazoo, MI, U.S.A.

TABLE III

PHARMACOKINETIC PARAMETERS CALCULATED FROM A 400-mg (13.8 mg/kg) INTRAVENOUS DOSE OF SODIUM IBUPROFEN IN A DOG

α (h^{-1})	$T½\alpha$ (h)	β (h^{-1})	$T½\beta$ (h)	Vd_{area} (l/kg)	Plasma clearance (ml/kg/min)	$AUC^{0\rightarrow\infty}$ * [(μg/ml) · h]
3.35	0.207	0.182	3.81	0.18	0.502	457.827

*$AUC^{0\rightarrow\infty}$ represents the area under the plasma level vs. time curve from time zero to infinity as calculated by the trapezoidal rule to time t, plus the $AUC^{t\rightarrow\infty}$ as calculated by the concentration at time t/β.

DISCUSSION

The HPLC method described herein greatly simplifies sample handling procedures and avoids derivatization as required by previous GLC methods [8—11]. Despite the recent development of GLC methods which obviate derivatization of IBU [12, 13], the identification of interfering peaks associated with certain GLC support packings [17] introduces possible errors with this method of analysis. The larger plasma volumes (i.e., > 1.0 ml) necessary for previously reported methods [8—14] could also limit their application to detailed pharmacokinetic or bioavailability studies in pediatric populations.

The method described has demonstrated appropriate sensitivity (range of linearity = 0.5—100.0 μg/ml) with as little as 0.5 ml of serum. The simple extraction procedure, isocratic composition of the solvent system, rapid elution characteristics and application to automated analysis systems make it well suited for the study of IBU disposition and possible therapeutic monitoring of this drug. This is emphasized by findings for extraction efficiency, stability, variability, selectivity and a demonstrated lack of potential interfering substances. These are advantages to previously published methods for the analysis of IBU [8—14, 17].

In addition to demonstrating the application of our method to the study of IBU disposition in the dog, we have also shown that the method is qualitatively and quantitatively identical when applied to human serum. This is in contrast to a previously reported HPLC method illustrating only the detectability of IBU from canine serum [14].

While hemolysis did not appreciably alter the chromatogram of the extracted sample, comparison of the results determined from a standard curve prepared in serum from hemolyzed samples were significantly lower than those obtained from the analysis of non-hemolyzed samples. We have not at this time determined the etiology of this disparity in resultant IBU concentrations from hemolyzed samples. The reliability of IBU determination from hemolyzed specimens is questionable and results should not be reported for such samples, or used for data analysis.

CONCLUSION

We have developed a specific HPLC method for the determination of IBU

from human serum and have demonstrated its utility in a limited study of IBU disposition. Suitable application of this method to pharmacokinetic and bioavailability studies of IBU in both adults and children is apparent. We are currently evaluating IBU disposition in man using this method and are investigating its application to microanalytical procedures for the determination of free vs. protein-bound IBU in plasma, and for the determination of IBU in other biological fluids.

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Note

Estimation of sugar alcohols by gas—liquid chromatography using a modified acetylation procedure

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The measurement of sugar alcohols in urine is of interest in patients with diabetes, and renal failure [1]. Urinary galactitol has been studied in patients with galactosaemia [2], and mannitol excretion for determining glomerular filtration rates [3] and in studies of intestinal permeability [4].

Although gas—liquid chromatography (GLC) has been used to measure sugar alcohols, resolution of isomeric mixtures may be difficult, particularly if packed columns are used. We recently described a procedure for mannitol estimation in which trimethylsilyl ethers were formed [5]. Although mannitol was separated from sorbitol and galactitol, the latter two hexitols were not resolved.

The present report describes a modified procedure for the measurement of sugar alcohols as acetyl esters. Isomeric hexitols are resolved and interferences due to monosaccharides overcome by forming methyloxime-acetyl derivatives of reducing sugars.

EXPERIMENTAL

Reagents

Sugar alcohols. Arabitol erythritol, inositol, pentaerythritol, perseitol (α-mannoheptitol), ribitol and xylitol were obtained from Sigma London (Poole, Great Britain). Mannitol and sorbitol were supplied by Koch-Light Labs. (Colnbrook, Great Britain).

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Sugars. D-Arabinose, D-fructose and D-fucose were obtained from Sigma, D-galactose and D-ribose from BDH (Poole, Great Britain). D-Glucose was supplied by Hopkins and Williams (Chadwell Heath, Great Britain) and D-xylose by Koch-Light Labs.

Chemicals. Analytical grade solvents were used. Pyridine was refluxed over potassium hydroxide pellets and redistilled before use. Zerolit DM-F (a mixed ion-exchange resin) was obtained from BDH, and converted to the H^+ acetate$^-$ form [5]. Methoxylamine hydrochloride was supplied by Pierce & Warriner (UK) (Chester, Great Britain).

Chromatograph

An F33 gas chromatograph (Perkin-Elmer, Beaconsfield, Great Britain) with a flame ionisation detector was used. This was fitted with a coiled glass column (2 m × 1.75 mm I.D.) which had been siliconised with 5% dimethyldichlorosilane in toluene, and filled with 3% XE-60 on Gas-Chrom Q (80—100 mesh), obtained from Phase Separations (Queensferry, Great Britain). The column was conditioned before use by heating overnight at 250°C with a carrier gas flow-rate of 50 ml/min.

Test procedure

To 2 ml urine, test solution or standard was added 1 ml internal standard solution (pentaerythritol, 50 mg/l). For quantitative studies a standard solution containing 20 mg/l of erythritol, xylitol, sorbitol and galactitol was used. Resin was added to occupy, after expansion, 60% of the total volume. The specimens were shaken for 3 min and centrifuged at 2000 *g* for 5 min. An aliquot (1 ml) of the clear supernate was transferred to a 10-ml conical glass tube in a water bath at 70°C, and evaporated to dryness under a stream of air. The tubes were transferred to a desiccator containing phosphorus pentoxide which was evacuated for a minimum period of 1 h. After removing the tubes from the desiccator, 0.5 ml methoxylamine hydrochloride in pyridine (10 mg/ml) was added to each sample, followed by incubation at 70°C for 30 min in a heating block (Techne Dri-Block DB-3; Techne, Duxford, Great Britain). The specimens were acetylated by adding 0.5 ml acetic anhydride to each and incubating for a further 10 min at 70°C. Two drops of methanol were added and the solvents evaporated by placing the tubes in a water bath at 30°C under a stream of air. The residues were desiccated for at least 1 h and then dissolved in 50 µl methanol. Using a microsyringe, 2 µl were injected onto the chromatographic column.

The chromatograph was operated with the injector port and detector oven at 250°C and a column temperature of 230°C. Nitrogen was used as carrier gas with a flow-rate of 50 ml/min. The inlet pressures of hydrogen and air were 1.1 bar and 1.6 bar respectively. The amplifier attenuation was 8×10^2. The chromatograph was linked to a Rikadenki Recorder (Rikadenki Kogyo Co., Tokyo, Japan).

Polyhydric alcohols were quantified by comparing the peak height ratios in standard and test samples.

Specimens from normal subjects were collected from ten healthy adult subjects for determination of sugar alcohol excretion. Urine was collected

for 5 h after an overnight fast; volumes were recorded and aliquots, preserved with thiomersal (100 mg/l), stored at 4°C prior to analysis.

RESULTS

Relative retention times

The retention times of monosaccarides and polyols relative to that of the internal standard were determined using pure aqueous solutions (1 g/l). The results are shown in Table I. The retention time of pentaerythritol was approximately 4 min. Pentaerythritol was selected as the internal standard because it does not occur naturally, and appears in an otherwise vacant position on the chromatogram. Fig. 1 shows a chromatogram obtained using an aqueous solution containing ten sugar alcohols.

TABLE I

RETENTION TIMES OF SUGAR ALCOHOLS AND SELECTED MONOSACCHARIDES RELATIVE TO THAT OF THE INTERNAL STANDARD, PENTAERYTHRITOL

The retention time of pentaerythritol was approx. 4 min.

Compound	Peak 1	Peak 2
Arabinose	0.64	0.70
Arabitol	1.17	
Erythritol	0.45	
Fucose	0.54	0.60
Fructose	1.84	
Galactitol	2.68	
Galactose	1.57	1.80
Glucose	1.23	1.93
Inositol	3.49	
Lyxose	0.73	
Mannitol	2.44	
3-O-Methylglucose	1.24	1.29
Pentaerythritol	1.00	
Perseitol	5.75	
Ribitol	1.09	
Ribose	0.68	
Sorbitol	3.03	
Xylitol	1.47	
Xylose	0.89	

Recovery

Known amounts of erythritol, xylitol, galactitol and sorbitol were added to urine and the concentrations of each determined in treated and untreated specimens. After subtracting the values for endogenous polyols, the concentrations were expressed as a percentage of the true values. Recoveries varied from 94 to 109%. The results are shown in Table II.

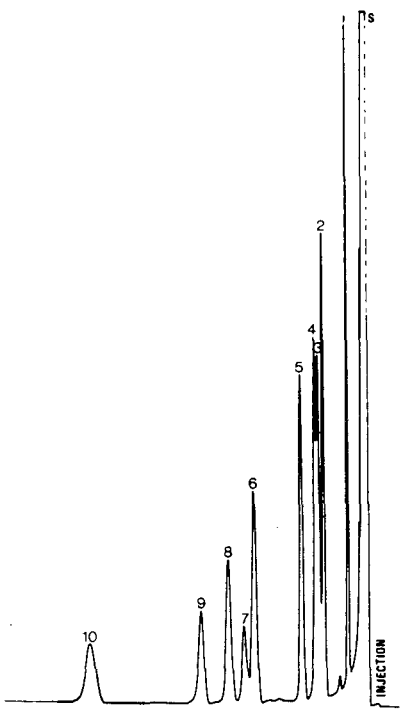

Fig. 1. Chromatogram of a standard solution of ten sugar alcohols. Peaks: S = solvent front; 1 = erythritol; 2 = pentaerythritol; 3 = ribitol; 4 = arabitol; 5 = xylitol; 6 = mannitol; 7 = galactitol; 8 = sorbitol; 9 = inositol; 10 = perseitol. The retention time of pentaerythritol was 4 min.

TABLE II

RECOVERY OF SUGAR ALCOHOLS ADDED TO URINE

Values for endogenous polyols have been subtracted after estimating the amounts in untreated urine.

Amount added (mg/l)	Recovery (%)			
	Erythritol	Galactitol	Sorbitol	Xylitol
20	94	105	109	109
200	98	102	95	105

Precision

Precision was assessed for erythritol, xylitol, galactitol and sorbitol by adding various amounts to urine and estimating the concentrations by single injection of samples which were analysed in three batches. These results are shown in Table III, coefficients of variation of 4.1—9.7% being obtained.

TABLE III

PRECISION OF SUGAR ALCOHOL MEASUREMENT IN URINE

The formula $\sqrt{\frac{\Sigma x^2 - (\Sigma x)^2}{n-1}}$ was used for calculating standard deviation.

Sugar alcohol	Mean (mg/l)	n	S.D.	Coefficient of variation (%)
Erythritol	194.7	15	28.7	9.7
	88.1	21	6.0	6.8
Galactitol	161.5	15	13.0	8.0
	26.9	21	2.3	8.5
Sorbitol	153.5	15	10.3	6.7
	20.2	21	1.4	6.9
Xylitol	179.3	15	7.7	4.3
	33.9	21	1.4	4.1

Linearity

Linearity was assessed for seven sugar alcohols by adding varying amounts to urine to a limit of 1 g/l. The concentration of internal standard solution remained constant (200 mg/l). Inositol measurement was linear to 700 mg/l, erythritol, sorbitol and perseitol to 800 mg/l and xylitol, mannitol and galactitol to 1 g/l.

Sensitivity

For erythritol, galactitol, sorbitol and xylitol, concentrations of 2 mg/l were quantified if the amplifier attenuation was reduced to 1×10^2. Although the limit of sensitivity was not determined absolutely, it is well below this level for most polyols and may be further improved if the sample volume is increased. With concentrations below 2 mg/l, erythritol was not well defined because of its proximity to the solvent peak. However, the resolution of erythritol was improved by temperature programming with the initial temperature 200°C for 4 min, the rate of increase 3°C/min and the final oven temperature 230°C. With such a modification erythritol concentrations lower than 2 mg/l may be measured.

Interferences

If monosaccharides were not converted to the corresponding methyloxime derivatives prior to acetylation, galactose and glucose interfered with galactitol and mannitol estimation, respectively. By including the additional derivatisation procedure the retention times of sugars were reduced and interferences prevented (Fig. 2). Sugars and polyols do not appear to cause interference.

Excretion of sugar alcohols in healthy subjects

Excretion rates of xylitol, mannitol, galactitol and sorbitol were determined, the results being expressed as mg/h (Table IV).

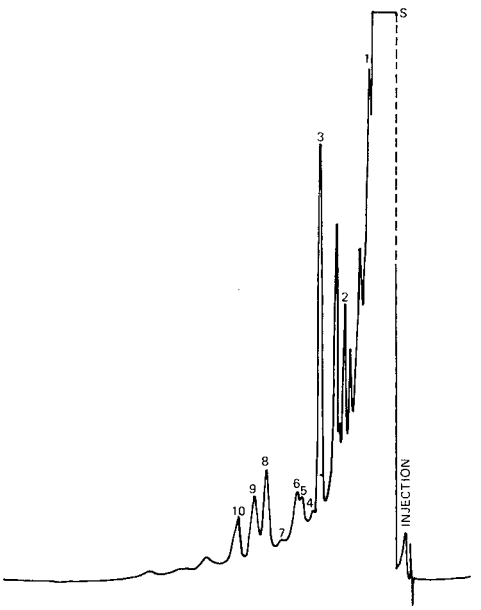

Fig. 2. Chromatogram of urine. Peaks: S = solvent front; 1 = erythritol; 2 = pentaerythritol; 3 = xylitol; 4 and 7 = glucose; 5 and 6 = galactose; 8 = mannitol; 9 = galactitol; 10 = sorbitol. For clarity, sugar alcohols were added to urine to give concentrations of 20 mg/l.

TABLE IV

RATE OF EXCRETION OF SUGAR ALCOHOLS IN TEN HEALTHY SUBJECTS

Sugar alcohol	Excretion (mg per 5 h)	
	Mean	Range
Xylitol	0.72	0.32—2.08
Mannitol	1.24	0.44—2.47
Galactitol	0.51	0.26—0.87
Sorbitol	0.46	0.22—0.86

DISCUSSION

Gas—liquid chromatography has been used to measure the low levels of sugar alcohols which occur in urine, since it is sensitive and capable of resolving the components of complex mixtures. However, isomeric polyols have proved difficult to separate, particularly if trimethylsilyl derivatives are formed. Although these derivatives have been widely used for sugar measurements complete resolution of isomeric trimethylsilyl sugar alcohols does not occur when packed columns are used [5—7].

Oades [8] demonstrated that sugar alcohol mixtures may be more satisfactorily resolved if acetyl esters are prepared and this procedure was adapted for

analysis of polyols in urine by Pitkänen [1]. Because it interfered with sorbitol and galactitol estimation, glucose was removed prior to chromatography by incubating samples with glucose oxidase, the resulting gluconate being removed with an ion-exchange resin. The present procedure avoids interference due to monosaccharides by the less cumbersome method of combining methyloxime formation with acetylation, thus modifying the retention times of sugars which would otherwise co-chromatograph with polyols. The retention times of sugar alcohols are not altered by including derivatisation with methoxylamine hydrochloride in addition to acetylation; thus polyols form only acetyl esters. Sugar alcohol excretion rates are comparable to those obtained by Pitkänen [1].

The method is precise, accurate, sensitive and linear over wide ranges. It appears to be free from interferences.

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Note

Silica Sep-Pak preparative chromatography for vitamin D and its metabolites

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Assay techniques for the accurate measurement of vitamin D and its more polar metabolites from a single lipid extract of human plasma or serum require preparative chromatography for separation and initial purification of vitamin D and its metabolites before assay [1—4]. At present, the most frequently employed preparative chromatographic method, open-column chromatography with Sephadex LH-20 [1—4], is cumbersome and requires considerable time in column preparation. We have developed a rapid, batch-elution, preparative chromatographic technique using disposable silica cartridges to achieve separation and initial purification of vitamin D_3, 25-hydroxyvitamin D_3 (25-OH-D_3), 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2$-D_3], and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3] from a lipid extract of 3 ml of human plasma or serum.

EXPERIMENTAL

Materials

[1,2-^{3}H] Vitamin D_3 (7.4 Ci/mmol), [26,27-^{3}H] 25-OH-D_3 (22.3 Ci/mmol), [23,24-^{3}H] 24,25-$(OH)_2$-D_3 (9.0 Ci/mmol), and [23,24-^{3}H] 1,25-$(OH)_2D_3$ (82 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, IL, U.S.A.) and were purified over either silica Sep-Pak or by high-performance liquid chromatography (HPLC) before use. Standard 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2$-D_3] was kindly provided by Drs. Milan Uskokovic and Enrico Baggiolini of Hoffmann-La Roche (Nutley, NJ, U.S.A.) and 25-hydroxyvitamin D_2 (25-OH-D_2) by Dr. John Babcock of Upjohn (Kalamazoo, MI, U.S.A.). Silica Sep-Pak cartridges (Lot P022721) were obtained from Waters Assoc. (Milford, MA, U.S.A.). Ethyl acetate and *n*-hexane (redistilled in glass) were purchased from Burdick and Jackson Labs. (Muskegon, IL, U.S.A.). Stop

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cock-regulated glass reservoirs equipped with nitrogen ports (Wilbur Scientific, Boston, MA, U.S.A.), or standard 50-ml, luer-lock glass syringes (Popper and Sons, New Hyde Park, NY, U.S.A.) were employed as solvent reservoirs.

Procedures

Tracer quantities (2000—3000 cpm) of radiolabelled vitamin D_3, 25-OH-D_3, 24,25-$(OH)_2$-D_3, and 1,25-$(OH)_2$-D_3 or standard 25-OH-D_2 and 25,26-$(OH)_2$-D_3 were added separately or in combination to 3 ml of pooled human plasma and allowed to equilibrate for 45 min at 4°C. After lipid extraction of plasma samples with methanol—methylene chloride [3], the lipid extract was taken to dryness under nitrogen and redissolved in 300 μl of 7% ethyl acetate in *n*-hexane. The sample and a 300-μl wash of the extraction vial were applied to a Sep-Pak cartridge after presaturation of the cartridge with 4 ml of starting solvent.

Batch elution of vitamin D_3, 25-OH-D_3, 24,25-$(OH)_2$-D_3, and 1,25-$(OH)_2$-D_3 was then performed at a constant flow-rate of 5 ml/min under positive pressure with nitrogen. To evaluate the resolving capacity of the system for each compound, 5-ml fractions were collected during sample elution with increasing concentrations of ethyl acetate in *n*-hexane as follows: 30 ml of 7% ethyl acetate in *n*-hexane (fraction I), 20 ml of 25% ethyl acetate in *n*-hexane (fraction II), and 30 ml of 60% ethyl acetate in *n*-hexane (fraction III). After establishing the elution profiles for individual compounds, plasma lipid extracts containing a mixture of tritiated vitamin D_3, 25-OH-D_3, and 1,25-$(OH)_2$-D_3 were chromatographed as described above. Tracer recovery was determined by liquid scintillation counting of radioactivity (Tricarb Model 3325, Packard Instrument Company, Downers Grove, IL, U.S.A.) recovered in each 5-ml fraction. Quench corrections were made according to an external standard. Overall recovery data from four samples are expressed as the percent (mean ± S.D.) of radioactivity applied to the column.

RESULTS AND DISCUSSION

The elution profiles for extracts of plasma lipid containing tracer amounts of vitamin D_3, 25-OH-D_3, 24,25-$(OH)_2$-D_3, and 1,25-$(OH)_2$-D_3 are shown in Fig. 1, panels A, B, C, and D, respectively. Recoveries of applied vitamin D_3 in fraction I (7% ethyl acetate in *n*-hexane), of 25-OH-D_3 in fraction II (25% ethyl acetate in *n*-hexane), and of 24,25-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3 in fraction III (60% ethyl acetate in *n*-hexane) were 86 ± 3%, 87 ± 2%, and 81 ± 2%, and 91 ± 5%, respectively. There was less than 1% carryover of each metabolite into adjacent batch fractions with the exception of 6 ± 1% of the total 24,25-$(OH)_2$-D_3, which eluted with 25-OH-D_2 and 25-OH-D_3 in fraction II. The elution profile of a mixture of tracer vitamin D_3, 25-OH-D_3, and 1,25-$(OH)_2$-D_3 in single plasma extracts (Fig. 1, Panel E) was similar to those obtained when just vitamin D_3 or its metabolites were evaluated separately. A total of 91 ± 3% of applied radiolabel was recovered in the 80 ml of the collected eluent. Similar recovery data were obtained for tracer added to serum.

Sep-Pak chromatography of plasma and serum lipid extracts is now the

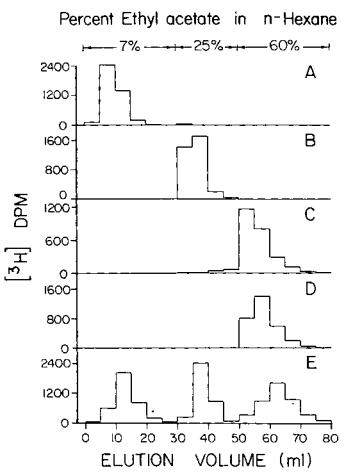

Fig. 1. Elution profiles of plasma lipid extracts containing (A) [^{3}H]vitamin D_3, (B) [^{3}H]25-OH-D_3, (C) [^{3}H]24,25-$(OH)_2$-D_3, (D) [^{3}H]1,25-$(OH)_2$-D_3, and (E) a mixture of [^{3}H]vitamin D_3, [^{3}H]25-OH-D_3, and [^{3}H]1,25-$(OH)_2$-D_3. Each bar represents the mean recovery of radioactivity in 5 ml of eluent (n = 4) during batch elution of sample with 7, 25, and 60% ethyl acetate in n-hexane.

method routinely used in our laboratory in the preparation of samples for assay of vitamin D, 25-OH-D, and 1,25-$(OH)_2$-D. Fraction I is subjected to final purification and direct spectrophotometric quantification of vitamin D on HPLC [5]. Fraction III containing 1,25-$(OH)_2$-D is further purified on straight-phase HPLC and then assayed in a competitive-protein-binding assay [6]. After evaporation under nitrogen, fraction II is assayed directly by competitive-protein-binding assay for 25-OH-D [7]. In as much as the vitamin-D-binding protein employed for routine assay of 25-OH-D has similar affinity for 24,25-$(OH)_2$-D [8], it is important to resolve these closely migrating compounds before assay. In this chromatographic system, only 6 ± 1% of tracer 24,25-$(OH)_2$-D_3 coeluted with 25-OH-D_3, whereas the remaining 81 ± 2% of recovered 24,25-$(OH)_2$-D_3 eluted in fraction III. The dihydroxylated metabolites 24,25-$(OH)_2$-D_3, 25,26-$(OH)_2$-D_3, and 1,25-$(OH)_2$-D_3, coelute in this system and can be separated and further purified on straight-phase HPLC before assay [3]. By these assay techniques, values in normal subjects for vitamin D, 25-OH-D, and 1,25-$(OH)_2$-D ranged from 0.5 to 20 ng/ml (n = 30), 10 to 65 ng/ml (n = 32), and 33 to 61 pg/ml (n = 36), respectively. Normal ranges, sample interassay and intra-assay variation and assay non-specific binding for vitamin D metabolites following Sep-Pak chromatography are comparable to assay data obtained from samples purified by Sephadex LH-20 chromatography [9].

The major advantages of using silica Sep-Pek preparative chromatography are convenience, reproducibility, cartridge disposability, and saving of time in column packing and standardization. Time required for cartridge preparation, sample loading, chromatography, and clean-up for fifteen samples is 30 min. Chromatography on the silica Sep-Pek cartridges is comparable in cost, efficiency of sample recovery, and resolution capabilities for vitamin D and its metabolites with that reported for Sephadex LH-20 [2, 3]. The use of silica Sep-Pak chromatography provides a rapid and efficient alternative to currently

employed open column chromatography for the initial separation and purification of vitamin D_3 and its mono- and dihydroxylated metabolites.

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Note

Analysis of adenosine, inosine and hypoxanthine in suspensions of cardiac myocytes by high-performance liquid chromatography

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Adenosine has been implicated as a physiological regulator in many biological systems, as amply reviewed by Arch and Newsholme [1]. In the heart, adenosine is a potent coronary vasodilator [2] and much evidence suggests that it is a mediator substance in the autoregulation of coronary blood flow [3—6]. In order to study adenosine release by heart cells, we sought a method of quantification for adenosine and its catabolites, inosine and hypoxanthine. It was necessary to obtain a method which would allow us to quantify the desired nucleosides in heart tissue where adenine nucleotide levels far exceed nucleoside levels. Although there are reported methods for the analysis of one or more nucleosides [6—10], each had limitations or presented special problems. The purpose of this note is to describe our high-performance liquid chromatographic (HPLC) method for the quantification of adenosine, inosine and hypoxanthine in nucleotide-rich tissue extracts.

EXPERIMENTAL

High-performance liquid chromatography

Our system consists of a Milton Roy mini-pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) 7120 sample injection valve and a Perkin-Elmer (Norwalk, CT, U.S.A.) LC-55 variable-wavelength detector set at 260 nm. Flow-rate under all conditions was constant at 1.12 ml/min. The analysis scheme used has two stages. In the first stage (clean-up stage), nucleosides and bases were isolated as a group completely free of nucleotides using a Whatman (Clifton, NJ, U.S.A.) Partisil SAX anion-exchange column (25 cm × 4.6 mm; 10-μm particle size). In the second stage, the separation of nucleosides and bases from one another was

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accomplished using a Whatman Partisil ODS-1 reversed-phase C_{18} column (25 cm × 4.6 mm; 10-μm particle size).

In the clean-up stage (Fig. 1) the group separation of nucleosides and bases, with complete removal of contaminating nucleotides, was accomplished by injecting 500 μl of tissue extract onto the Partisil SAX anion-exchange column equilibrated with 0.005 *M* ammonium phosphate buffer, pH 2.8. The peak containing all of the nucleosides and bases (hereafter referred to as the nucleoside peak) was eluted isocratically and collected in a measured time interval, 55 sec (volume, 1.03 ml), while the bulk of the nucleotides remained bound to the column. Four individual 500-μl samples of tissue extract could be processed in this manner before it was necessary to purge the column of accumulated nucleotides. Purging was accomplished with a step gradient of ammonium phosphate buffers as follows: 0.20 *M*, pH 3.7, 2 min; 0.75 *M*, pH 3.7, 15 min; 0.20 *M*, pH 3.7, 2 min; and re-equilibration with 0.005 *M*, pH 2.8, 15 min.

Samples of collected nucleoside peak material were stored at −20°C until analyzed by reversed-phase chromatography. Nucleoside and base analysis was carried out with isocratic elution, using the Whatman ODS-1 reversed-phase column equilibrated with a solution of 0.010 *M* potassium phosphate (pH 5.5)—methanol (90:10, v/v). Sample injection volume on the ODS-1 column was usually 200 μl.

Concentrations of adenosine, inosine and hypoxanthine in tissue samples were calculated by comparing peak areas from chromatograms of tissue extract with peak areas from chromatograms of standard solutions of authentic compounds (Fig. 2). Peak area was determined as peak height × peak width at half peak height. Standard solutions of authentic adenosine, inosine and hypoxanthine were prepared to have a concentration of approximately $3 \cdot 10^{-5}$ *M*. The exact concentrations of these compounds were then determined spectrophotometrically using millimolar extinction coefficients, $ml \cdot \mu mole^{-1} \cdot cm^{-1}$, of 15.4 at 259 nm [11], 12.2 at 248.5 nm [12], and 10.6 at 250 nm [12] for adenosine, inosine and hypoxanthine, respectively. Standards, prepared in a buffer of 0.05 *M* 3-(N-morpholino)propanesulfonic acid with 0.13 *M* sodium chloride, pH 5.2, were subjected to exactly the same chromatographic scheme as the tissue extracts.

Proof of the identity of the chromatographic peaks of interest (inosine, hypoxanthine and adenosine) was obtained by three independent means: (1) retention time as compared with pure known substance, (2) comparison of UV spectra (220—300 nm) of peak material in question with that of a known substance and (3) enzymic peak shift [13], using adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase.

Preparation of tissue

Protein-free tissue extracts were prepared from cell suspensions obtained from adult rat hearts dissociated with crude collagenase [14]. Ice-cold trichloroacetic acid (TCA), 1 g/ml, was added to the cell suspensions to give a concentration of 10% TCA and the sample was homogenized immediately. After centrifugation to remove solids, the TCA was removed with five extractions of equal volumes of cold, water-saturated diethyl ether. The resulting

aqueous extract was bubbled with water-saturated air for 5 min to remove the dissolved ether. Finally, the pH was adjusted to 4.5—5.0 with potassium hydroxide, and the sample was frozen at −20° C until HPLC analysis.

The efficiency of recovery of nucleosides from tissue during tissue extract preparation was determined by comparing the amount of added nucleoside that can be recovered from a TCA tissue extract versus a non-extracted standard solution. Recovery was 111.3 ± 0.6% and 105.7 ± 4.0% (mean ± S.D.) for adenosine and inosine, respectively.

Reagents and chemicals

Ammonium hydroxide and phosphoric acid were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Ammonium dihydrogen phosphate was from Baker (Phillipsburg, NJ, U.S.A.) and methanol (acetone-free) was obtained from Fisher (Fair Lawn, NJ, U.S.A.). Nucleosides, related bases and enzymes were obtained from Sigma (St. Louis, MO, U.S.A.). Collagenase was obtained from Worthington (Freehold, NJ, U.S.A.).

RESULTS

A chromatogram representative of the first stage (clean-up stage) of analysis is shown in Fig. 1. The compounds eluting in the broad peak following each nucleoside peak are nucleotides which were not specifically identified. The bulk of the nucleotides in each sample remain bound to the column. As shown in Fig. 1, for each sample injected, the nucleoside peak (numbered peaks) was reasonably narrow allowing this material to be collected in a rela-

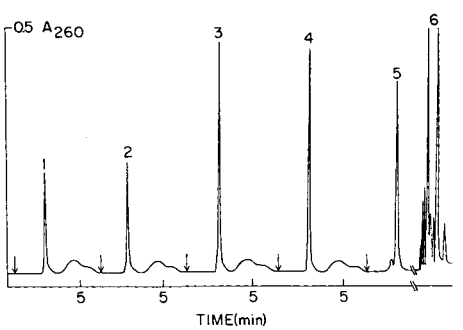

Fig. 1. The first stage of nucleoside analysis: removal of nucleotides. Representative chromatogram of multiple injections (indicated by arrows) of cardiac myocyte extracts on anion-exchange column chromatography. Peaks 1, 2, 3, and 4, containing the nucleosides and bases (free of nucleotides) were each collected in a volume of 1.03 ml (collection time, 55 sec) and saved for reversed-phase chromatography (Fig. 2). Upon the fifth sample injection, the usual result was a split peak (labeled 5) which, if collected would be undesirably diluted. Purging the column of accumulated nucleotides (peak 6, shown on a compressed time scale) usually after the fourth sample injection, allowed a new cycle of four sample injections to be made. Sample injection volume, 500 μl; column, Whatman SAX at ambient (ca. 22° C) temperature; detector at 260 nm; flow-rate, 1.12 ml/min. Isocratic elution, 0.005 *M* ammonium phosphate (pH 2.8); nucleotide purging (6) accomplished by changing eluent from 0.005 *M* to 0.750 *M* ammonium phosphate (pH 3.7) for 15 min.

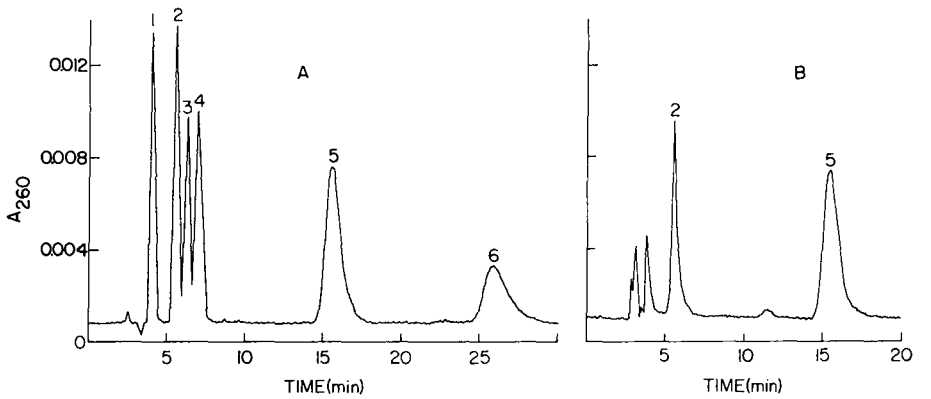

Fig. 2. (A) Chromatogram of authentic compounds, uridine (1), inosine (2), hypoxanthine (3), thymidine (4), adenosine (5) and adenine (6); quantities injected were 0.5, 1.0, 0.6, 0.7, 0.9, and 0.6 nmole, respectively. (B) Chromatogram of peak material collected from the anion-exchange column (Fig. 1), showing the presence of inosine (2) and adenosine (5), but an absence of hypoxanthine in cardiac myocyte suspensions. Sample injection volume, 200 μl; column, Whatman ODS-1 C_{18} at ambient (ca. 22° C) temperature; detector at 260 nm; flow-rate, 1.12 ml/min. Isocratic elution, 0.010 *M* potassium phosphate (pH 5.5)—methanol (90:10, v/v). Retention times vary with individual columns.

tively small volume (1.03 ml). However, peak splitting usually occurred upon the fifth in a series of injections (Fig. 1, peak 5). Thus, we usually inject and collect four samples sequentially and then purge before making further injections. Purging the column of bound nucleotides restores the unity of the nucleoside peak, allowing another series of four injections and peak collections to be made.

Each collected nucleoside peak from the first stage was subsequently analyzed by reversed-phase column chromatography. The results, shown in Fig. 2, were obtained using an elution scheme optimized for the separation of inosine, hypoxanthine and adenosine. Specifically, we found that isocratic elution with 0.010 *M* potassium phosphate (pH 5.5)—methanol (90:10, v/v) allowed inosine and hypoxanthine to be almost baseline resolved, while adenosine was completely separated from all compounds present. Varying the phosphate concentration from 0.005 *M* to 0.020 *M* and the pH from 5.5 to 6.0 did not alter the results. Retention time decreased with increasing methanol concentration. Better resolution was not obtained using 5% or 20% methanol buffer or a linear gradient elution to 30% methanol.

In the clean-up stage of analysis we routinely injected 500 μl of tissue extract onto the anion-exchange column and collected the nucleoside peak in 1.03 ml (Fig. 1). However, we determined that we could inject up to 1.0 ml of tissue extract onto this column and still collect the resulting nucleoside peak in a volume of 1.03 ml. Also routinely, we injected only 200 μl (of the 1.03 ml collected peak) onto the reversed-phase column to separate and quantify inosine, hypoxanthine and adenosine (Fig. 2). We found, however, that virtually all (1.00 ml out of 1.03 ml) of the collected nucleoside peak from the anion-exchange column could be injected onto the ODS-1 column with no ill effects on peak separation or linearity of peak area response. Thus,

if 1.0 ml of tissue extract is loaded onto the anion-exchange column, almost all of it (ca. 97%) can be collected and subsequently loaded onto the reversed-phase column for quantitation of inosine, hypoxanthine and adenosine. This capability allowed us to maintain the maximum sensitivity possible in the HPLC analysis of purines, when using a spectrophotometric detector (λ = 260 nm), about 50 pmole [10].

The observed retention times (Fig. 2) for inosine, hypoxanthine and adenosine were 5.44 ± 0.04, 6.19 ± 0.04 and 15.56 ± 0.07 min, respectively (mean ± S.D., n = 10). An ODS-1 column used in earlier work exhibited shorter retention times during its useful life (e.g. 4.73, 5.36 and 12.0 min for inosine, hypoxanthine and adenosine, respectively).

Comparison of Fig. 2A and B indicates that cardiac myocyte suspensions possess adenosine (1.14 ± 0.25 nmole per mg cell protein) and inosine (0.73 ± 0.32 nmole per mg cell protein), but usually no measurable hypoxanthine. Occasionally, a cardiac myocyte sample exhibited a small hypoxanthine peak (ca. 5% of the inosine peak). Fig. 2A shows that uridine, thymidine and adenine although not measurable in heart cell extracts, can be separated under the isocratic elution conditions utilized.

DISCUSSION

Several analytical methods for one or more nucleosides have been reported [6—10] and each has its advantages and disadvantages. We sought a method which would allow us to quantify not only adenosine but also its catabolites, inosine and hypoxanthine, in suspensions of cardiac myocytes. Certain methods were excluded because they were applicable only to adenosine [8, 9]. Others required equipment which was not available to us (a dual-wavelength spectrophotometer) [6, 7] or extensive sample clean-up [7, 8]. We examined one potential HPLC method [10] in which the tissue extract was injected directly onto a C_{18} reversed-phase column and the nucleosides and bases eluted with a methanol gradient. Two problems were encountered. Firstly, the method required gradient elution, an operation which we wished to avoid. Secondly, we found that with heart tissue, which is extremely rich in nucleotides, the inosine and hypoxanthine chromatographic peaks eluted high along the trailing edge of the large early-eluting nucleotide region. Eluting in this manner with the nucleotides, the inosine and hypoxanthine peaks varied erratically in size (area) upon repetitive injection of the same sample, making quantification impossible.

In the present study using an isocratic HPLC method, we show that inosine, hypoxanthine and adenosine are easily separated and quantified in heart cell suspensions where potentially interfering nucleotides are present in amounts considerably in excess of nucleosides. Because of the high nucleotide levels in myocardial tissue extracts, the initial anion-exchange HPLC clean-up (nucleotide removal) was necessary in order to allow quantitation of inosine and hypoxanthine using reversed-phase HPLC. Although sample clean-up can be accomplished with thin-layer chromatography or with small open columns [7, 8] utilization of anion-exchange HPLC for this process allowed us to monitor (Fig. 1) the isolation and collection of the nucleosides and bases in

a sample, with no risk of loss of peak material or unnecessary dilution thereof and with a minimum of sample manipulation. The sample injection—collection procedure is rapid and allows four samples to be processed before the accumulated nucleotides must be purged from the column. If greater dilution of the peak material can be tolerated, the peak-splitting (Fig. 1, peak 5) can be ignored and a greater number of samples can be processed before column purging is necessary.

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Note

Rapid assay of spermidine synthase activity by high-performance liquid chromatography

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The final steps of spermidine and spermine biosynthesis involve the addition of an aminopropyl group from S-methyladenosylhomocysteamine (decarboxylated S-adenosyl-L-methionine) to putrescine and spermidine respectively [1—3]. The reactions, forming 5′-methylthioadenosine as a secondary product, are catalyzed by two distinct synthases [4—7]. Thus far, studies concerning polyamine metabolism have addressed primarily the decarboxylations of ornithine and S-adenosylmethionine (SAM), which are considered the rate-limiting steps in this metabolic process. Recent methodological improvements, including techniques for the preparation of decarboxylated SAM as well as for the separation of reactants from reaction products have provided important information regarding spermidine and spermine synthases [8—10]. Our interest in polyamines derives from our findings that brain levels of SAM are decreased prior to seizures elicited by a single administration of the chemical convulsant L-methionine-*d,l*-sulfoximine (MSO) [11]. This depletion of SAM is due to its increased utilization via transmethylation reactions [12]. Since the depletion of SAM levels by MSO could also limit the availability of decarboxylated SAM, the precursor of spermidine, we decided to investigate polyamine biosynthesis in the MSO epileptogenic mouse brain. In this paper we describe a rapid and economic high-performance liquid chromatographic (HPLC) method for the separation and quantitation of spermidine formed upon incubation of putrescine with a crude extract of mouse brain. This method thus permits the rapid measurement of spermidine synthase (EC 2.5.1.16) activity.

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EXPERIMENTAL

Equipment

A Waters Model ALC/GPC-204 liquid chromatograph equipped with a Model 6000A high-pressure pump, a U6K loop injector and Model 440 absorbance detector (254 nm) was used throughout the study. The chromatographic runs were recorded on an Omniscribe recorder (Texas Instruments, Austin, TX, U.S.A.). A pre-packed (30 × 0.4 cm I.D.) μBondapak C_{18} (10-μm particle size) column (Waters Assoc., Milford, MA, U.S.A.) was used to separate the benzoylated polyamines.

Materials

Putrescine dihydrochloride and spermidine trihydrochloride were from Sigma (St. Louis, MO, U.S.A.). [1,4-^{14}C] Putrescine dihydrochloride (122 mCi/mmol) and [^{14}C] spermidine (122 mCi/mmol) were from Amersham/Searle (Arlington Heights, IL, U.S.A.). Decarboxylated SAM was kindly supplied by Dr. G. Stramentinoli (Bio Research, Liscate, Italy). All other chemicals were of analytical grade quality.

Enzyme source

Brains from adult male Swiss—Webster mice (25—30 g) were homogenized in 5 volumes (w/v) of 25 m*M* potassium phosphate buffer (pH 7.2). The mixture was centrifuged at 100,000 *g* for 60 min and the supernatant used as the source of spermidine synthase. Protein content was measured by the method of Lowry et al. [13].

Spermidine synthase assay

Unless otherwise stated a standard reaction mixture (0.5 ml) contained 0.1 *M* sodium phosphate buffer (pH 7.4), 0.05 m*M* putrescine, 2.0 μ*M* [1,4-^{14}C] putrescine, 0.06 m*M* decarboxylated SAM and 2 mg of enzyme protein. Blanks contained no decarboxylated SAM. After incubation at 37°C for 90 min the reaction was stopped by addition of 0.25 ml of 0.6 *M* perchloric acid. After centrifugation (10 min at 10,000 *g*), the supernatants were subjected to the benzoylation procedure and the extraction of the benzoylated polyamines as previously described [14,15]. The benzoylated polyamines were dissolved in 200 μl of methanol and aliquots (50 μl) were injected onto the μBondapak C_{18} column in order to separate putrescine from spermidine [15]. Elution was with 60% methanol (v/v) at a flow-rate of 2 ml/min. The quantitative determination of spermidine was carried out in the following two ways: (a) by radiometric determination of the [^{14}C] spermidine formed from [^{14}C] putrescine. In this case the HPLC eluates were collected (0.8 ml/fraction) into scintillation vials and counted after addition of ACS (a tissue solubilizer—scintillant mixture from Amersham/Searle), and (b) by measuring the increase in UV absorbance (254 nm) of the spermidine peak with respect to the blank (this correction is necessary when crude extracts, containing endogenous spermidine, are used as enzyme source). Calibration curves were generated by using [^{12}C] putrescine and spermidine in the presence of tracer amounts of [^{14}C] putrescine and spermidine to determine recoveries for each polyamine.

RESULTS AND DISCUSSION

Fig. 1 illustrates a typical HPLC pattern of benzoylated polyamines as derived from the enzymatic assay sample and its corresponding blank. The separation of putrescine and spermidine was highly satisfactory and the HPLC run was completed in 6 min. The increase in absorbance of the spermidine peak as well as the appearance of a radioactive peak corresponding to spermidine are well evident in the experimental sample. It should be noted that since the sensitivity of the detection of the benzoylated spermidine by UV absorbance is sufficiently high (about 0.1 nmol) it is possible to avoid, if desired, the use of radio-labelled putrescine in the enzymatic assay. Fig. 2 shows the time course of spermidine formation. The reaction was linear for up to 90 min using the enzymatic assay conditions described in the text, whether spermidine formation is determined radiometrically or by UV absorbance. Also, the reaction was linear with up to 2 mg of protein in the reaction mixture (Fig. 3). Fig. 4 shows the apparent K_m of the enzyme for putrescine. The apparent K_m values (2.2 and 3.3 · 10^{-5} M), calculated using either procedure of product quantitation, are in good agreement with those previously reported for mammalian spermidine synthase [16,17].

The recent widespread interest in the aminopropyltransferase reactions has led to various attempts to develop new and more rapid procedures for their in vitro assays. Among the various assay procedures recently reviewed [16], those using HPLC separation of polyamines require a flow cell for radioactivity measurements of the formed spermidine or spermine and a total elution time of at least 15 min for a single analysis [18,19]. Benzoylation appeared to be a much simpler and economical tool for the derivatization, separation and quantitation of polyamines produced in vitro upon incubation of the appro-

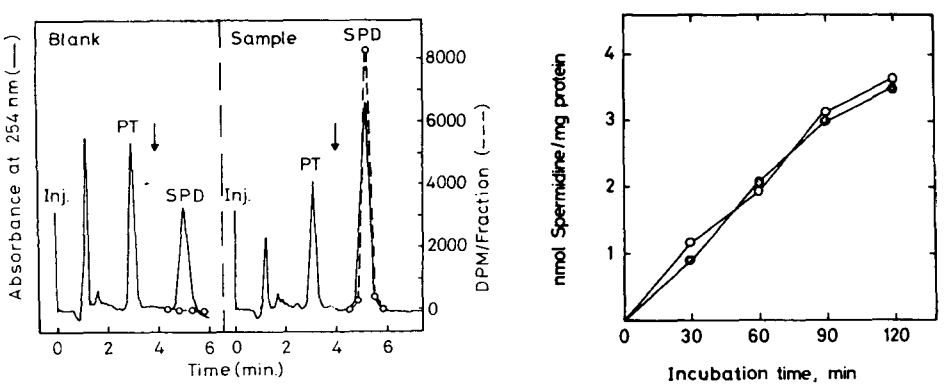
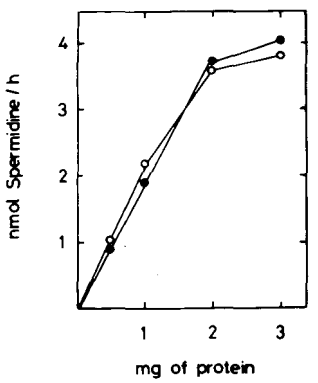


Fig. 1. HPLC separation of the benzoyl derivatives of putrescine (PT) and spermidine (SPD) contained in a standard reaction sample and in the corresponding blank after 60 min of incubation at 37°C. Details of the enzymatic assay and of the chromatographic run are described in the text and also in ref. 15. Fractions (0.8 ml) were collected for determination of radioactivity. The arrows indicate a change in detector sensitivity from 0.2 to 0.05 a.u.f.s.

Fig. 2. Mouse brain spermidine synthase activity as a function of time. The assay conditions are as described in the text. Both the absorbance (○——○) and the radiometric (●——●) determination of product formation represent the mean value of duplicate assays. The reproducibility was within 10%.



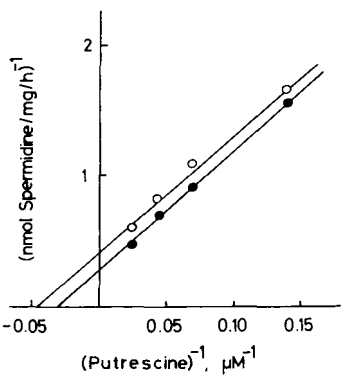


Fig. 3. Spermidine production as a function of protein concentration. The details of the assay conditions are as described in the text. Both the absorbance (○——○) and the radiometric (●——●) determination of product formation represent the mean value of duplicate assays. The reproducibility was within 10%.

Fig. 4. Graphic estimation of the apparent K_m for putrescine (Lineweaver—Burke plot). The assay conditions were as described in the text. Both the absorbance (○——○) and the radiometric (●——●) determination of product formation represent the mean value of duplicate assays. The reproducibility was within 10%.

priate aminopropyltransferase assay mixture. This paper specifically describes an assay procedure for spermidine synthase although, as shown elsewhere [15], it is theoretically possible to use the same methodology for the determination of spermine synthase activity. The purity of the samples after derivatization, the brevity of the chromatographic run and the isocratic elution schedule are the favorable characteristics of the assay technique described herein. The most significant improvement over existing methods, however, is that the high sensitivity of the assay obviates the determination of the radioactivity in the spermidine peak for an accurate quantitation of spermidine synthase activity. Indeed, as shown by our findings, there was virtual identity between spermidine synthase activity values obtained with the non-isotopic and the isotopic procedure of product quantitation.

Results of applications of the HPLC method in studies of polyamine biosynthesis in the brain of animals subjected to convulsant stimuli [15,20] have been presented elsewhere.

ACKNOWLEDGEMENT

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Note

Sensitive gas chromatographic method for the determination of alphadolone in plasma

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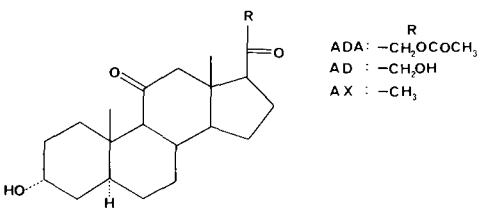
(First received March 16th, 1981; revised manuscript received May 27th, 1981)

Alphadolone acetate (21-acetoxy-3α-hydroxy-5α-pregnane-11,20-dione; ADA) is one of the two active ingredients of Althesin, a steroidal intravenous anaesthetic [1]. While a number of workers have published assays for alphaxalone (AX) [2—7] an assay for alphadolone (AD) has not been described previously. The aim of the present work was to develop an assay for AD in the plasma of experimental animals as a preliminary to obtaining pharmacokinetic data. It will be shown later that it is important to assay AD rather than its 21-acetate because ADA is rapidly hydrolysed to AD in rat plasma.



EXPERIMENTAL

Standards and reagents

Alphadolone (3α,21-dihydroxy-5α-pregnane-11,20-dione), alphadolone 21-acetate, and 3α-hydroxy-2β-*n*-butoxy-5α-pregnane-11,20-dione, the internal standard (IS), were obtained from the Organic Chemistry Department of Glaxo Group Research Ltd. Heptafluorobutyrylimidazole (HFBI) was obtained from Pierce (Chester, Great Britain). A solution (2%, v/v) was prepared in SLR grade toluene (Fisons, Loughborough, Great Britain). Diethyl ether

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(anaesthetic ether, Macfarlan Smith, Edinburgh, Great Britain) was used without further treatment.

Ammonium chloride buffer was prepared by the dropwise addition of concentrated hydrochloric acid (BDH, Poole, Great Britain) to 0.5 *M* aqueous ammonia (Fisons) until pH 9 was attained.

Phosphate buffer was prepared by adding potassium dihydrogen orthophosphate solution (0.5 *M*) to disodium hydrogen orthophosphate solution (0.5 *M*; BDH), until pH 7.4 was attained. The mixture was then diluted to 0.1 *M* total phosphate with water.

Stock solutions of steroids were prepared in ethanol to give concentrations of 10 μg/ml (AD), 50 μg/ml (IS) and 500 μg/ml (ADA).

Apparatus

A Perkin-Elmer Model F33 gas chromatograph equiped with a ^{63}Ni electron-capture detector was used. The glass column (2 m × 4 mm I.D.), packed with 2% Dexsil 300 on Gas-Chrom Q (100—200 mesh), had been conditioned for 24 h at 270°C. For subsequent analytical operation the column temperature was 240°C, the injection port and detector temperatures were 275°C, the carrier-gas was a mixture of argon—methane (9:1) (flow-rate 65 ml/min), the detector pulse setting was 5, and the amplifier attenuation setting was ×256—×64. Peak areas were measured using a Trivector Triton 3 computing integrator (Trivector Systems, Sandy, Bedfordshire, Great Britain).

The temperature of the column was raised to 265°C and that of the detector to 300°C each night to maintain detector sensitivity and minimise adsorption.

For mass spectrometry a Varian Aerograph series 2740 gas chromatograph combined with a Varian MAT 311A mass spectrometer were used. The electron energy was 70 eV and the temperature of the ion source 200°C. The carrier gas was helium (flow-rate 40 ml/min), but all other conditions were the same as those used for the conventional gas chromatography (GC) described previously.

Extraction and derivatisation

Internal standard solution (25 μl = 1.25 μg) was added to 1 ml of plasma in a 50-ml glass-stoppered test-tube, then 1 ml of ammonium chloride buffer and 10 ml of diethyl ether were added. The mixture was shaken by hand for 1 min. The organic layer was transferred by pipette to a conical test-tube, an anti-bumping granule was then added, and the solution was evaporated to dryness at 45°C.

The residue was dissolved in HFBI solution (100 μl), the tube was stoppered, and the mixture was incubated for 10 min in a 55°C water-bath. Phosphate buffer (0.5 ml) was then added and mixed by vortexing for 15 sec and the mixture was then centrifuged at 300 *g* for 5 min at room temperature. The aqueous phase was discarded, the organic layer was diluted with 700 μl toluene and 1 μl of this solution was injected into the GC column.

Calibration graph

Using the procedure described above, a calibration graph was obtained by

running plasma samples spiked with AD at concentrations varying from 10—600 ng/ml and with IS at a fixed concentration of 1.25 μg/ml.

RESULTS AND DISCUSSION

Typical chromatograms obtained from blank rat plasma, and plasma spiked with AD (300 ng/ml) and IS (1.25 μg/ml) are shown in Fig. 1. Similar chromatograms obtained using human plasma are shown in Fig. 2. The retention times of AD and IS were 6.5 and 8.5 min respectively. ADA had a retention time of 12 min but is very unstable in rat plasma. The success of the derivatisation of AD and IS with HFBI was confirmed by gas chromatography—mass spectrometry (GC—MS, Fig. 3).

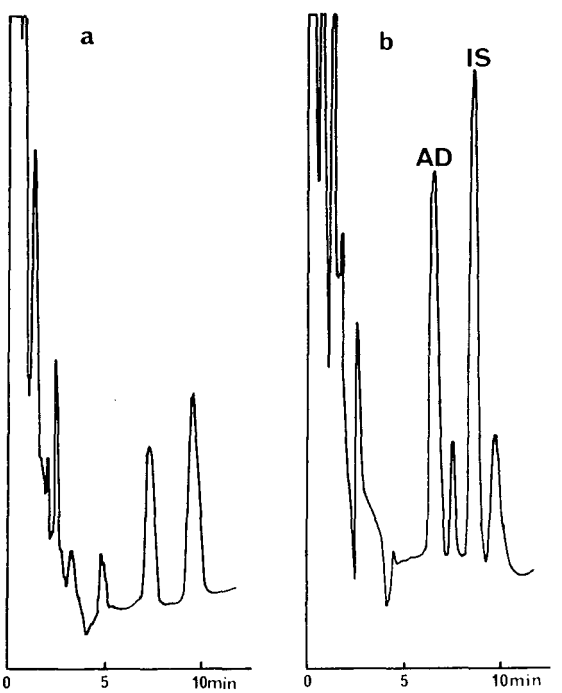

Fig. 1. Gas chromatograms of extracts from (a) normal rat plasma and (b) rat plasma spiked with 300 ng/ml of AD and 1.25 μg/ml of IS.

A calibration graph for extracted AD obtained by plotting the ratio of the peak area of AD to that of IS against the concentration of AD in rat plasma is shown in Fig. 4. The graph was slightly sigmoidal in the range studied, and the minimum measurable concentration (as determined by inspection) was 10 ng/ml. The corresponding value in human plasma is likely to be somewhat higher than this due to the less favourable blank chromatogram.

The accuracy and precision of the assay were determined by analysing samples of plasma spiked with several concentrations of AD. The results are presented in Table I.

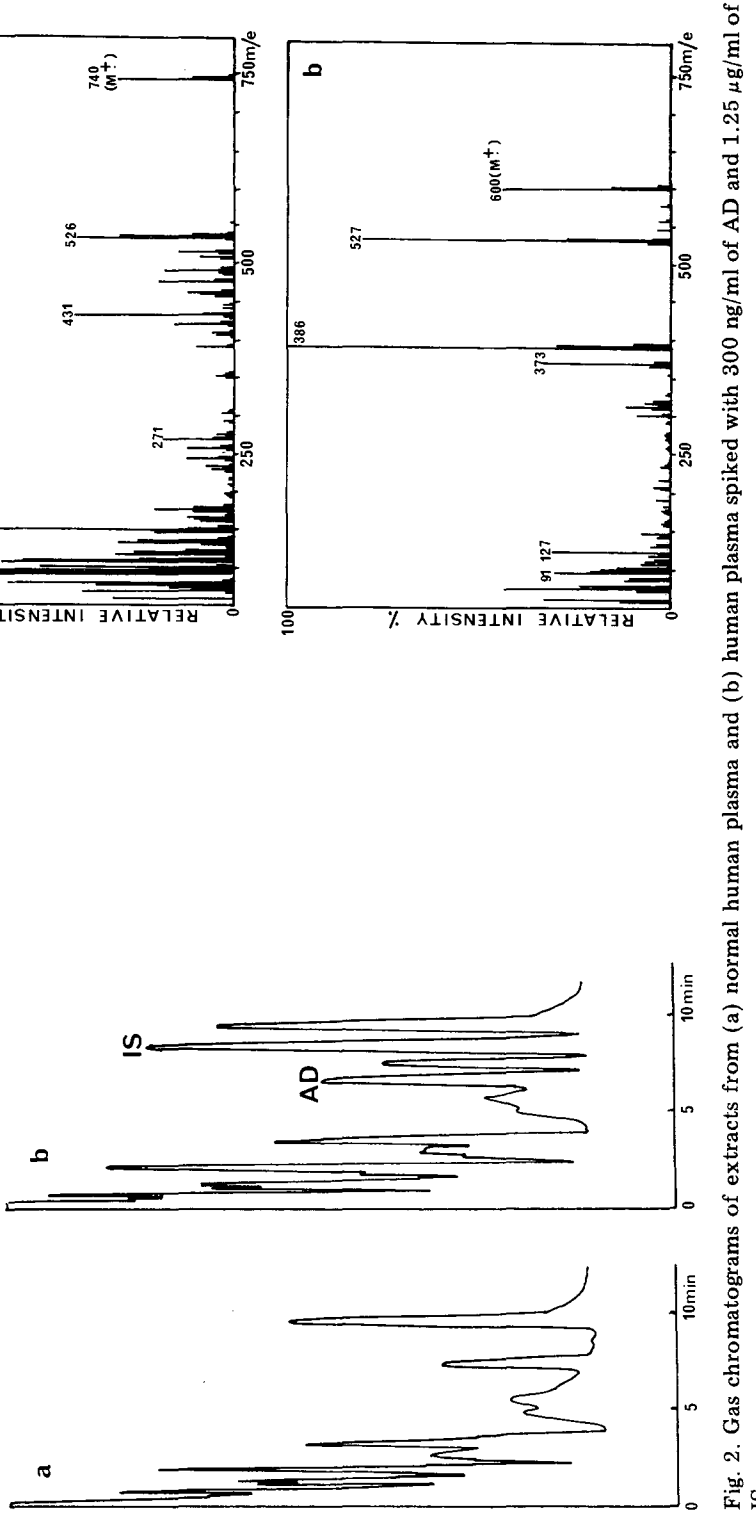

Fig. 2. Gas chromatograms of extracts from (a) normal human plasma and (b) human plasma spiked with 300 ng/ml of AD and 1.25 μg/ml of IS.

Fig. 3. Mass spectra obtained during GC–MS analysis for (a) AD-diHFB and (b) IS-monoHFB.

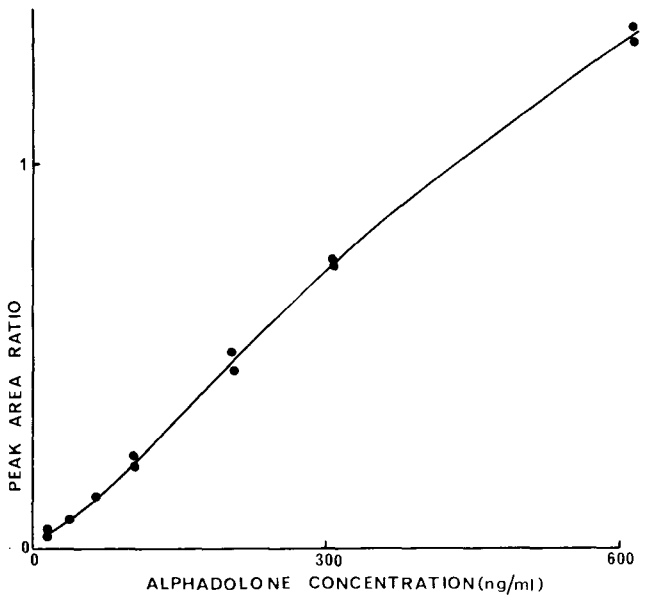

Fig. 4. Calibration graph for AD with IS.

TABLE I

RECOVERY OF ADDED ALPHADOLONE FROM PLASMA

Alphadolone (ng/ml)	
Added	Found (± S.E.M.)*
50	47 ± 1
200	181 ± 4
600	584 ± 18

*Each value is the mean of 5 estimations; S.E.M. = standard error of the mean.

When rat plasma, to which ADA had been added at a concentration of 1 μg/ml, was assayed it was found that within 5 min the amount of AD formed corresponded to complete hydrolysis of the ester. This indicates that AD, and not ADA, is the appropriate steroid to assay after the administration of Althesin to rats.

Fig. 5 shows that the method allowed the determination of the plasma concentration—time curve after a single intravenous anaesthetic dose of Althesin (= 3 mg/kg ADA) to rats. The plasma concentration of AD 2 min after dosing was 1.8 μg/ml, and at this time the concentration of ADA was less than 0.05 μg/ml, the lowest concentration of ADA that could be detected under these conditions. The half-life of AD, when measured between 2 and 50 min after dosing, was 7 min. This is very similar to the half-life of AX in rats [2]. Beyond 50 min the plasma concentration rose slightly from 0.015 μg/ml to 0.028 μg/ml and then resumed its fall. This disturbance in the plasma level—time curve has been observed for other steroidal anaesthetics (un-

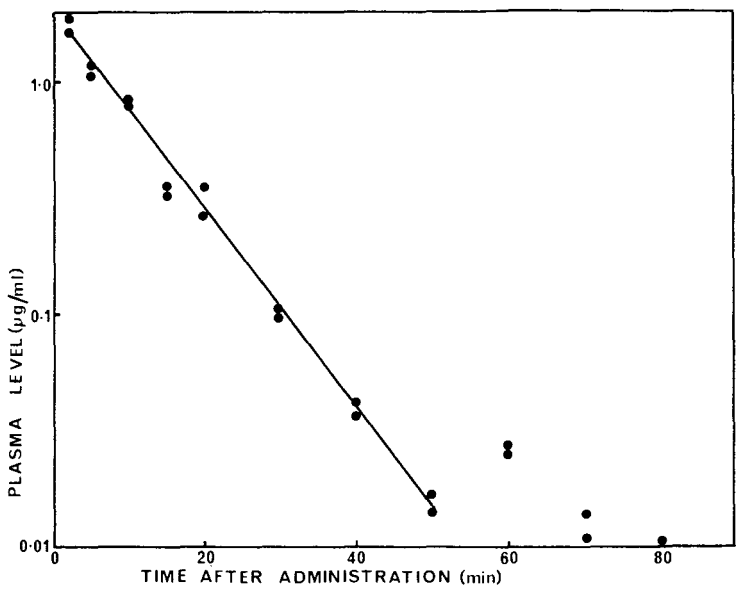

Fig. 5. Plasma levels of alphadolone in rats after a single intravenous dose of Althesin of 1 ml/kg. A separate animal was used for each time point. Samples were assayed in duplicate, and both values are shown.

published observations) and is probably not spurious. It could reflect the release of AD from a tissue depot into the blood stream or be a result of biliary recirculation.

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Note

Gas chromatographic determination of clioquinol (Vioform) in human plasma

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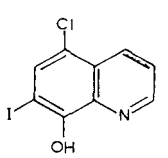
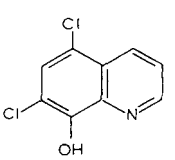
(First received March 30th, 1981; revised manuscript received May 26th, 1981)

Clioquinol* (5-chloro-8-hydroxy-7-iodoquinoline) is used in the treatment of infections of the gastrointestinal tract and of the skin. Several methods for the



clioquinol



5,7-dichloro-8-hydroxyquinoline
(internal standard)

assay of clioquinol have been described. Clioquinol in pharmaceutical preparations has been determined by the formation of copper complexes followed by iodometric titration [1]. Thin-layer chromatographic determination of clioquinol and/or its conjugate in plasma at concentrations down to 40 ng/ml has been described [2]. Several gas chromatographic (GC) methods have already been published [3—8] but they suffer from certain disadvantages. In particular, methods using the acetyl derivative of clioquinol for electron-capture detection (ECD) [4, 5] require a laborious extraction procedure, and methods using extractive methylation followed by GC—ECD analysis [6, 8] present an instability problem. A high-performance liquid chromatographic method is available [9] for measurement of conjugate levels but it is not applicable to the parent drug. A qualitative method utilizing gas chromatography—mass spectrometry has been published and used to detect a new iodine-containing metabolite [10].

This paper reports a rapid procedure, particularly adapted to a large series of samples, which permits the determination of clioquinol down to 50 ng/ml of

*Vioform®, Ciba-Geigy.

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plasma and which has been used successfully in this laboratory for seven years.

EXPERIMENTAL

Chemicals and reagents

Clioquinol and 5,7-dichloro-8-hydroxyquinoline were supplied by Ciba-Geigy (Basle, Switzerland). All reagents and solvents were of analytical grade. A pH 5 buffer (Titrisol, Merck 9885, E. Merck, Darmstadt, G.F.R.) was prepared by diluting the contents of seven vials with 1000 ml of water. Sodium sulfate (Merck 6649) solution was prepared by diluting 300 g of sodium sulfate with 1000 ml of water. Acetic anhydride (Fluka 45830; Fluka, Buchs, Switzerland) was purified before use by adding 20 g of sodium acetate to 100 ml of acetic anhydride and boiling under reflux for 15 min. Then, the acetic anhydride was distilled off and collected between 135 and 140°C. Pyridine (Fluka 82702) was distilled at 115—116°C with potassium hydroxide pellets and stored over the same reagent. The solution of internal standard contained 400 ng of 5,7-dichloro-8-hydroxyquinoline in 20 μl of methanol—water (1 : 1, v/v).

Equipment

A Hewlett-Packard, Model 5713A, gas chromatograph equipped with a Hewlett-Packard electron-capture detector (Model 18713A) was used. The peak areas were estimated by a Hewlett-Packard electronic integrator. The column was operated at 195°C, the injector at 250°C and the detector at 300°C with an argon—methane (90 : 10) flow-rate of 60 ml/min. The glass column was washed as described previously [11]. The column packing was 3% OV-101 on Chromosorb W HP 80—100 mesh (Applied Science Labs., State College, PA, U.S.A.). The filled-column (2 m × 3 mm I.D.) was conditioned as described previously [11].

Extraction

Twenty microlitres of the internal standard solution were measured into a glass tube, to which 1 ml of plasma, 2 ml of sodium sulfate solution and 5 ml of diethyl ether—dichloromethane (4 : 1, v/v) were then added. The tube was shaken mechanically (Infors shaker) for 25 min at 300 rpm and centrifuged at 4800 *g* for 10 min.

An aliquot of the organic phase was transferred to another tube and 3 ml of pH 5 buffer were added. The tube was stoppered and shaken mechanically for 10 min at 300 rpm, and then centrifuged for 3 min at 2450 *g*. An aliquot of the organic phase was transferred to another tube and dried under a nitrogen stream in a water-bath at 37°C.

Derivatization and chromatography

To the dry residue were added 50 μl of 10% pyridine in toluene and 50 μl of acetic anhydride. The tube was stoppered tightly, agitated and put in a dry heating block (Grant Instruments) at 70°C for 1 h. Excess reagent was removed by evaporation to dryness under a nitrogen stream in a water-bath at 37°C; 1 ml of ethyl acetate was added, and the tube was shaken on a Vortex mixer. A 2-μl portion of the ethyl acetate solution was injected into the gas chromato-

graph. The clioquinol content was calculated from the peak area ratio by reference to a calibration curve. This curve was obtained by extraction of plasma spiked with increasing amounts of clioquinol (from 50 to 10,000 ng/ml) and a constant amount of internal standard (400 ng/ml of plasma).

Study in man

Eight healthy fasted subjects, who had been advised to take no drugs during the week preceding the experiment and none besides clioquinol throughout the duration of the study, received 400 mg of clioquinol as Entero-Vioform powder with 150 ml of water. Blood samples were collected before and 2, 4, 8, 24, 48 and 72 h after the administration. Samples were centrifuged and the plasma was removed and stored at −20°C until analysis.

RESULTS AND DISCUSSION

Precision and recovery

Table I gives the results obtained when the described procedure was applied to spiked plasma samples. The results demonstrate the good reproducibility of the assay down to concentrations of 50 ng of clioquinol per ml of plasma, which may be taken as the sensitivity limit of the method. Lower concentrations could still be detected.

TABLE I

PRECISION AND RECOVERY OF THE DETERMINATION OF CLIOQUINOL APPLIED TO SPIKED HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Number of assays	Amount found (mean, ng/ml)	Standard deviation (±)	Recovery (mean, %)
50	9	50.3	6.0	100.6
100	10	99.4	9.5	99.4
500	6	511	38	102.2
1000	8	1004	58	100.4
5000	8	5024	260	100.5
10000	8	9924	527	99.2
				100.4 ± 1.1

Plasma interference

Fig. 1 shows the chromatograms of an extract of human plasma and of the same extract spiked with 100 ng of clioquinol and 400 ng of internal standard. There is no interference from the normal components of the plasma extract.

Scope and limitations of the described method

Like some already published methods [4, 5, 7], the described assay makes use of the acetyl derivative of clioquinol for GC—ECD, but it is much less laborious, and particularly adapted to large series of samples. It has a sensitivity comparable with that of the already published acetyl-derivative methods. Ex-

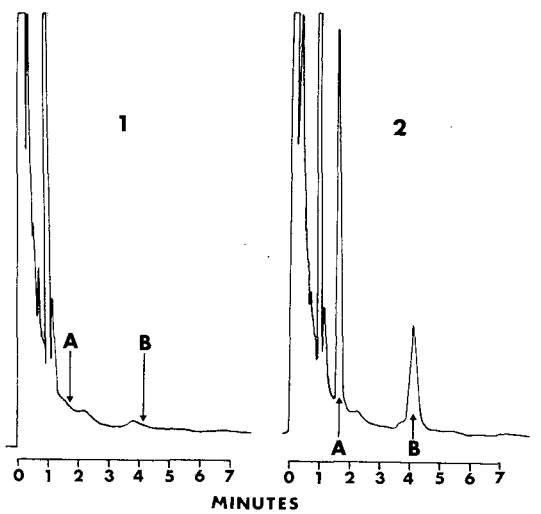

Fig. 1. Examples of chromatograms: (1) human plasma blank (1 ml of plasma); (2) the same plasma spiked with 400 ng/ml internal standard (A), and 100 ng/ml clioquinol (B).

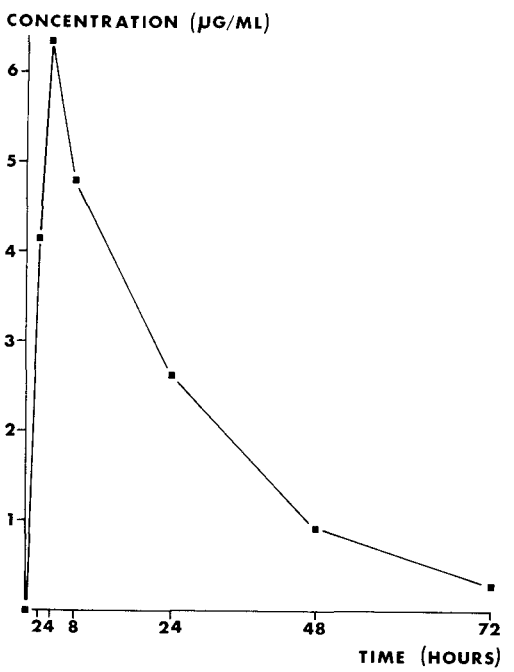

Fig. 2. Average clioquinol plasma concentrations obtained in eight healthy subjects after administration of 400 mg of clioquinol as Entero-Vioform powder.

tractive methylation methods [6, 8] allow determination of about 20 ng of clioquinol per ml of plasma by GC—ECD, but it seems that stability problems were encountered with the methyl derivative and the methylating reagent. The

described method has been used successfully in our laboratory for seven years by different analysts to assay more than 6000 plasma samples with good precision and accuracy.

Study in man

Fig. 2 shows the average curve obtained from the plasma samples of the eight subjects given 400 mg of clioquinol as Entero-Vioform powder. The sensitivity of the method thus appears sufficient to determine clioquinol in bioavailability or pharmacokinetic studies.

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Note

High-performance liquid chromatographic determination of acetaminophen in plasma: single-dose pharmacokinetic studies

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Acetaminophen (paracetamol) is extensively used as a non-prescription analgesic and antipyretic agent [1, 2]. Recent studies suggest that excessive doses and/or excessively high plasma concentrations of acetaminophen may be associated with hepatotoxicity [1—4]. Thus understanding of its pharmacokinetic properties in humans might be of value in preventing clinical toxicity.

High-performance liquid chromatography (HPLC) is applicable to quantitation of acetaminophen in human plasma [5—14]. However, a sensitivity range of 0.1—0.2 μg per ml of plasma is needed for evaluation of acetaminophen pharmacokinetics following single therapeutic doses. The present paper describes an HPLC method with this degree of sensitivity that is applicable to single-dose pharmacokinetic studies as well as to quantitation of acetaminophen following overdosage.

EXPERIMENTAL

Materials

Acetaminophen and the internal standard 2-acetaminophenol (2-AAP) (Fig. 1) were obtained from Aldrich (Milwaukee, WI, U.S.A.). All other reagents, analytical grade or better, were purchased from commercial sources and were used without further purification.

Apparatus and chromatographic conditions

A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph was used. The instrument was equipped with a Model 6000A solvent delivery system,

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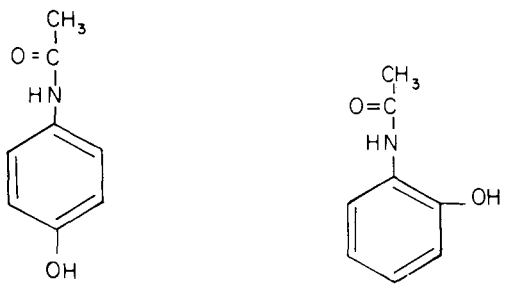

ACETAMINOPHEN 2-ACETAMINOPHENOL
(internal standard)

Fig. 1. Structural formulae of acetaminophen and of 2-acetylaminophenol, the internal standard.

a Model 440 Ultraviolet detector operated at 254 nm, and a 30 cm × 3.9 mm stainless steel μBondapak C_{18} reversed-phase column. A 10 cm × 8 mm Radial-Pak radial compression C_{18} cartridge was used alternatively. The injection system was a Model U6K sampling loop, or a WISP Model 710B automatic sample processor. Detector output was quantitated using either a strip chart recorder or a Model 730 data module.

The mobile phase consisted of either: (a) 3.5% acetonitrile—sodium acetate buffer (pH 4.0), operated at a flow-rate of 2 ml/min; or (b) acetonitrile—methanol—water (6:6:88), operated at a flow-rate of 1.8—2.2 ml/min. All analyses were performed at room temperature.

Stock solutions

Standard solutions of acetaminophen and of 2-AAP were prepared by dissolving 100 mg of each compound in 100 ml of methanol. Working solutions were prepared by appropriate dilution with methanol. Solutions are stable for at least one year when stored at 4°C.

Preparation of samples

Internal standard (15 μg) was added to a series of 13-ml round-bottom culture tubes equipped with PTFE-lined screw-top caps. The organic solvent was evaporated to dryness at 40—50°C under mildly reduced pressure. To a series of calibration tubes were added variable amounts of acetaminophen ranging from 0.1—15 μg. Again, the organic solvent was evaporated to dryness. Drug-free control plasma (0.5—1 ml) was added to each of the calibration tubes; 0.2—1.0 ml of unknown plasma was added to all other tubes.

Ethyl acetate (5 ml) was added to each tube, and the tubes were gently agitated on a Vortex-type mixer for 30 sec. After centrifugation for 10 min at 400 *g*, an aliquot (approximately 4.5 ml) of the organic phase was transferred to a 13-ml tapered glass centrifuge tube. The organic solvent was evaporated to dryness at 40—50°C under mildly reduced pressure. The residue was redissolved in 100 μl of methanol, of which 10—20 μl were injected into the sampling loop.

Clinical pharmacokinetic study

A healthy 26-year-old male volunteer (68 kg) participated after giving written informed consent. He received single 650-mg doses of acetaminophen on two occasions separated by one week. On one trial, a sterile solution of acetaminophen (13 ml of a 50 mg/ml solution [propylene glycol—ethyl alcohol—5% dextrose (40:10:50, v/v)] diluted to 50 ml with 5% dextrose) was infused into an antecubital vein over a period of 5 min by a constant-rate infusion pump. For the other trial, the subject ingested two 325-mg tablets of acetaminophen (Parke-Davis, Ann Arbor, MI, U.S.A.) in the fasting state with 100—200 ml of tap water.

Venous blood samples were drawn into heparinized tubes from an indwelling cannula, or by venipuncture, at multiple points in time during the 12 h after each dose. Plasma concentrations of acetaminophen in all samples were determined using the method described above.

Plasma acetaminophen concentrations were analyzed by weighted iterative non-linear least-squares regression techniques described in detail previously [15]. After intravenous administration, data points were fitted to the following function:

$$C = Ae^{-\alpha t} + Pe^{-\pi t} + Be^{-\beta t} \quad (1)$$

where C is the plasma acetaminophen concentration at time t after dosage. A, P, and B are hybrid intercept terms having units of concentration; they were appropriately corrected for the 5-min infusion period [16]. The exponents α, π, and β are hybrid quantities having units of reciprocal time. Using standard pharmacokinetic methods [17], coefficients and exponents from the fitted function were used to determine the following kinetic variables for acetaminophen: initial distribution half-life, intermediate distribution half-life, elimination half-life, volume of the central compartment, total volume of distribution using the area method, and total clearance. Assuming that all of acetaminophen clearance is accounted for by hepatic biotransformation, the predicted extraction ratio was calculated as the quotient of hepatic clearance and estimated hepatic blood flow (21 ml/min/kg) [18].

After oral acetaminophen administration, plasma concentrations were fitted to the following function:

$$C = -(A + B)e^{-ka(t - t_0)} + Ae^{-\alpha(t - t_0)} + Be^{-\beta(t - t_0)} \quad (2)$$

where C is the plasma concentration at time t after dosage. As in eqn. 1, A and B are hybrid intercept terms, and α and β are hybrid exponents; ka is also a hybrid exponent, representing the apparent phase of drug absorption; t_0 is the lag time elapsing prior to the start of first order absorption. The absolute bioavailability of orally administered acetaminophen was calculated as the area under the plasma concentration curve (extrapolated to infinity) after oral dosage divided by the area under the curve following intravenous administration [17].

RESULTS

Evaluation of the method

Under the described chromatographic conditions, acetaminophen and its internal standard yielded two symmetric, well-resolved peaks (Fig. 2). Endogenous plasma substituents did not interfere with peaks corresponding to acetaminophen or the internal standard.

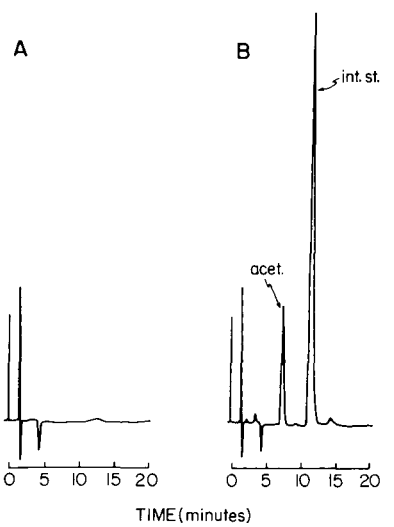

Fig. 2. Chromatograms of plasma extract from a subject (A) prior to administration of acetaminophen; (B) 2.5 h after a single intravenous dose of acetaminophen, showing peaks corresponding to acetaminophen (acet.) and to the internal standard (int. st.).

Either peak area ratio or peak height ratio can be used to quantitate detector response. The relation of detector response ratio to plasma acetaminophen concentration is linear. The day-to-day coefficient of variation in the slope of calibration curves (n = 10) was 6.0%. The sensitivity of the method is approximately 0.1—0.2 μg of acetaminophen per ml of plasma. Coefficients of variation for identical samples ranging in concentration from 0.25—15 μg/ml did not exceed 5% (Table I). The mean deviation between pairs of duplicate samples analyzed during pharmacokinetic studies (n = 45) was 2.4%. Mean recovery of acetaminophen was 90.1% (S.D. = ± 4.5%; range 79—100%). Recovery of the internal standard was 93.8 ± 6.0%.

A series of fourteen samples (concentration range: 0.26—48.8 μg/ml) were analyzed using mobile phase (a) and the stainless-steel column, with quantitation of detector response by peak height ratio. Results were compared with analysis of the same samples using mobile phase (b) and the radial compression cartridge, with detector response quantitated by peak area ratio. The correlation coefficient was 0.999, with a regression line slope of 0.94. The mean deviation between the two methods for identical samples was 3.4%.

TABLE I

REPLICABILITY OF IDENTICAL SAMPLES

Concentration (μg/ml)	Coefficient of variation* (%) (n = 6 at each concentration)
0.25	3.6
0.5	2.0
1.0	1.6
2.5	2.4
5.0	1.8
10.0	4.7
15.0	0.6

*Standard deviation divided by mean, expressed in percent.

TABLE II

KINETICS OF INTRAVENOUS AND ORAL ACETAMINOPHEN

Kinetic variables	Route of administration	
	Intravenous	Oral
Lag time (min)	—	4.7
Absorption half-life (min)	—	11.6
Initial distribution half-life (min)	1.6	—
Intermediate distribution half-life (h)	0.12	—
Elimination half-life (h)	2.6	2.5
Total clearance (ml/min/kg)	4.46	—
Total area under the curve (μg/ml·h)	35.7	26.7
Predicted extraction ratio	0.21	—
Absolute bioavailability	1.00	0.75

Clinical pharmacokinetic study

Table II shows kinetic variables for acetaminophen after intravenous and oral administration to the same volunteer. Values of elimination half-life were 2.6 and 2.5 h, respectively, by the two routes of administration (Fig. 3). After a lag time of 4.7 min, oral acetaminophen was absorbed with an apparent half-life of 11.6 min. Based on comparison of areas under the curve following oral and intravenous administration, absolute bioavailability of oral dosage was 75%. This is very close to the predicted extraction ratio of 79% based on the ratio of hepatic clearance after intravenous dosage to hepatic blood flow.

DISCUSSION

The present paper describes a rapid and sensitive quantitative assay for acetaminophen in plasma. The drug and its internal standard are readily extracted into ethyl acetate at neutral pH with no special sample preparation. Since drug-free plasma samples yield no contaminating peaks, clean-up pro-

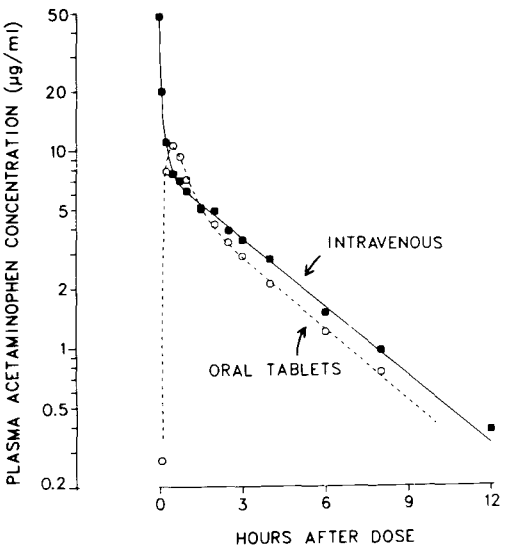

Fig. 3. Plasma acetaminophen concentrations following intravenous and oral administration of 650 mg to a healthy volunteer subject. Solid and dashed lines represent computer-determined pharmacokinetic functions consistent with eqns. 1 and 2, respectively. See Table II for kinetic analysis.

cedures are not required. The organic extract is evaporated to dryness, reconstituted, and chromatographed directly. The method is adaptable to an automatic sampling system, such that up to 100 samples can be analyzed in a 24-h period.

Meaningful data on the pharmacokinetics of acetaminophen following single therapeutic doses in humans require reliable routine quantitation of plasma concentrations as low as 0.1—0.2 μg per ml of plasma. The described method achieves this level of sensitivity, and its application to single-dose pharmacokinetic studies of acetaminophen is illustrated. Disappearance of acetaminophen from plasma following intravenous infusion was consistent with a sum of three exponential terms. The elimination half-life was 2.6 h. Total acetaminophen clearance was 4.5 ml/min/kg, predicting an extraction ratio of 21% assuming hepatic blood flow to be 21 ml/min/kg. The absolute bioavailability of oral acetaminophen in tablet form was 75%. Thus, the systemic availability of oral acetaminophen is less than 100%, and appears to be accounted for by first pass extraction rather than incomplete absorption.

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Note

Reversed-phase high-performance liquid chromatographic determination of caffeine and its N-demethylated metabolites in dog plasma

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Theophylline (1,3-dimethylxanthine) is a bronchodilator used extensively in the treatment of chronic obstructive pulmonary diseases. Because of the relatively narrow and well defined therapeutic index associated with this drug [1], monitoring of its circulating levels in patients has become a common and important clinical practice.

While numerous assay methods have been reported for theophylline analysis, few have shown the capability of simultaneous determination of this drug and other dietary xanthines such as caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine), and their various metabolites. A first step in the metabolism of caffeine in man is N-demethylation, which may occur at either the 1, 3 or 7 position to form theobromine, 1,7-dimethylxanthine and theophylline, respectively, the last two being the major pathways [2]. Theophylline and 1,7-dimethylxanthine can be separated when ion-exchange [3, 4] or normal-phase [5, 6] chromatographic columns are used, but peak resolution is poor on most reversed-phase chromatographic columns [7—11]. Interference by 1,7-dimethylxanthine can be a serious concern in the determination of theophylline when the consumption of beverages containing caffeine is difficult to control. Recent developments to solve this problem include the use of ion-pair complexing methods coupled with reversed-phase high-performance liquid chromatography (HPLC) [12, 13]. While these assays are capable of separating theophylline and 1,7-dimethylxanthine, they often lack the speed and efficiency desired in the routine analysis of these compounds. Farrish and Wargin [12] reported retention times of 22 and 12 min, respectively, for theophylline and

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1,7-dimethylxanthine. The method of Muir et al. [13], which requires ion-pair complexing and extraction together with gradient-elution systems, has retention times of 17 min for 1,7-dimethylxanthine, 20 min for theophylline, and 30 min for caffeine, plus a 17-min re-equilibration time. The complexity of the five-step ion-pairing gradient and a partially sloping baseline are further drawbacks of this system.

Miksic and Hodes [14] used a reversed-phase column with a 5-μm support or a radial compression module with a reversed-phase cartridge to resolve theophylline and 1,7-dimethylxanthine. The retention time for theophylline is 18 min on the 5-μm reversed-phase column with a mobile phase (7% methanol, 1% tetrahydrofuran, in 0.01 *M* sodium acetate pH 5 buffer) flow-rate of 1.5 ml/min at 24.2 MPa (3500 p.s.i.) pressure. The analysis time is reduced to 12 min using the radial compression unit with a mobile phase (6% methanol, 1.2% tetrahydrofuran, in 0.01 *M* pH 5 sodium acetate buffer) flow-rate of 3 ml/min at approximately 6.9 MPa (1000 p.s.i.). However, the high initial equipment cost of the radial compression module may limit the accessibility of this method.

In the present paper, an improved method using reversed-phase HPLC for the simultaneous determination of caffeine and its N-demethylated metabolites theophylline, 1,7-dimethylxanthine and theobromine is described. The method was used to analyze plasma samples from a beagle dog after receiving multiple oral doses of caffeine.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Waters Model 6000 pump, a Waters Model U6K universal injector, a Waters Model 440 UV detector, a 30 cm × 3.9 mm I.D. 10-μm μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.), a 7 cm × 2.1 mm I.D. precolumn (Co:Pell ODS, Whatman, Clifton, NJ, U.S.A.), and a 10-mV Fisher Series 500 recorder (Fisher Scientific, Pittsburgh, PA, U.S.A.) operated at a chart speed of 0.5 cm/min.

Chemicals

Ultrapure water, glacial acetic acid and tetrahydrofuran (HPLC grade, J.T. Baker, Phillipsburg, NJ, U.S.A.); methanol and acetonitrile (UV grade, Burdick and Jackson Labs., Muskegon, MI, U.S.A.); sodium acetate and trichloroacetic acid (certified ACS grade, Fisher Scientific); theophylline (Pfaltz and Bauer, Stanford, CT, U.S.A.); theobromine, 1,7-dimethylxanthine, β-hydroxyethyltheophylline and caffeine (Sigma, St. Louis, MO, U.S.A.) were all used as received.

Assay procedure

To 0.5 ml of plasma in a 2-ml centrifuge tube were added 0.05 ml of 40% trichloroacetic acid and 0.05 ml of an internal standard solution (200 μg/ml of β-hydroxyethyltheophylline in pH 5 buffer). After mixing on a vortex mixer for 30 sec, samples were allowed to stand for 5 min, followed by centrifugation at 1000 *g* for 10 min. A 25-μl aliquot of the supernatant was injected into the chromatograph.

The mobile phase for liquid chromatography was 0.005 *M* sodium acetate buffer adjusted to pH 5—methanol—acetonitrile—tetrahydrofuran (92.5 : 3 : 2.8 : 1.7, v/v). This solution was filtered through a 0.45-μm filter and degassed under vacuum with sonication. The chromatograph was operated at ambient temperature with a flow-rate of 1.5 ml/min at 10.4 MPa (1500 p.s.i.). The UV detector was set at 280 nm and 0.02 a.u.f.s.

A standard curve for each compound studied was prepared by assaying dog plasma samples containing caffeine, theophylline, 1,7-dimethylxanthine and theobromine at concentrations of 0.5, 1, 2, 4, 8, 16 and 32 μg/ml. All samples were analyzed in quadruplicate. Quantitation was achieved by the drug:internal standard peak height ratio method.

Experiment in the dog

A one-year-old male beagle dog weighing 11.4 kg was given a 100-mg tablet of caffeine (NoDoz, Bristol-Myers, New York, NY, U.S.A.) twice daily at 9:00 a.m. and 9:00 p.m. for 8 days. Serial venous blood samples were collected during the 12 h after the morning doses on days 1 and 8. The procedures for drug administration and plasma collection and storage were previously described in detail [15].

RESULTS

Assay

Chromatograms of control dog plasma and plasma spiked with the test compounds are shown in Fig. 1A and B, respectively, while Fig. 1C is the result of analysis of a plasma sample obtained from the dog 1 h after the morning dose of caffeine on day 8.

Complete peak resolution was achieved by the HPLC system in 14 min, with retention times of 5.1, 7.6, 8.3, 9 and 13.1 min for theobromine, 1,7-dimethylxanthine, theophylline, β-hydroxyethyltheophylline and caffeine, respectively. Other xanthines did not interfere. Plots of peak height ratios versus plasma drug concentrations over the range of 0.5—32 μg/ml were linear, with correlation coefficients better than 0.999 and *y*-intercepts not significantly different from zero for all compounds. The accuracy and precision of the method, determined by assaying plasma samples containing known quantities of the drugs, are shown in Table I. The limits of detection were 0.1 μg/ml for theophylline and theobromine, and 0.2 μg/ml for 1,7-dimethylxanthine and caffeine.

Plasma levels of caffeine and metabolites in the dog

Plasma concentration—time data after single and multiple oral doses of caffeine to the dog are presented in Fig. 2. Caffeine was rapidly absorbed from a single oral dose, reaching a peak plasma level of 10.9 μg/ml in 1 h. Elimination half-life calculated by linear regression of the 3—12 h data was 4.3 h. Theophylline and 1,7-dimethylxanthine appeared in plasma within 1 h, and slowly reached peak concentrations of 2.38 and 1.00 μg/ml, respectively, at 8 h after caffeine administration.

After repeated oral administration of caffeine, twice daily for eight days, there were slight increases in the peak plasma level (17.5 μg/ml) and half-life

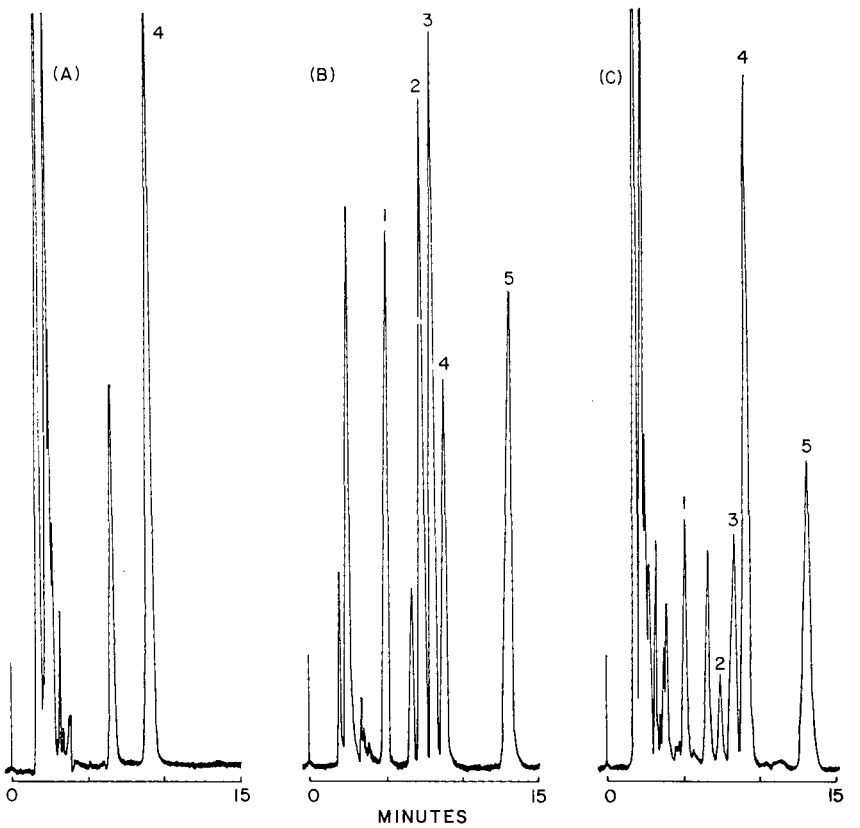

Fig. 1. Chromatograms of (A) control dog plasma with β-hydroxyethyltheophylline (internal standard); (B) plasma spiked with (1) theobromine at 16 μg/ml, (2) 1,7-dimethylxanthine, (3) theophylline, (5) caffeine, each at 32 μg/ml, and (4) β-hydroxyethyltheophylline at 20 μg/ml; and (C) plasma obtained from a dog 1 h after the morning dose on day 8 during a 100-mg caffeine twice daily regimen.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY

Concentration (μg/ml)	Theobromine		1,7-Dimethylxanthine		Theophylline		Caffeine	
	C_{obs}* (μg/ml)	C.V.** (%)	C_{obs} (μg/ml)	C.V. (%)	C_{obs} (μg/ml)	C.V. (%)	C_{obs} (μg/ml)	C.V. (%)
0.5	N.D.***	N.D.	0.47	6.38	0.54	5.56	0.62	12.0
1	0.84	0.59	1.10	0.27	1.12	6.79	1.15	10.4
2	N.D.	N.D.	2.14	1.87	2.13	3.29	2.11	6.16
4	4.04	0.71	4.05	0.25	4.05	1.98	4.03	3.47
8	N.D.	N.D.	7.85	0.38	7.82	1.41	7.86	2.54
16	16.3	0.63	15.8	2.86	15.6	3.46	15.8	1.08
32	31.8	1.16	32.1	0.78	32.2	1.83	32.1	1.71

*Mean observed concentration, $n = 5$.

**Coefficient of variation.

***N.D. = not determined.

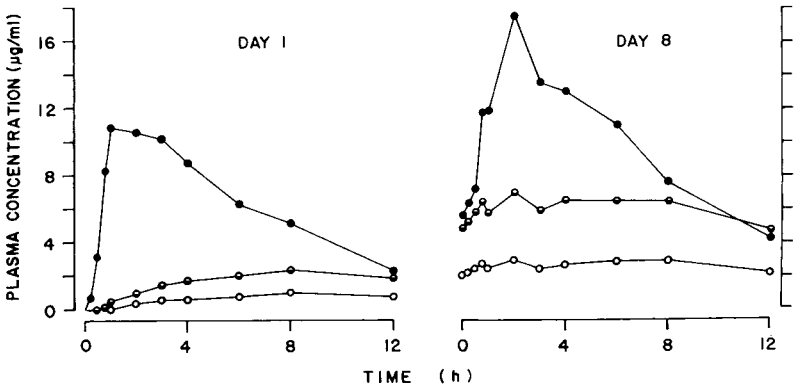

Fig. 2. Plasma concentrations of caffeine (•), theophylline (⊖), and 1,7-dimethylxanthine (○) after the morning doses on day 1 and day 8 during a 100-mg caffeine twice daily regimen.

(5.2 h) of the drug. On the other hand, significant accumulation of the metabolites was observed. Peak plasma concentrations were 6.99 μg/ml for theophylline and 2.98 μg/ml for 1,7-dimethylxanthine, both occurring at 2 h after dosing with caffeine.

Theobromine plasma concentrations similar to the levels of 1,7-dimethylxanthine were also observed. The data have been reported elsewhere [16].

DISCUSSION

In the present HPLC method, protein-precipitated plasma was injected directly into the liquid chromatograph. The simplicity of the method allowed quantitative analysis of small plasma samples (0.25—0.5 ml). This procedure is superior to solvent extraction methods which are more time consuming and frequently show low recovery and poor reproducibility due to emulsion formation. A regular 10-μm C_{18} column was used in the HPLC system, together with a precolumn to prolong column life and maintain maximum resolution.

Optimal resolution of theobromine, 1,7-dimethylxanthine, theophylline, β-hydroxyethyltheophylline and caffeine was achieved rationally by careful examination of the physicochemical properties and elution characteristics of these compounds in methanol, acetonitrile and tetrahydrofuran. The polarity of the xanthine analogues are, in descending order, theobromine, 1,7-dimethylxanthine, theophylline, β-hydroxyethyltheophylline and caffeine. Methanol, the most polar organic solvent used in the mobile phase, effectively accelerated the elution of the more polar interfering substances in the deproteinated plasma relative to the less polar xanthine analogues. Acetonitrile, a less polar solvent than methanol, increased the rate of elution of theophylline with respect to 1,7-dimethylxanthine. Similarly, tetrahydrofuran, the least polar of the three solvents, accelerated the elution of β-hydroxyethyltheophylline with respect to theophylline. The composition of the mobile phase was selected to give optimal resolution of the xanthine analogues in the shortest time possible.

Compared to the isocratic methods [14] and the ion-pair complexing methods [12, 13], the HPLC assay described here provides significant advantages in terms of speed and efficiency. It is sensitive and reproducible, and can be read-

ily applied to ordinary HPLC equipment without modification. The method was used successfully in the analysis of plasma from a dog during a multiple dosing experiment with caffeine, when significant accumulation (ca. three-fold) of both theophylline and 1,7-dimethylxanthine was observed. Higher steady-state levels of theophylline were noted in our previous paper [15], which was due apparently to interference by 1,7-dimethylxanthine. Nevertheless, the removal of the 7-methyl group appears to be the major metabolic pathway of caffeine in the dog, which is in agreement with previous observations in other laboratories [8].

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CHROMBIO. 1007

Note

Determination of allopurinol and oxipurinol in biological fluids by high-performance liquid chromatography*

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Allopurinol (4-hydroxy-3,4-*d*-pyrazolopyrimidine), a structural analogue of hypoxanthine, is a useful drug for the treatment of gout and hyperuricemia [1—3]. Allopurinol and its major metabolite oxipurinol (3,4-dihydroxy-3,4-*d*-pyrazolopyrimidine) decrease uric acid production by inhibiting xanthine oxidase, the enzyme that converts hypoxanthine to xanthine and xanthine to uric acid [4, 5]. Additionally, allopurinol and oxipurinol depress de novo purine biosynthesis through feedback inhibition of amidophosphoribosyltransferase and by depletion of the essential substrate, phosphoribosylpyrophosphate [6, 7].

Adverse effects of allopurinol therapy are reported to occur more frequently in patients with renal insufficiency [8]. Therefore, a knowledge of blood levels of allopurinol and oxipurinol will be helpful in establishing adequate drug dosage, especially in patients with renal impairment.

Previously described methods for the determination of allopurinol and oxipurinol include radioactive detection after paper or column chromatographic separation [9], column chromatography on Dowex resins [9, 10], gas chromatography [11], mass spectrometry [12], and direct electrochemical determination [13]. A competitive binding assay [14], photometric enzyme-inhibition assays [15, 16], and, more recently, a fluorimetric enzyme-inhibition test [17] have been described. Several high-performance liquid chromatographic (HPLC) methods for the determination of allopurinol and oxipurinol in biological fluids have been published [18—26]. However, their applicability to clinical monitoring remains questionable. The disadvantages of these methods

*G.G. will present this work as thesis to the Fachbereich Medizin der Justus Liebig-Universität Giessen.

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include difficult sample preparation [19, 25], long analysis time [18, 19, 26], insufficient sensitivity [23, 24] or selectivity [25], and restricted applicability for allopurinol [20, 22] or serum [21, 26] only.

Therefore, a rapid, simple and sensitive HPLC assay procedure for allopurinol and its active metabolite, oxipurinol, in plasma and urine, which is suitable for clinical studies, has been developed in our laboratory.

EXPERIMENTAL

Chemicals

Allopurinol, oxipurinol and uric acid were purchased from Henning, Berlin, G.F.R. 6-Thioguanine, guanine and 8-azaguanine were delivered from Burroughs Wellcome, London, Great Britain. All other substances were analytical grade reagents and were used without further purification.

Apparatus

The high-performance liquid chromatograph was equipped with a Gynkotek HPLC pump Model 600/200 (Gynkotek, Munich, G.F.R.), a modified automatic sample injector ASI 45 (Kontron, Eching, G.F.R.), a variable-wavelength ultraviolet detector Uvikon 720 LC (Kontron, Eching, G.F.R.), a computing integrator SP 4100 (Spectra-Physics, Darmstadt, G.F.R.), and a two-channel electronic recorder BD9 (Kipp and Zonen, Kronberg/Ts., G.F.R.).

Assay procedure

A short-alkyl reversed-phase material (SAS-Hypersil, 5 μm, 30 cm × 4.1 mm I.D.) was used as stationary phase. The eluent was prepared by mixing 190 ml of 0.1 *M* citric acid monohydrate with 810 ml of 0.2 *M* disodium phosphate and 2 l of distilled water. The flow-rate was 2.0 ml/min at a back pressure of 270 bar and at room temperature. Detection was set at 0.02 a.u.f.s. and at 252 nm, the absorption maximum of allopurinol at pH 7 [27].

Blood samples (1 ml) were taken into heparinized tubes and centrifuged at 8000 *g* for 5 min. Plasma was withdrawn, diluted 1:2 in the eluent, and injected into the chromatograph (20-μl samples). Plasma not immediately analyzed was stored frozen at −16°C. Urine samples were diluted 1:20 in distilled water before aliquots of 20 μl were chromatographed.

For quantitation the areas under the curves were computed by an integrator. Calibration was performed by the method of external standardization. Each sample was analyzed in duplicate. A third analysis was performed if the peak areas of the compounds differed by more than 5%.

Protein binding

For determination of protein binding, ultrafiltration was performed using a Model MM 302 ultrafiltration system (Amicon, Düren, G.F.R.) and Type PM 10 Diaflo® membranes. In controls there was no adsorption of allopurinol and oxipurinol to this membrane.

Stability

Stability of allopurinol and oxipurinol in human blood was tested in heparinized fresh blood samples, which were spiked with both compounds (1 μg/ml). Each sample was gently shaken at 37°C. After various time intervals aliquots were withdrawn, centrifuged and plasma samples subjected to HPLC.

RESULTS AND DISCUSSION

SAS-Hypersil was found to be a suitable reversed-phase material with regard to selectivity and stability. Since the quality of the SAS-Hypersil column did not noticeably decline with untreated plasma or urine (up to 200 injections of 20-μl samples), samples of plasma or urine were analyzed directly avoiding all deproteinization and extraction procedures. Optimal resolution was obtained with a citrate—phosphate buffer eluent (50 m*M*, pH 7.0). The addition of the citrate component provided sharp, symmetrical and well-defined peaks of allopurinol and oxipurinol (Fig. 1). Constituents of plasma (Fig. 1d and e) or urine (Fig. 1f and g) did not interfere with the resolution of either compounds.

The retention times of allopurinol (10 min) and oxipurinol (7.8 min) were quite stable with a relative standard deviation of less than 5%, as demonstrated from day to day with standard test solutions.

Separation was not disturbed by other purine analogues, such as uric acid,

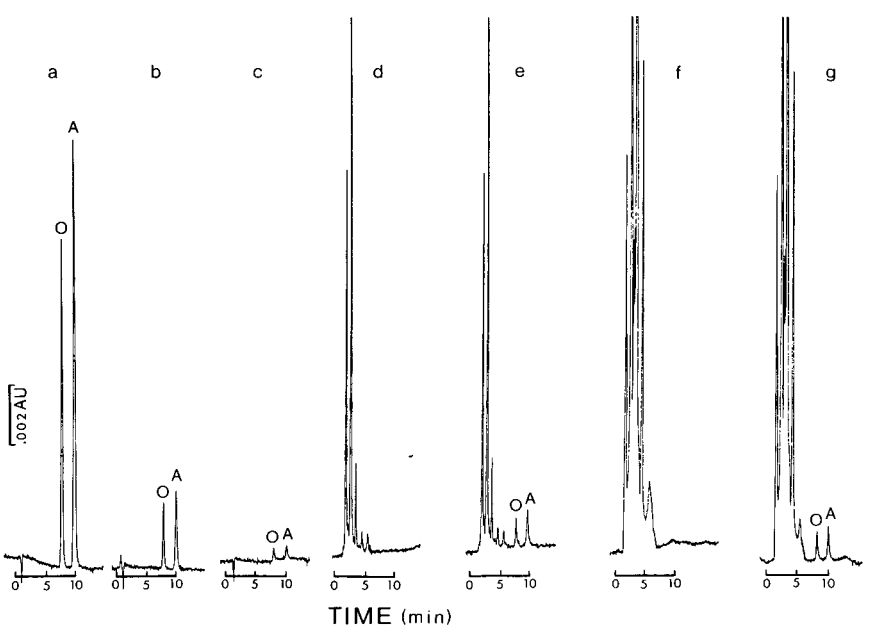

Fig. 1. Chromatograms of standards containing (a) 2.5, (b) 0.5, (c) 0.1 μg of allopurinol (A) and oxipurinol (O) per ml, (d) blank plasma, (e) standard plasma, (f) blank urine, and (g) standard urine. Plasma was diluted 1:2 and urine 1:20 in distilled water. Stationary phase: SAS-Hypersil (5 μm, 30 × 0.41 cm I.D.). Mobile phase: citrate—phosphate buffer (50 m*M*, pH 7.0). Sample volume: 20 μl. Flow-rate: 2 ml/min. Back pressure: 270 bar. Room temperature. Detection at 252 nm.

xanthine, hypoxanthine, guanine, 8-azaguanine, 6-thioguanine, and 6-mercaptopurine. No interfering peaks were found in the plasma of patients 2 h after application of the following drugs: acetylsalicylic acid, azathioprine, benzbromarone, caffeine, cotrimoxazole, cytarabine, diazepam, dihydralazine, dipyridamole, fluorouracil, methotrexate, procarbazine, propranolol, spironolactone, sulfinpyrazone, aminophylline, 6-thioguanine, and 6-mercaptopurine.

Calibration curves for allopurinol and oxipurinol were linear from 0.1 to 50 μg/ml with intercepts not significantly different from zero. The limit of detection was 0.1 μg/ml with a coefficient of variation of less than 5% for both compounds. The day-to-day precision as determined on six consecutive days for frozen plasma samples (1 μg/ml) was found to be 6.9% for allopurinol and 7.8% for oxipurinol.

Recovery from spiked samples of plasma or urine was 97—102% for both compounds when compared with the peak areas of aqueous standards. Since recovery from plasma or urine was highly reproducible and preliminary-clean-up procedures were eliminated, the method of external standardization was used for quantitation. Stability of allopurinol and oxipurinol in human blood, plasma and urine at 37°C was excellent. Contrary to the preliminary report of Kramer and Feldman [28], no evidence of erythrocyte metabolism of the drug was observed during the incubation period (2 h).

The plasma protein binding of allopurinol was found to be 2.0 ± 3.7% (mean ± S.D.) for the concentration range 0.5—50 μg/ml. In contrast to Elion et al. [9], who reported no significant protein binding of either allopurinol or oxi-

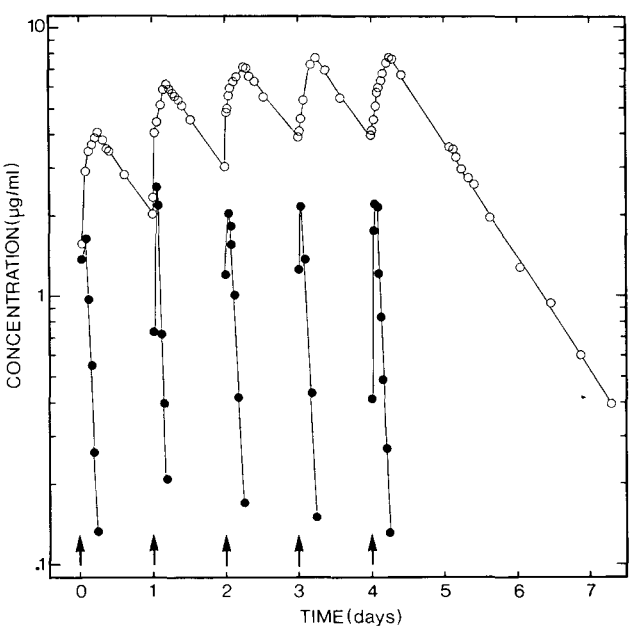

Fig. 2. Time course of plasma concentration of allopurinol (•) and oxipurinol (○) during and after oral application (↑) of 300 mg of allopurinol (3.2 mg/kg) on five consecutive days. Each point represents the mean value of two determinations. A third analysis was performed if the difference was more than 5%.

purinol, the binding of oxipurinol to plasma proteins was 16.8 ± 4.4% (mean ± S.D.).

The HPLC method was applied to pharmacokinetic studies in plasma (Fig. 2) and urine (Fig. 3). The plasma kinetics after oral application of 300 mg of allopurinol on five consecutive days demonstrate the rapid disappearance of allopurinol from plasma, with a half-time of 1.13 ± 0.13 h (mean ± S.E., $n = 5$ days). The plasma levels (1.5—8 μg/ml) and half-time of oxipurinol (18.45 ± 1.32 h) were comparable to those found by others [9, 16, 20]. The urinary recovery of intact allopurinol was 23% and that of oxipurinol 71% of the total dose administered.

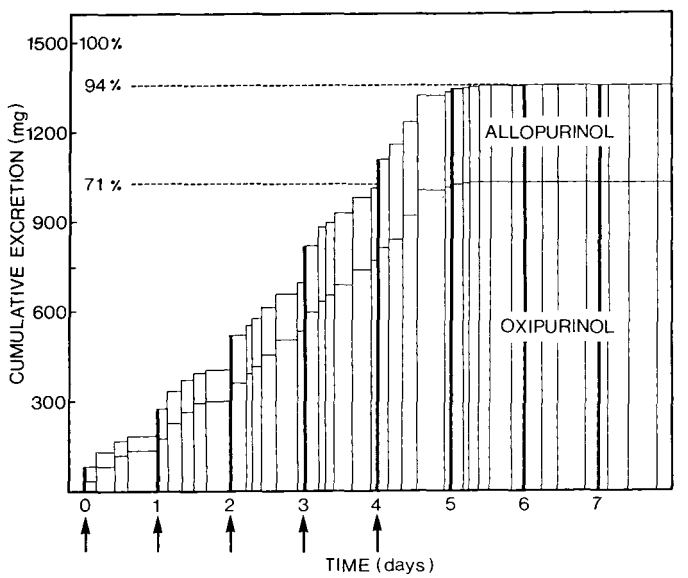

Fig. 3. Cumulative excretion of allopurinol and oxipurinol in urine. A total dose of 1500 mg of allopurinol was orally administered, given in five single doses of 300 mg on five consecutive days (↑).

In summary, a rapid and simple HPLC assay for the analysis of the hypouricemics allopurinol and oxipurinol in plasma and urine is described. Without time-consuming clean-up procedures, plasma and urine samples were analyzed directly by isocratic reversed-phase chromatography providing complete separation from constituents of plasma or urine. Both compounds can be precisely determined in human plasma and urine in concentration ranges usually encountered after therapy with allopurinol. This methods offers significant advantages in terms of rapidity, simplicity, specificity and reproducibility over previously published methods [9—26].

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Note

High-performance liquid chromatographic analysis of 5′-methylthioadenosine in rat tissues

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5′-Methylthioadenosine (MTA) is a naturally occurring nucleoside originating from S-adenosylmethionine (AdoMet) by several biochemical pathways [1, 2]. In mammalian tissues MTA is produced stoichiometrically with the polyamines spermidine and spermine [3, 4] and by direct enzymatic cleavage of AdoMet [5, 6].

As reported by several authors, the thioether exerts a variety of regulatory effects on a number of biological systems [2, 7—11] and is a potent inhibitor of mitogen- and antigen-stimulated lymphocyte proliferation [12]. An accurate measurement of the MTA concentration in the various tissues is a condition for a correct evaluation of the physiological significance of the above effects.

Only a few data are reported in the literature on the cellular content of the thioether [13—15]. This paper describes a method for the estimation of MTA in biological samples using high-performance liquid chromatography (HPLC) combined with an isotope dilution technique. Two alternative procedures have been developed. The first involves chromatography on Dowex-50 followed by HPLC separation on a reversed-phase column. The second method requires two chromatographic steps before cation-exchange HPLC analysis.

Preliminary results with this procedure have already been published [16, 17].

EXPERIMENTAL

Chemicals

Since commercial AdoMet is contaminated by numerous impurities, the

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compound was routinely prepared from cultures of *Saccharomyces cerevisiae* [18], isolated by ion-exchange chromatography [19] and analyzed by HPLC [20]. S-Adenosyl-L-[methyl-^{14}C]methionine (58 mCi/mmol) was supplied by The Radiochemical Centre, Amersham, Great Britain. 5'-[Methyl-^{14}C]-methylthioadenosine and unlabeled MTA were prepared by acid hydrolysis (pH 4.5, 100°C for 30 min) of AdoMet [21]. The reaction has been monitored spectrophotometrically using adenosine deaminase from *Aspergillus oryzae* (Sanzyme; Calbiochem, Los Angeles, CA, U.S.A.) [22]. 5'-Methylthioinosine (MTI) was obtained by enzymatic deamination of MTA [23]. Adenine (Ade), adenosine (Ado) and S-adenosylhomocysteine (AdoHcy) were supplied by Sigma, St. Louis, Mo, U.S.A. The chemical and radiochemical purity of the standards were checked by thin-layer chromatography, high-voltage electrophoresis and HPLC [20]. Dowex-50 resin (8 × 100 cross-linkage, 100—200 mesh) and Affi-gel 601 were obtained from Bio-Rad Labs., Richmond, CA, U.S.A. Other chemicals, analytical grade, were obtained from conventional commercial sources.

Analytical procedures

Sample preparation. Fig. 1 summarizes the multi-step sequence of the two proposed analytical procedures. Male Sprague—Dawley rats weighing 150—300 g were decapitated; the organs were removed quickly and weighed. Tissue portions (0.5 g) were homogenized with ice-cold 1.5 *M* perchloric acid (PCA) (1:4, w/v). Methyl-^{14}C-labeled MTA was added directly to the tissue during homogenization with PCA (2 nmol/g, 140,000 cpm/nmol). After centrifugation the deproteinized supernatant was neutralized with 1 *M* potassium hydroxide, centrifuged and chromatographed through a Dowex-50 (H^+) column (resin bed 2 × 0.2 cm) previously equilibrated with 0.1 *M* hydrochloric acid. Elution was carried out stepwise with 30 ml of 0.5 *M* hydrochloric acid to remove contaminating nucleotides and nucleosides and with 1.5 *M* PCA to collect MTA. The PCA eluate was analyzed (as reported in Fig. 1) either with procedure A or according to procedure B.

Procedure A. The PCA eluate was neutralized with potassium hydroxide and after centrifugation the supernatant was concentrated to 0.5 ml under reduced pressure. A 100-μl aliquot of this sample was analyzed by reversed-phase (Partisil 10 ODS) HPLC.

Procedure B. The PCA eluate from the Dowex-50 column, adjusted to pH 8.8, was applied to a 0.5 × 2 cm column of phenylboronate resin (Affi-gel 601) equilibrated with 0.25 *M* ammonium acetate (pH 8.8). The elution was carried out with 20 ml of 0.25 *M* ammonium acetate (pH 8.8) to remove all the non-*cis*-diol compounds. MTA was eluted with 10 ml of 0.1 *M* acetic acid and concentrated to 500 μl under vacuum on a rotary evaporator. A 100-μl aliquot of this sample was analyzed by ion-exchange (Partisil 10 SCX) HPLC.

High-performance liquid chromatography. A Perkin-Elmer liquid chromatograph, Model LC 65T, equipped with an ultraviolet detector operating at 254 nm, was used. The columns (25 × 4.6 mm I.D.) were prepacked with Partisil 10 ODS RP-18 (10 μm particle size), or Partisil 10 SCX (10 μm particle

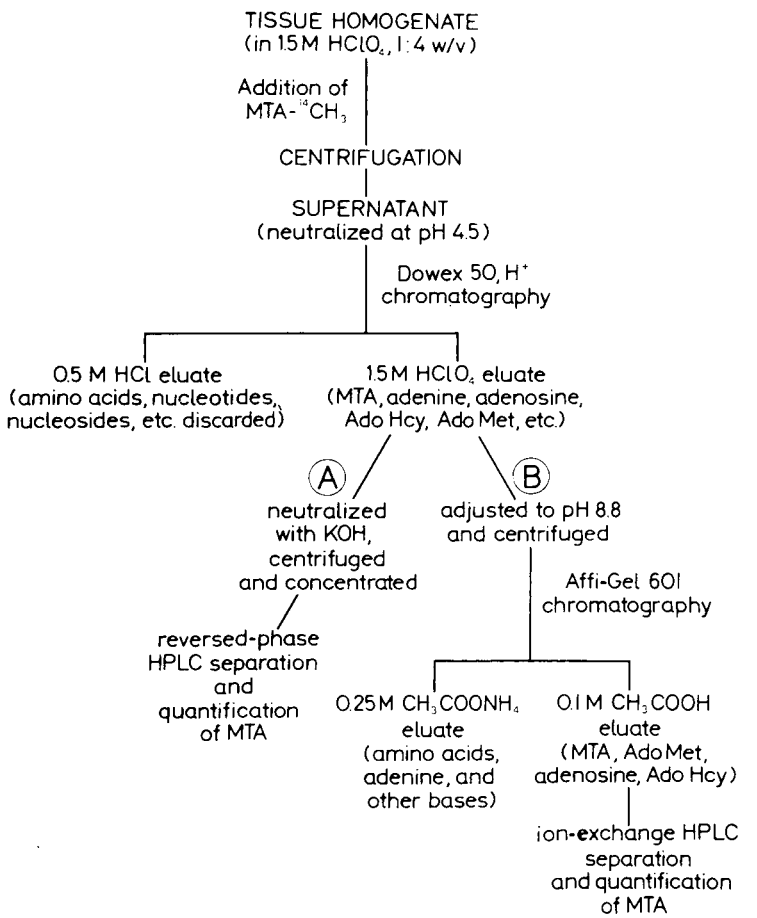

Fig. 1. Procedures for estimation of 5'-methylthioadenosine (MTA) in tissues. For abbreviations, see text.

size (Whatman, Clifton, NJ, U.S.A.). Integration was performed electronically using a Spectra-Physics Minigrator. Injection was performed via a Model 7120 sample injector valve (Rheodyne Inc.).

Elution from the reversed-phase column (Partisil 10 ODS) was carried out with a 7:93 (v/v) mixture of anhydrous methanol and 7 m*M* acetic acid at a flow-rate of 1.5 ml/min. Ammonium formate buffer (0.25 *M*, pH 4) was used as eluent for the Partisil 10 SCX column, with a flow-rate of 0.6 ml/min.

MTA quantitation. MTA concentration can be determined by comparison of absorbance integrated peak areas (or peak height) to standard curves obtained for solutions of pure MTA over the concentration range 0.1—5 nmol.

In order to calculate the recovery, methyl-^{14}C-labeled MTA was added directly to the homogenate, and after HPLC analysis 0.5-ml fractions of the eluates were collected and mixed in the scintillation vials with 4 ml of Instagel (Packard, Downers Grove, IL, U.S.A.). The radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard, Model 3380).

RESULTS AND DISCUSSION

This paper describes two alternative procedures for the analysis of MTA in rat tissues (Fig. 1). The extraction and separation conditions were optimized to avoid degradation of MTA and interference from other naturally occurring constituents.

Experiments not reported here demonstrated that no significant amount of MTA is produced by degradation of AdoMet, which appears quantitatively stable under the conditions employed in both analyses. The preliminary fractionation procedure of the PCA extracts on Dowex-50 does not involve any laborious or time-consuming manipulation of the samples. Procedure B, which implies an additional chromatographic step, appears useful for the tissues (i.e. lung and heart) where unknown compounds interfere with reversed-phase analysis.

For the quantitation of MTA an isotope dilution technique was used because of the small amounts of the thioether present in the tissues. The average recovery of ^{14}-C-labeled MTA added to the rat tissues was 82—85% for procedure A and 75—80% for procedure B. An isotope dilution technique appears to be necessary because of variability of yields.

A representative chromatogram of a standard mixture of MTA and related compounds (Ade, Ado, AdoHcy, MTI, AdoMet) is shown in Fig. 2a. The chromatographic separation was carried out on a reversed-phase column processed as described. All the compounds were eluted within 20 min of injection. Fig. 2b is a typical chromatogram from an analysis of rat liver. The thioether was extracted from 500 mg of wet tissue according to method A. Samples equivalent to 100 mg of tissue were injected for HPLC analysis. The chromatogram shows a peak which can be identified as MTA according to its retention time. Co-injection with a non-labeled MTA standard resulted

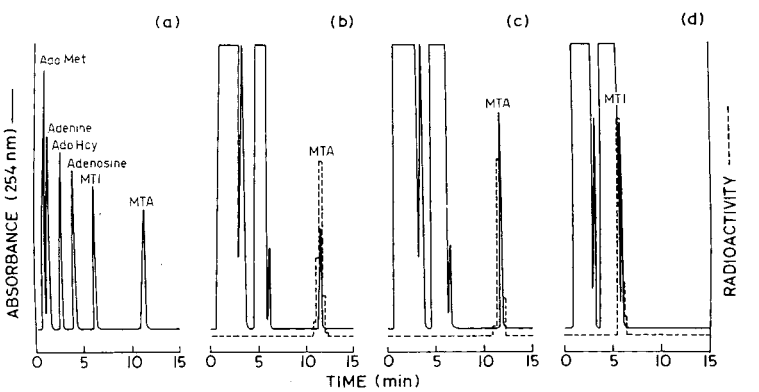

Fig. 2. (a) Separation of MTA and related reference compounds detected at 254 nm. The compounds were added in the amount of 0.2 nmol each. Column, Partisil 10 ODS; temperature, ambient; sensitivity, 0.5 a.u.f.s.; eluent, 7:93 (v/v) mixture of anhydrous methanol and 7 m*M* acetic acid; flow-rate, 1.5 ml/min. (b) Chromatogram of a rat liver extract after Dowex-50 chromatography. (c) Chromatogram of the sample shown in Fig. 2b coinjected with MTA. (d) Chromatogram of the sample shown in Fig. 2b, incubated with adenosine deaminase (incubation time, 30 min). For abbreviations, see text.

in an increase of the peak area coincident with the radioactive peak (Fig. 2c). To check the purity of the identified thioether, an aliquot of the sample was incubated for 30 min with non-specific adenosine deaminase just before the HPLC analysis. The chromatogram of the resulting mixture shows the absence of UV-absorbing material and radioactivity at the retention time corresponding to MTA (10.8 min) and the presence of a new radioactive peak with the elution time of MTI (6.4 min) (Fig. 2d). This evidence excludes the presence of UV-contaminating compounds in the MTA peak, allowing the application of the isotope dilution technique.

The alternative procedure proposed involves a strong cation-exchange HPLC method. Fig. 3a shows the elution profile of a standard mixture of

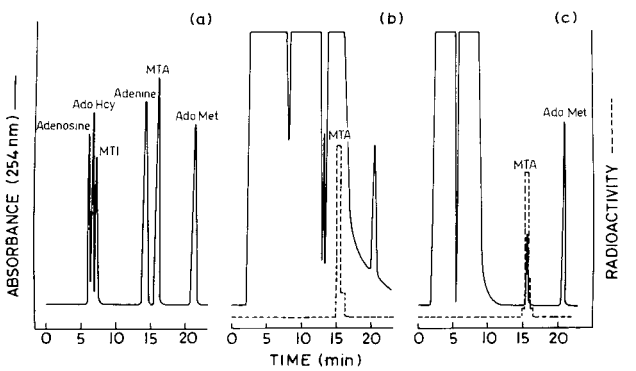

Fig. 3. (a) Separation of reference compounds detected at 254 nm. The compounds were added in the amounts of 0.2 nmol each. Column, Partisil 10 SCX; temperature, ambient; sensitivity, 0.5 a.u.f.s.; eluent, ammonium formate buffer (0.25 *M*, pH 4.0); flow-rate, 0.6 ml/min. (b) Chromatogram of rat liver extract after Dowex-50 chromatography. (c) Chromatogram of a rat liver extract after Dowex-50 plus Affi-gel 601 treatment. For abbreviations, see text.

TABLE I

MTA CONTENT OF RAT TISSUES

The values are the mean of five determinations (± standard deviation). MTA analysis was performed by: (A) PCA extraction, Dowex-50 chromatography and reversed-phase HPLC analysis as indicated in Materials and Methods (see legend to Fig. 2); (B) PCA extraction, Dowex-50 and Affi-gel 601 chromatography and ion-exchange HPLC separation as indicated under Materials and Methods (see legend to Fig. 3).

Tissue	MTA (nmol/g wet tissue)	
	A	B
Liver	2.5 ± 0.2	2.9 ± 0.3
Testis	2.8 ± 0.3	—
Brain	1.9 ± 0.2	1.7 ± 0.2
Heart	—	1.7 ± 0.2
Kidney	2.2 ± 0.2	—
Lung	—	0.7 ± 0.08

MTA and related compounds processed using Partisil 10 SCX, while in Fig. 3b and c are compared the chromatograms of rat liver extracts obtained with (c) or without (b) a pre-run on an Affi-gel 601 column. The lack of resolution of MTA in chromatogram b indicates the necessity of Affi-gel 601 chromatography in this case.

The quantitative results of the analysis of rat tissues obtained with both the procedures are reported in Table I. The MTA concentration averaged from five separate experiments, for each tissue, agreed to within 15%, demonstrating the accuracy and reproducibility of the two analyses. The levels of the thioether in the tissues examined ranged from 0.6 to 3 nmol/g.

The sensitivity of the method, which permits the determination of MTA concentrations as low as 200 pmol/g, is sufficient for its application in biological samples.

ACKNOWLEDGEMENTS

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Note

Hydrophilic ion-pair reversed-phase high-performance liquid chromatography for the simultaneous assay of isoniazid and acetylisoniazid in serum: a microscale procedure

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(First received March 27th, 1981; revised manuscript received June 16th, 1981)

Since acetyl isoniazid (AINH) generates stoichiometrically acetyl hydrazine which is a potent hepatotoxin [1], the simultaneous determination of serum isoniazid (INH) and AINH is of value in acetylator phenotyping and drug monitoring. Gas chromatographic assays for these compounds via their silylated [2] or hydrazone [3] derivatives as well as ion-pair high-performance liquid chromatographic (HPLC) assay [4] are tedious because of the large sample (3 ml) and reagent volumes involved. In this paper, a sensitive (0.5 mg/l) micro method for the simultaneous determination of serum INH and AINH using 0.5 ml of sample and hydrophilic ion-pair reversed-phase HPLC is described. Electron-impact (EI) and chemical-ionization (CI) mass spectrometry of the peaks of interest (INH, AINH) are used in assessing the selectivity of this assay. The total assay time (extraction and chromatography) is shortened from 90 to 30 min.

EXPERIMENTAL

Instrumentation

HPLC was performed on a model ALC/GPL 244 liquid chromatograph (Waters Assoc., Paris, France) equipped with a Model 440 UV (254 nm) detector and a column 30 cm × 3.9 mm packed with μBondapak C_{18} (10 μm particle size) (Waters Assoc.). Five per cent of methanol in 95% of 0.1 *M* KH_2PO_4 (pH 6.9) were used (degassed) as the optimal isocratic mobile phase with a flow-rate of 2 ml/min.

The mass spectrometry was performed on a Jeol 300 D EI—CI magnetic mass spectrometer using a Jama 2000 computer system (Jeol Europe, Rueil Malmaison, France) by direct probe insertion. In the ion source of the mass

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spectrometer, AINH and INH volatilized at about 85°C and 110°C, respectively. In the CI mode, methane was used as reactant gas at a pressure of 1 bar.

Chemicals

Pure INH was obtained from Roche (Neuilly/Seine, France). AINH was from Sigma (St. Louis, MO, U.S.A.) and nicotinic amide (NA) from Lemat et Boinot (Paris, France). Other chemicals were of analytical reagent grade from E. Merck (Darmstadt, G.F.R.).

Assay procedure

To 500 μl of blank human serum spiked with 0.25, 0.5, 1.0, 2.5, 3.5 μg of INH or AINH, both in the form of freshly prepared aqueous solution (10 mg/l), or to 500 μl of serum sample, in a 10-ml screw-capped glass tube were added 2.5 μg of NA (aqueous solution of 100 mg/l) as internal standard (IS), 150 μl of 0.1 *M* sodium hydroxide and 0.5 g of solid ammonium sulfate in three small portions, followed by gentle shaking. The mixture was shaken for 5 min with 3 ml of chloroform and centrifuged at 520 *g* for 5 min. To 2—2.5 ml of organic phase in a 4-ml tapered tube were added 200 μl of 0.05 *M* sulfuric acid. The mixture was shaken for 5 min then centrifuged; 30 μl of clear supernatant were injected into the chromatograph.

In the selectivity study, eluted INH and AINH peaks from the HPLC of samples from patients given INH + ethambutol + rifampin were collected, extracted as previously described, and 100 μl of the chloroform phase were transferred into the capillary quartz tube then evaporated for direct insertion into the mass spectrometer.

RESULTS AND DISCUSSION

Extraction

Due to their polar nature, INH, AINH and NA were extracted on the basis of ion-suppression according to the satisfactory method of Saxena et al. [4] using sodium hydroxide and solid ammonium sulfate, which also precipitate plasma proteins, except with the use of chloroform alone as organic solvent. Once extracted on the day of sampling, the extract can be stored at 4°C before chromatography without significant loss for two weeks. Under these conditions recoveries and coefficients of variation (CV) at concentration levels of 0.5—7.0 mg/l INH and AINH were 101 ± 4% and 98 ± 5%, respectively. Otherwise, only 75 ± 18% recovery of INH was obtained if the serum samples were kept for longer than 24 h, even at 4°C, before the extraction step, while the recovery of AINH remained unchanged. The reason for this erratic loss is not clear. Indeed, if INH can be generated from its labile hydrazone as previously quoted [5], the serum INH after storage should be rather higher.

Chromatograms and the results of a patient sample

In the chromatographic step, 0.1 *M* phosphate was used as a hydrophilic counter-ion in an optimalized pH 6.9 condition. All peaks were well resolved within 7 min (Fig. 1). Retention times (t_R) were 3.3 ± 0.2, 3.9 ± 0.3, 5.8 ± 0.3

min for AINH (I), INH (II) and NA (III), respectively. HPLC elution profiles of AINH and INH in the serum of a patient 2 h (Fig. 1a) and 4.5 h (Fig. 1b) after a 5 mg/kg oral dose of INH are shown for comparison. The large "lump" after the elution of III in fig. 1b was regularly present and is due to serum components; the t_R of this, as in the chromatogram of blank serum (not shown), was 19.8 ± 0.3 min. The data for this example are summarized in Table I.

The elimination half-life of INH from serum was 2.8 h. Depending upon both phenotype and physiopathological factors, these data vary largely from one patient to another and suggest a measure of individual kinetic parameters of INH and their use in the calculation of dose regimen.

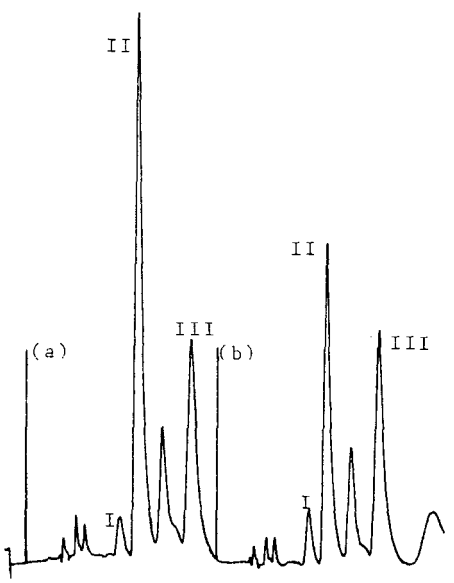

Fig. 1. Elution profiles of AINH (I) and INH (II) from patient serum at 2 h (a) and 4.5 h (b) after an oral dose of INH, using NA (III) as internal standard.

TABLE I

SERUM AINH AND INH CONCENTRATIONS IN A PATIENT AFTER AN ORAL DOSE OF INH

See text for experimental details.

Sampling time after dosing (h)	INH (mg/l)	AINH (mg/l)	AINH/INH ratio
2	7.4	1.4	0.189
4.5	4.0	2.0	0.50

Standard curve

The calibration curves [i.e. peak height ratios of INH/IS and AINH/IS versus appropriate amounts (see assay procedure) of drug and AINH in the therapeutic concentration range (1—3 mg/l) of INH in serum] were linear: for INH = $0.693x - 0.04$ ($r = 1$), and for AINH = $0.226x + 0.04$ ($r = 0.977$). Minimal detectable amounts calculated as greater than two standard deviations from zero dose of INH and AINH were 0.3 ± 0.15 and 0.25 ± 0.1 mg/l. The coefficient of variation for within-run and between-run of INH and AINH were in the same range, 6 ± 0.5% and 8 ± 0.7%, respectively.

Mass spectra

Typical mass spectra of peaks I and II obtained from one patient exceptionally treated with the three first-line anti-tuberculosis drugs INH + ethambutol + rifampin (in which serum AINH must be closely monitored because of intrinsic hepatotoxicity and microsomal enzymatic induction of rifampin) are shown in Fig. 2. The EI mass spectrum of peak I (Fig. 2a)

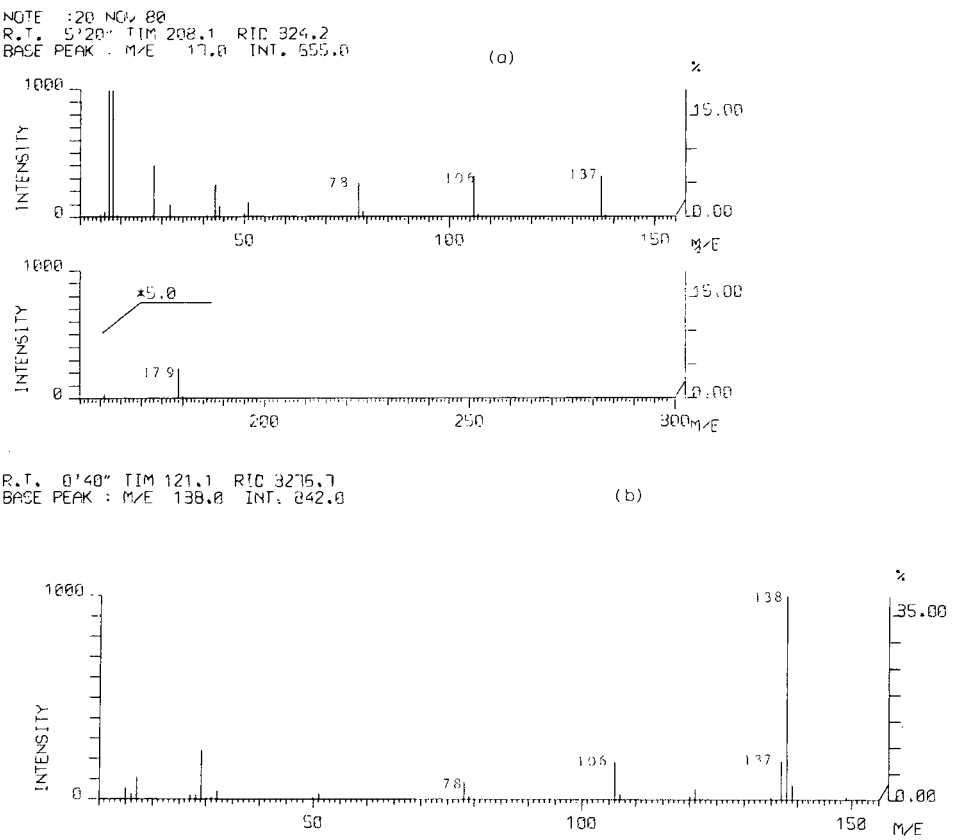

Fig. 2. Typical EI mass spectrum of peak I (a) and methane CI mass spectrum of peak II (b) eluted and extracted from the serum of a patient given INH + ethambutol + rifampin. The spectra are consistent with those of pure AINH and INH, respectively.

containing a molecular ion (M^+) at *m/e* 179 and ions 137 (INH), 106, 78, 51 and 43, was consistent with pure AINH. The identification was easier with CI because of minimal sample ion fragmentations [6] as shown for peak II (Fig. 2b). This methane CI mass spectrum presented a quasi-molecular ion (M+1) at *m/e* 138 (base peak) and 137, 106 and 78 as prominent ions; this was consistent with pure INH. The typical ion M + 29 due to methane CI was small but visible. This indicated lack of intereference in the assay by ethambutol, rifampin, their metabolites, and serum components.

This convenient and rapid assay has been tested for eight months in our laboratory and is of great interest in the routine profiling of serum INH and AINH at therapeutic levels of INH for patients with a high risk of developing hepatitis [7]. Most interesting is the fact that this method might easily be suitable if needed to quantitate INH and AINH in urine.

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Note

Quantitation of D-tubocurarine in human plasma using high-performance liquid chromatography

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D-Tubocurarine is a natural non-depolarizing neuromuscular blocking agent largely used in anesthetic practice. Pharmacological data indicate a neuromuscular blockage with very low serum concentrations [1, 2].

Several methods have been used to assay D-tubocurarine in biological fluids in order to determine its pharmacokinetic and pharmacodynamic properties in man. D-Tubocurarine plasma concentrations have been determined by radioimmunoassay [3], fluorimetry after formation of a complex with a dye [4, 5], and more recently by high-performance liquid chromatography (HPLC) [6, 7]; however, the latter techniques have never been applied to clinical situations.

This report describes a new method for the selective quantitation of nanogram amounts of D-tubocurarine in human plasma using ion-pair extraction and reversed-phase liquid chromatography. Preliminary results concerning the pharmacokinetics of D-tubocurarine in man using this assay are also given.

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MATERIALS AND METHODS

Chemicals and glassware

D-Tubocurarine was purchased from Bruneau, Boulogne sur Seine, France. ORG NC 45 is a gift from Organon, Oss, The Netherlands. Alloferin was purchased from Roche SA, Neuilly sur Seine, France. Methanol and 1,2-dichloroethane were of analytical grade quality and purchased from Merck (Darmstadt, G.F.R.). Phosphoric acid, hydrochloric acid and triethylamine were of analytical grade quality and purchased from Merck. Pentanesulfonic acid was purchased from Waters Assoc. as Pic B5 (Waters, Paris, France).

The glycine stock solution contained 750 mg of glycine and 585 mg of sodium chloride per 100 ml of water.

The potassium iodide—glycine buffer solution [4] was prepared freshly each day of analysis by mixing 4 ml of 0.1 *N* sodium hydroxide, 6 ml of glycine stock solution and 12.8 g of potassium iodide.

All determinations were done in polypropylene tubes.

Sample processing

To 1 ml of plasma or standard in a polypropylene tube was added 0.1 ml of glycine buffer. The contents were mixed for 2 min on a Vortex mixer, then 1 ml of ethylene dichloride was added and mixed for 30 min on a mechanical rotary-type shaker. This mixture was centrifuged at 1000 *g* for 10 min (4°C); then exactly 750 μl of the ethylene dichloride layer were added to a second conical polypropylene tube (Beckman) with 200 μl of 0.01 *N* hydrochloric acid. The contents of this second test tube were mixed by swirling for 30 sec and centrifuged for 2 min. Exactly 120 μl of the aqueous phase were pipetted for injection into the chromatograph.

HPLC apparatus and phase system

The HPLC set-up consisted of a Waters Model 6000A pump, a Waters U6K universal injector and an UV detector Model 440 (Waters). All chromatograms were obtained using a linear potentiometric recorder (one channel) (Kipp & Zonen).

Throughout the investigation a radial compression system with a μC_{18} column (particle size 10 μm, 15 cm × 8 mm I.D.; Rad-Pack A, Waters) used in an isocratic mode. The UV detector was set at 280 nm with the appropriate filter on 0.005 absorbance unit. The mobile phase consisted of 40% of methanol in an aqueous mixture of triethylamine (10 g/l), Pic B5 (1 ml), phosphoric acid (2 ml) and distilled water to 1 liter. The final pH of the aqeuous mixture was 3.4. The eluent was filtered through a Millipore filter (0.2 μm) prior to use. The eluent flow-rate was 2 ml/min, and the recorder was 1 cm/min on 10 mV.

RESULTS

Using the described conditions D-tubocurarine has a retention time of approximately 4.4 min (k' = 1.75). Fig. 1 shows chromatograms of a plasma sample with D-tubocurarine and a blank plasma.

The concentration of D-tubocurarine standard and the peak height were linearly related over the range 25—500 ng/ml. The standards were obtained by dissolution of D-tubocurarine in a plasma blank.

The limit of sensitivity for D-tubocurarine was 0.025 μg/ml. The reproducibility was in a range of 6—8% within the standard curve (0.025—0.5 μg/ml). The recovery, tested by adding D-tubocurarine to known samples, was never below 95%. The linearity, tested by dilution of plasma samples, was above 97%.

Other non-depolarizing muscle relaxants were tested in this chromatographic system and showed very different retention times (NC 45, $k' = 5$; alloferine, $k' = 5.68$).

The evolution of the plasma concentration of D-tubocurarine was followed after a single intravenous injection in three patients undergoing abdominal surgery (Fig. 2).

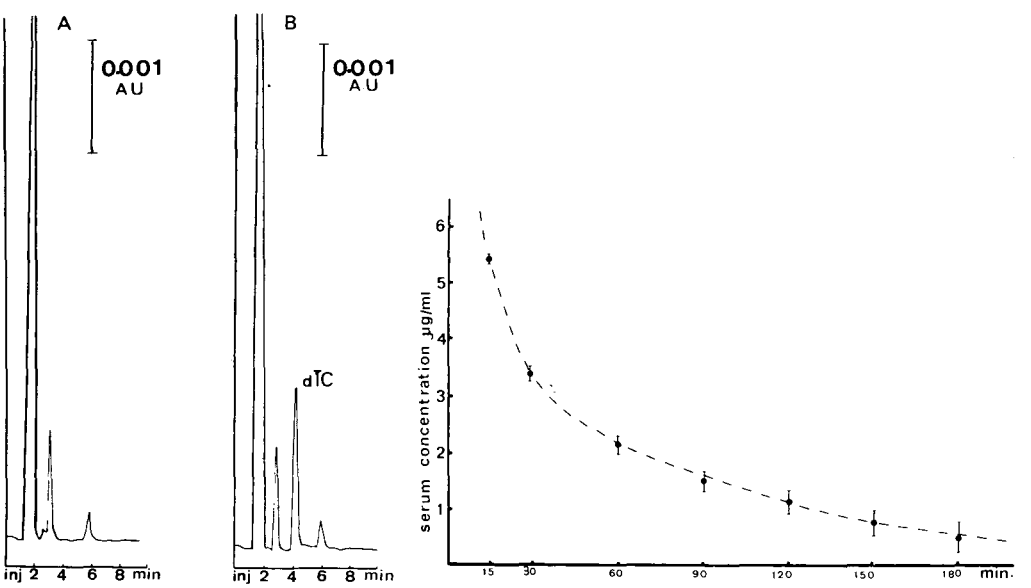

Fig. 1. Chromatograms of blank human plasma (A) and a human plasma sample from a clinical study (B). D-Tubocurarine (dTC) concentration is 95 ng/ml.

Fig. 2. Profile of D-tubocurarine plasma concentration vs. time after an intravenous bolus dose of 0.3 mg/kg. Each point is the mean of the plasma concentration of three patients. The plasma must be diluted with distilled water (1:10) for such elevated values.

DISCUSSION

Firstly, the extraction of D-tubocurarine from plasma is very important in this procedure. This two-step extraction was established by Cohen [4]. The first ethylene dichloride extraction gives a constant coefficient of 85% and the second acidic extraction gives a constant coefficient of 89%. The ratio of

the solvents' volume in the two cases determined to a large extent the limit of sensitivity of the assay.

Secondly, the chromatographic eluent was chosen to shorten the duration of the procedure on the radial compression system. The chromatogram (Fig. 1) shows that one assay lasted 5 min. The reliability of the assay was not increased when we added alloferine as internal standard before the first extraction.

CONCLUSION

This procedure is a selective and sensitive chemical assay which permits the quantitation of D-tubocurarine in the plasma of patients undergoing therapy with this drug.

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Note

Sensitive high-performance liquid chromatographic method for the determination of labetalol in human plasma using fluorimetric detection

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Since 1977, labetalol {2-hydroxy-5-[1-hydroxy-2-(1-methyl-3-phenyl propylamino)ethyl] benzamide hydrochloride} has been in use as an antihypertensive agent. Its pharmacological effect is based on the fact that it antagonizes both α- and β-adrenoceptors [1, 2]. The effective plasma levels are reported to be 100—300 ng/ml, but inter-individual variations are considerable in this respect [3].

Several methods are at present available for monitoring labetalol levels [4—6]. Detection limits ranging from 40 to 80 ng per sample were reported, which means that at least 1—2 ml of plasma are required for a single analysis. Some fluorimetric methods do not include a chromatographic separation and are therefore expected to be less reliable. Detailed pharmacokinetic studies using such methods will be troublesome.

In the recent literature there is growing support for the usefulness of labetalol in the treatment of pregnancy-induced hypertension [7—9]. No detrimental effects to the mother or foetus have been observed. Additionally, there are indications that labetalol enhances early foetal lung maturation. For pharmacokinetic studies during the perinatal period a sensitive and reliable method for the determination of labetalol could be useful.

The present method, which involves separation by ion-pair reversed-phase high-performance liquid chromatography (HPLC) and fluorimetric detection of labetalol and the internal standard chloroquine after on-line post-column buffering of the eluent, meets these sensitivity and selectivity requirements.

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EXPERIMENTAL

Chemicals

All aqueous solutions were prepared with double-distilled water. Labetalol hydrochloride was kindly supplied by Glaxo (Hoofddorp, The Netherlands); chloroquine sulfate was obtained from Nogepha (Alkmaar, The Netherlands). All other chemicals were obtained from E. Merck (Darmstadt, G.F.R.) and were of analytical reagent grade, except for the trimethylammonium chloride, (TMA) which was of synthetical reagent grade. All chemicals were used as received.

Standard aqueous solutions were prepared of labetalol (10.0 mg/l, of the base) and of chloroquine (2.0 mg/l, of the base). These solutions were slightly acidified with formic acid (± 0.01%) for better preservation. The carbonate buffer (pH 9) contained 90 g of $NaHCO_3$ and 32 g of K_2CO_3 per liter. The borate buffer (pH 10.3), which was used for buffering the eluent post column, contained 110 g of Borax and 17 g of sodium hydroxide per liter. A mixture of ethylene dichloride—diethyl ether—isopropanol (45:45:10) was used as the extraction solvent.

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 100-μl loop. The borate buffer was delivered by a 60-ml syringe infusion pump (Dascon B.V., Uden, The Netherlands) and mixed with the eluent in a T-union (15 MTA; Inacom, Veenendaal, The Netherlands) with the column effluent inflow opposite to that of the infusion pump's effluent. A Schoeffel FS 970 LC fluorometer (Schoeffel, Westwood, NJ, U.S.A.) was used as the detector with an excitation wavelength of 335 nm and emission wavelength of 370 nm. An Aminco-Bowman spectrophotofluorometer was used for scanning the fluorescence spectra.

Chromatographic system

Separations were performed on LiChrosorb 10 RP-18 or Nucleosil 10 C-18 obtained as prepacked columns (250 × 4.6 mm) from Chrompack (Middelburg, The Netherlands). The particle size of both columns is 10 μm. The composition of the eluent was water—acetonitrile (68:32) containing 40 g of $NaClO_4$, 40 g of trimethylammonium chloride and 4 g of sodium acetate per liter. The pH of the eluent was about 4.5. The system was operated at ambient temperature with a flow-rate of 1.0 ml/min. The borate buffer was added post-column with a flow-rate of 0.23 ml/min, adjusting the pH of the eluent to about 9.3.

Sample preparation

In a glass-stoppered tube 1.0 ml of plasma sample was mixed with 25 μl (50 ng) of the internal standard solution, and 200 μl of the carbonate buffer (pH 9) were added. If less than 1.0 ml of plasma was used, this needed to be diluted

with water to that volume. Extraction was carried out with 5.0 ml of the extraction mixture by shaking for 10 min.

After centrifugation the organic layer was evaporated under nitrogen at about 40°C. The residue was then redissolved in 120 μl of eluent and 50 μl of this solution were injected into the liquid chromatograph. Calibration curves using labetalol concentrations in the range 12.5—250 ng/sample were obtained by adding 25 μl of the (appropriately diluted) standard solution to 1.0 ml of blank human plasma and utilizing the same procedure.

RESULTS

The results presented in this section were obtained on a LiChrosorb 10 RP-18 column. Chromatograms of plasma samples are shown in Fig. 1. In most cases the baseline was hardly disturbed by a front, even at high detector sensitivity. A representative plot of peak height ratio vs. labetalol concentration was described by the equation $y = 0.0084x + 0.0234$. The coefficient of correlation of this calibration curve was 0.9992. The within-day coefficient of variation, calculated from fifteen determinations of a plasma sample spiked with 25 ng of labetalol, was found to be 7.2%. In the same experiment the recovery of labetalol was 95.3 ± 10.6%. The day-to-day coefficient of variation was calculated from duplicate measurements for the concentration ranges 8—25 ng/ml and 25—250 ng/ml and was found to be 8.08% ($n = 7$) and 2.22% ($n = 8$), respectively. The recovery of the internal standard was 96.0 ± 7.7% ($n = 5$). Although we did not measure concentrations below 8.0 ng/ml, the signal-to-noise ratio indicates a detection limit down to 1 ng/ml.

Interference with the method was excluded for the following antihypertensive and diuretic drugs: clonidine, diazoxide, hydralazine, propranolol, chlorthalidone, chlorthiazide, frusemide and triamterene. Interference with the method by prazosin can be circumvented by choosing an alternative eluent

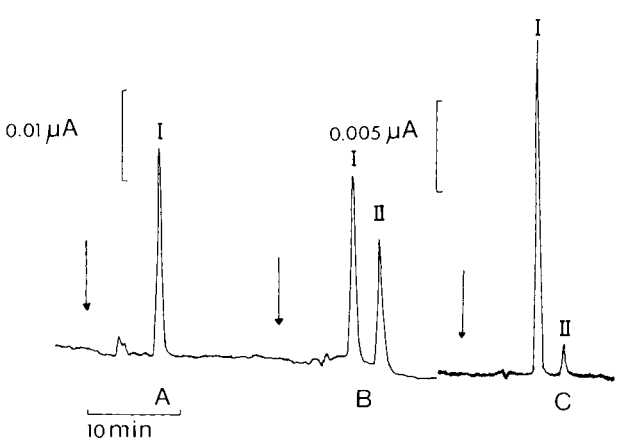

Fig. 1. Chromatograms of plasma samples from the same patient as in Fig. 2. I = internal standard, II = labetalol. (A) Blank plasma with internal standard; (B) 33 ng/ml labetalol, 0.5 ml of plasma used; (C) 8 ng/ml labetalol, 1.0 ml of plasma used.

composition as follows: 27.5% of acetonitrile instead of 32% and 1.5% $NaClO_4$ instead of 4%. Under the latter conditions, however, triamterene does interfere with the method.

In Fig. 2 a plasma concentration—time curve is shown for a patient who received 100 mg of labetalol orally. Plasma samples of 0.5 ml were used for analysis, except for the last four observations when 1.0-ml samples were used.

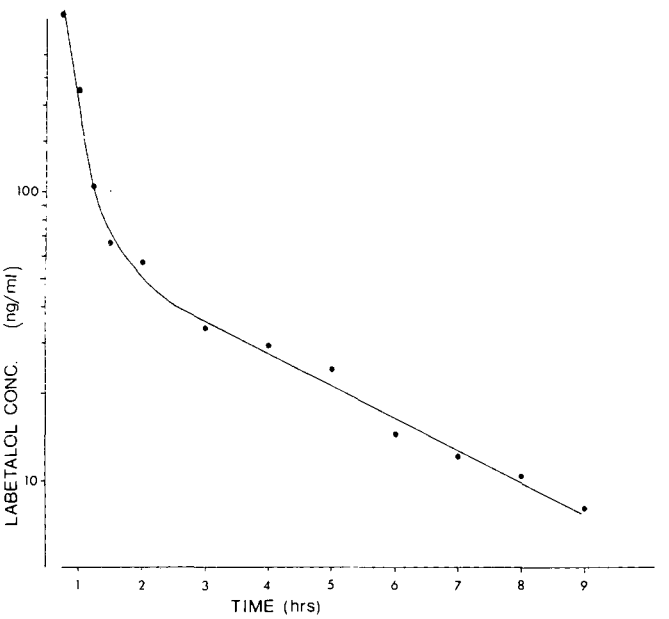

Fig. 2. Semilogarithmic plot of plasma labetalol concentration vs. time after oral administration of 100 mg of labetalol to a patient. The line represents that of the best visual fit.

DISCUSSION

Chloroquine was chosen as the internal standard because its fluorescence properties are very similar to those of labetalol, although it belongs to quite another therapeutic class of drugs. The precision of the method was not significantly improved by the use of the internal standard; however, its use was maintained out of considerations of practicality.

The extraction of labetalol and chloroquine has been investigated by other workers [5, 6, 10]. Both compounds require a rather polar extraction solvent. The composition of our solvent was adjusted in order to obtain about the same recovery for both compounds, and to get a floating organic layer. Raising the extraction pH beyond a value of 9.0 caused a decrease of the recovery of labetalol due to its phenolic hydroxyl group, whereas the recovery of chloroquine was not further improved.

When chloroquine was eluted on a LiChrosorb 10 RP-18 column with buffered acetonitrile—water or methanol—water mixtures it gave extremely

broad bands. We observed an increase in retention time and tailing with decreasing pH, indicating that particularly the protonated form of chloroquine interacts strongly with the unshielded residual silanol groups of the LiChrosorb RP-18 [11]. As we associated this effect with the tertiary amino group in the chloroquine molecule [12], we examined the influence of trimethylammonium chloride (TMA) as a possible adsorption competitor. Addition of this compound to the eluent indeed caused a dramatic improvement in peak shape as well as a decrease in the elution volume and plate height. With increasing TMA concentration the asymmetry factor [13] and the plate height, along with the elution volume, did approach a constant value, giving support to the idea of adsorption competition (Fig. 3). As it is a well-known fact that RP columns of the same type but from different producers can differ strongly in this respect [11], we investigated whether the same effect could be obtained with a Nucleosil 10 C-18 column. The results of this experiment are demonstrated in Fig. 4. When no TMA was added to the eluent, only a rather broad labetalol band could be discerned in the case of the LiChrosorb whereas a good separation is obtained on Nucleosil. However, when TMA was added to the eluent, the chromatograms on LiChrosorb and Nucleosil became very comparable. The efficiency of the Nucleosil column seems somewhat better. In this particular case the Nucleosil column in combination with an eluent without TMA seems preferable. On the other hand, these observations suggest that if our method is used with other octadecyl RP columns an eluent with TMA should be used. The unpleasant odour of trimethylamine can be neutralized by eluent efflux into dilute acetic acid.

Due to ion-pair formation [14] the capacity factor of both labetalol and chloroquine increases when sodium perchlorate is added to the eluent. As this effect was stronger in the case of chloroquine, it was utilized to adjust the resolution between the chloroquine and labetalol bands. Changes in pH between 2 and 5 appeared not to influence the separation. With sodium acetate

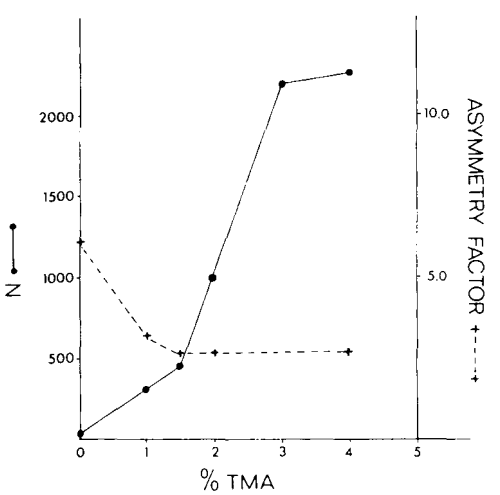

Fig. 3. A plot of plate number (N) and asymmetry factor vs. TMA concentration in the eluent (calculated for the chloroquine peak on the LiChrosorb 10 RP-18 column).

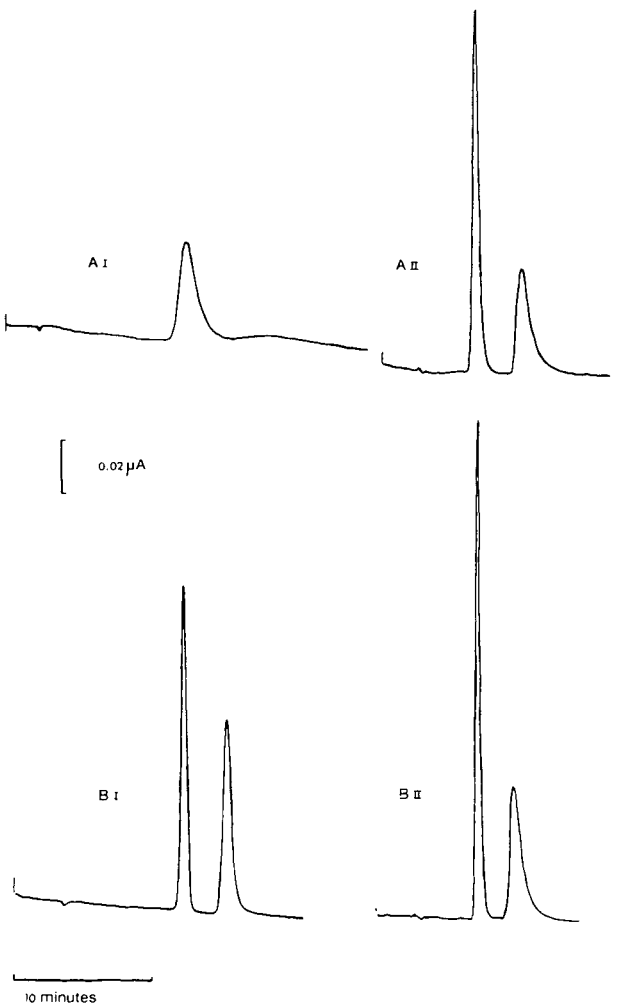

Fig. 4. Chromatograms of a standard sample containing 125 ng of labetalol and 100 ng of chloroquine on a LiChrosorb 10 RP-18 column (A) and a Nucleosil 10 C-18 column (B). In the chromatograms on the left (AI, BI) TMA was omitted from the eluent (pH corrected with HCl). In the chromatograms on the right (AII, BII) the eluent contained 4% TMA.

the pH of the eluent was adjusted to 4.5. The analytical column showed no important loss of performance during six months of intensive use.

Optimum conditions for the fluorescence detection of labetalol were investigated by Martin et al. [6]. Under our conditions there was an excitation maximum at 340 nm and an emission maximum at 415 nm.

We examined the influence of pH and composition of the post-column buffer mixture on the fluorescence yield of both labetalol and chloroquine. Our observations did not confirm the advantage of ammonium hydroxide over buffer solutions [6]. Optimum pH values were less critical for labetalol

($9 < pH < 10$) than for chloroquine ($9.2 < pH < 9.3$). The fluorescence yield of labetalol was almost identical with the different buffer mixtures we tested, but for chloroquine it was optimum with the borate buffer in comparison to a carbonate or a glycine buffer.

The fluorimetric detection under the above conditions appears to improve the selectivity of the method [15, 16], which is particularly important for antihypertensive agents which are frequently given in combination.

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JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETING

1982 LCEC SYMPOSIUM: BIOMEDICAL APPLICATIONS OF LCEC AND VOLTAMMETRY

The third International LCEC Symposium will be held at the Indianapolis Hyatt Regency Hotel in Indianapolis, IN, U.S.A., on May 16–18, 1982. Titles for papers and poster sessions at the LCEC Symposium are now being accepted for consideration by the Symposium committee. Qualified speakers will have applied the techniques of either liquid chromatography with electrochemical detection (LCEC) or voltammetry to research problems of a biomedical nature.

As in past years, the purpose of this meeting will be to provide a forum for the exchange of technical information on sample preparation techniques, chromatographic conditions, data processing, and instrumentation. A short course pertaining to the Biomedical Applications of LCEC will be presented prior to the Symposium. The LCEC Symposium is held each May and alternates between topics of Biomedical or Environmental/Industrial interest.

Inquiries, abstracts and requests for information should be addressed to: The LCEC Symposium, P.O. Box 2206, West Lafayette, IN 47906, U.S.A. Tel.: (317) 463-2505.

CALENDAR OF FORTHCOMING EVENTS

Jan. 19–20, 1982 Amsterdam, The Netherlands	**Symposium on "Detection in High-Performance Liquid Chromatography"** Contact: Mrs. Peschier, Hewlett-Packard Nederland B.V., Analytical Department, van Heuven Goedhartlaan 121, 1181 KK Amstelveen, The Netherlands (Tel.: 020-47 20 21). (Further details published in Vol. 212, No. 2)
March 8–12, 1982 Atlantic City, NJ, U.S.A.	**1982 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Linda Briggs, Program Secretary, Pittsburgh Conference, Department J-057, 437 Donald Road, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 212, No. 2)
March 28–April 2, 1982 Las Vegas, NV, U.S.A.	**183rd American Chemical Society National Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.

April 5–8, 1982 Las Vegas, NV, U.S.A.	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623. (Further details published in Vol. 212, No. 3)
April 14–16, 1982 Amsterdam, The Netherlands	**12th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Prof. Dr. Roland W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 206, No. 1)
April 15–17, 1982 Tokyo, Japan	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.
April 19–22, 1982 Barcelona, Spain	**International Congress on Automation in Clinical Laboratory** Contact: Dr. R. Galimany, Sección de Automatización, Laboratorio de Analisis Clinicos, C.S. "Principes de Espana", Hospitalet de Llobregat, Barcelona, Spain.
April 20th, 1982 Loughborough, Great Britain	**Electrochemical Analysis in the Pharmaceutical Industry** Contact: The Royal Society of Chemistry, Analytical Division (Electroanalytical Group), Burlington House, London W1V 0BN, Great Britain.
April 20th, 1982 Belfast, Northern Ireland, U.K.	**Derivative Spectroscopy and its Applications in Bioanalytical and Environmental Chemistry** Contact: The Royal Society of Chemistry, Analytical Division (Northern Ireland Region), Burlington House, London W1V 0BN, Great Britain.
April 21–23, 1982 Neuherberg near Munich, G.F.R.	**Second International Workshop on Trace Element Analytical Chemistry in Medicine and Biology** Contact: Dr. P. Schramel, Gesellschaft fuer Strahlen- und Umweltforschung, Institut fuer Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstaedter Landstrasse 1, D-8042 Neuherberg, G.F.R.
April 27–30, 1982 Munich, G.F.R.	**Biochemische Analytik Conference** Contact: Prof. I. Trautschold, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, 3000 Hannover 61, G.F.R. (Further details published in Vol. 224, No. 3)
May 11–14, 1982 Ghent, Belgium	**4th International Symposium on Quantitative Mass Spectrometry in Life Sciences** Contact: Prof. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, De Pintelaan 135, B-9000 Ghent, Belgium.
May 16–18, 1982 Indiannapolis, IN, U.S.A.	**1982 LCEC Symposium: Biomedical Applications of LCEC and Voltammetry** Contact: LCEC Symposium, P.O. Box 2206, West Lafayette, IN 47906, U.S.A. Tel.: (317) 463-2505; Telex: 276 141.
May 20th, 1982 Bath, Great Britain	**Development of Chromatographic Techniques in Cancer Therapy** Contact: The Royal Society of Chemistry, Analytical Division (Western Region), Burlington House, London W1V 0BN, Great Britain.

June 6–11, 1982 Kansas City, MO, U.S.A.	**International Symposium on the Synthesis and Application of Isotopically Labeled Compounds** Contact: Dr. Alexander Susán, Scientific Secretary of the Symposium, c/o Midwest Research Institute, 425 Volker Boulevard, Kansas City, MO 64110, U.S.A. Tel: (816) 753-7600, extension 268. (Further details published in Vol. 225, No. 1.)
June 7–11, 1982 Philadelphia, PA, U.S.A.	**VI International Symposium on Column Liquid Chromatography** Contact: R.A. Barford, ERRC – SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A. (Further details published i Vol. 211, No. 3).
June 17 + 18, 1982 St. Andrews, Scotland	**Advances in Immunoassay: Techniques and Applications** Contact: The Royal Society of Chemistry, Analytical Division (Scottish Region), Burlington House, London W1V 0BN, Great Britain.
June 18–21, 1982 Lund, Sweden	**Flow Analysis II** Contact: Flow Analysis II, c/o The Swedish Chemical Society, Upplandsgatan 6A, 1 tr., S-111 23 Stockholm, Sweden. (Further details published in Vol. 216.)
June 20–23, 1982 Bordighera (near San Remo), Italy	**International Symposium on Chromatography and Mass Spectrometry in Biomedical Sciences** Contact: Dr. Alberto Frigerio, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62 – 20517 Milan, Italy. Tel.: 35.54.546; Telex: 331268 NEGRI I. (Further details published in Vol. 225, No. 1.)
June 20–24, 1982 Toronto, Canada	**North American Medicinal Chemistry Symposium** Contact: Symposium Secretariat, North American Medicinal Chemistry Symposium, c/o Ayerst Laboratories, P.O. Box 6115, Montreal, Quebec H3C 3J1, Canada.
July 11–16, 1982 Washington, DC, U.S.A.	**6th International Conference on Computers in Chemical Research and Education (ICCCRE)** Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.
Aug. 15–21, 1982 Perth, Australia	**The 12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia
Aug. 22–28, 1982 Vancouver, Canada	**The XIth International Carbohydrate Symposium** Contact: Mr. K. Charbonneau, Executive Secretary, XIth International Carbohydrate Symposium, c/o National Research Council of Canada, Ottawa, Ontaria, Canada K1A 0R6. Tel.: (613) 993-9009; Telex: 053-3145.
Aug. 30–Sept. 3, 1982 Vienna, Austria	**9th International Mass Spectrometry Conference** Contact: Interconvention, P.O. Box 105, A-1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.

Sept. 5–9, 1982 Liège, Belgium	**Eighth European Workshop on Drug Metabolism** Contact: Professor Jacques E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, Université de Liège, Bâtiment B 23, B-4000 Sart Tilman par Liège 1, Belgium. Tel.: (32-41)-56.24.80/81. (Further details published in Vol. 225, No. 2.)
Sept. 6–9, 1982 Bath, Great Britain	**4th European Symposium on Chemical Structure – Biological Activity: Quantitative Approaches** Contact: Dr. J.C. Dearden, School of Pharmacy, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, Great Britain.
Sept. 7–11, 1982 Hradec Králové, Czechoslovakia	**8th International Symposium on Biomedical Applications of Chromatography** Contact: Dr. K. Macek, Institute of Physiology, Czechoslovakian Academy of Sciences, Vídeňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia. (Further details published in Vol. 225, No. 2.)
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)
May 30–June 3, 1983 Melbourne, Australia	**International Conference on Chromatographic Detectors** Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria 3052, Australia.
July 17–23, 1983 Edinburgh, Scotland, Great Britain	**SAC '83, International Conference and Exhibition on Analytical Chemistry** Contact: The Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel.: 01-734-9971. (Further details published in Vol. 214, No. 2.)
Aug. 28–Sep. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459

PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	212/3 213/1 213/2	213/3 214/1 214/2	214/3 215 216	217 218 219/1	219/2 219/3
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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

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