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STATISTICAL TREATMENT OF EXPERIMENTAL DATA

By J.R. GREEN, *Lecturer in Computational and Statistical Science, University of Liverpool, U.K. and* D. MARGERISON, *Senior Lecturer in Inorganic, Physical and Industrial Chemistry, University of Liverpool, U.K.*

PHYSICAL SCIENCES DATA 2

This book first appeared in 1977. In 1978 a revised reprint was published and in response to demand, further reprints appeared in 1979 and 1980. Intended for researchers wishing to analyse experimental data, this work will also be useful to students of statistics. Statistical methods and concepts are explained and the ideas and reasoning behind statistical methodology clarified. Noteworthy features of the text are numerical worked examples to illustrate formal results, and the treatment of many practical topics which are often omitted from standard texts, for example testing for outliers, stabilization of variances and polynomial regression.

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CHROMBIO. 935

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC PROFILE OF ORGANIC ACIDS IN URINE AND SERUM OF DIABETIC KETOTIC PATIENTS

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(First received January 27th, 1981; revised manuscript received April 2nd, 1981)

SUMMARY

The organic acids in the urine and serum of diabetic patients with ketoacidosis and disturbance of consciousness were studied using acidification, extraction, evaporation, methoxime formation and trimethylsilylation, gas chromatographic separation and mass spectrometric identification procedures. The organic acid profile of 1 ml of serum ultrafiltrate was obtained with good separation using a gas chromatograph equipped with a glass capillary column and a splitless injector. 5-Hydroxyhexanoic acid and 3-hydroxyvaleric acid were identified for the first time in the urine of diabetic patients with ketoacidosis. Urinary excretion and serum concentrations of 2,3-dideoxypentonic acid were increased in diabetic patients.

INTRODUCTION

The well known abnormal metabolites occurring in diabetic ketoacidosis are 3-hydroxybutyric acid, acetoacetic acid and acetone. Several studies on the organic acid profile of diabetic ketotic urine by gas chromatography—mass spectrometry (GC—MS) have been reported. Pettersen and co-workers [1, 2]

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reported that the urinary excretion of adipic acid and suberic acid in diabetic patients with ketosis was increased, and that this phenomenon was due to the increased liberation of free fatty acids from peripheral fat deposits and also to ω-oxidation of the fatty acids followed by β-oxidation. Landaas has shown that the urinary excretion of 3-hydroxyisovaleric acid [3] and of 3-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid [4] is increased during ketoacidosis. Landaas and co-workers reported that the urinary excretion of 2-hydroxybutyric acid [5, 6] and 2-hydroxyisovaleric acid [7] was increased in patients with lactic acidosis and ketoacidosis. Liebich and co-workers [8, 9] studied the volatile organic metabolites in the diabetic urine using GC—MS.

We describe here the organic acid profile of diabetic patients with ketoacidosis using GC—MS, which has been employed for the investigation of organic acids and phenols in uraemic blood ultrafiltrate [10—14]. By use of a GC—MS system equipped with a glass capillary column and a splitless injector, the organic acid profile of a small volume of serum ultrafiltrate can be studied with good separation and good sensitivity. About 50 compounds were identified in the urine of diabetic patients with ketoacidosis. 5-Hydroxyhexanoic acid and 3-hydroxyvaleric acid were detected in diabetic ketotic urine for the first time.

EXPERIMENTAL

Samples

Urine samples were obtained from four diabetic patients with ketoacidosis, three diabetic patients without ketosis and five healthy adults. Serum samples were obtained from four diabetic patients with ketoacidosis, six diabetic patients without ketosis and six healthy adults. The four diabetic patients with ketoacidosis had suffered from disturbance of consciousness, coma or precoma, hyperglycaemia (400—600 mg/dl), glycosuria, ketonuria and dehydration.

The urine samples and the serum samples were kept at −20°C prior to analysis.

Sample preparation

Serum was ultrafiltered using a CF25 Amicon Filter. A 1-ml volume of serum ultrafiltrate or urine was acidified to pH 1 with 6 *N* hydrochloric acid and saturated with sodium chloride. After the addition of *p*-(*n*-amyl)benzoic acid as an internal standard (10 μg in serum ultrafiltrate or 50 μg in urine), the organic acids were extracted three times with 3 ml of ethyl acetate. The organic phase was dehydrated over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen. A 1-mg amount of methoxylamine hydrochloride in 50 μl of ethyl acetate was added to the extract and allowed to react for 30 min at 60°C. The extract was concentrated to dryness under a stream of nitrogen and trimethylsilylated with N,O-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical, Rockford, IL, U.S.A.; 20 μl for serum samples, 100 μl for urine samples).

A 3-μl volume of the sample was subjected to GC—MS.

Gas chromatograph—mass spectrometer—computer system

Mass spectral data were obtained by directly coupling a Hewlett-Packard

5710A gas chromatograph to the source of a JMS D-300 double-focusing mass spectrometer (JEOL, Tokyo, Japan). The data were stored and processed by a JMA 2000 data system (JEOL). The gas chromatograph was equipped with a 30 m × 0.25 mm I.D. OV-101 open-tubular glass capillary column and a splitless injector. The injection temperature was 250°C and the column temperature was programmed from 70 to 250°C at 3°C/min.

Low-resolution electron impact (EI) spectra were obtained under the following conditions: ionizing energy, 70 eV; ionization current, 300 μA; accelerating voltage, 3kV; and ion source temperature 210°C; scanning was over the range m/z 40—650 in 1 sec. Chemical ionization (CI) spectra were obtained at an ionizing energy of 260 eV by using methane as a reactant gas. High-resolution spectra were obtained by scanning and peak matching with a resolution of 5000.

RESULTS

Fig. 1 shows typical profiles of organic acids in the urine and serum of a diabetic patient with ketoacidosis and a healthy control. There are several high

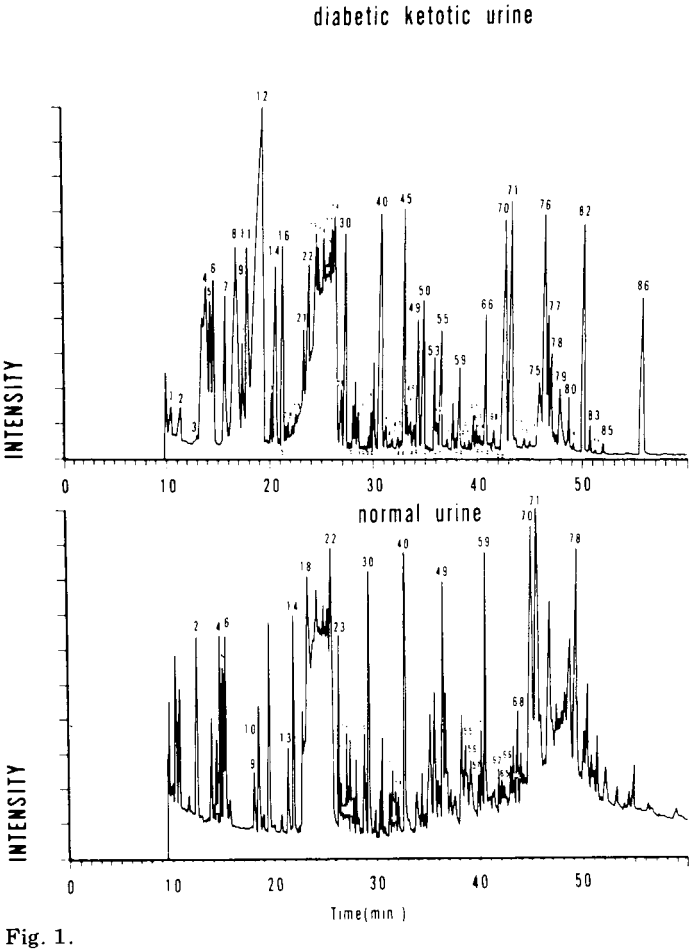

Fig. 1.

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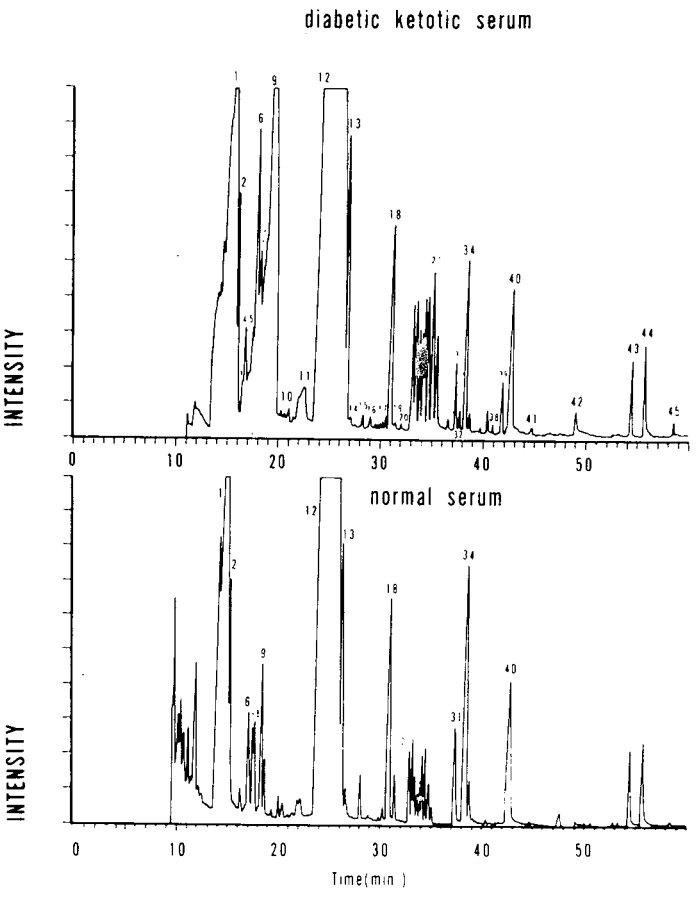

Fig. 1. Gas chromatographic profiles of organic acids in the urine and serum of a diabetic ketotic patient and a healthy subject. The extract was subjected to methoxime formation and trimethylsilylation and separated on an OV-101 open-tubular glass capillary column (30 m × 0.25 mm I.D.). The column temperature was programmed from 70° to 250°C at 3°C/min. The peaks that differed in the normal and ketoacidotic urine were identified as follows: 4, lactic acid; 7 and 8, acetoacetic acid; 9, 2-hydroxybutyric acid; 12, 3-hydroxybutyric acid and 2-hydroxyisovaleric acid (minor component); 14, 3-hydroxyisovaleric acid; 15, $C_3H_4O_3$; 17, 3-hydroxyvaleric acid; 28, fumaric acid; 29, 5-hydroxyhexanoic acid (one of two components of peak 29); 39, $C_6H_{10}O_4$; 45, adipic acid; 50, 2,3-dideoxypentonic acid. The peaks that differed in the normal and ketoacidotic serum were identified as follows: 1, lactic acid; 4, acetoacetic acid; 6, 2-hydroxybutyric acid; 9, 3-hydroxybutyric acid; 27, 2,3-dideoxypentonic acid.

peaks in the profiles of the diabetic ketotic patient compared with the healthy subject. The occurrence of acetoacetic acid and the high peak of 3-hydroxybutyric acid indicate the ketotic state of the patient. Each component of the profile was identified by EI and CI mass spectra, and high-resolution data in conjunction with its GC relative retention time. The mass spectra of the unknown compounds were compared with the published mass spectra and the mass spectra obtained in our laboratory from the derivatives.

Peak 29 in Fig. 1 (diabetic ketotic urine) was composed of two compounds.

The mass spectrum of a component of the peak is presented in Fig. 2. The molecular ion was found to be m/z 276 by recording the CI spectrum. High-resolution data revealed that the molecular formula was $C_6H_{12}O_3$. The compound was then identified as 5-hydroxyhexanoic acid by comparison with its published mass spectrum [15]. 5-Hydroxyhexanoic acid was detected in the urine from three out of four diabetic ketotic patients, but could not be detected in the patients' sera. 5-Hydroxyhexanoic acid was not detectable in the urine of the patients up to day 4 after admission, when the patients became non-ketotic with insulin therapy. The compound was not detected in the urine and the serum of the other non-ketotic diabetic patients or healthy subjects.

The EI mass spectrum of peak 17 in Fig. 1 (diabetic ketotic urine) is presented in Fig. 3. The CI mass spectrum revealed that the molecular ion of the compound was m/z 262. The molecular composition of the compound was found to be $C_5H_{10}O_3$. A fragment due to a loss of 29 a.m.u. at m/z 233 suggests the presence of an ethyl group. A fragment ion at m/z 205 due to a loss of CH_2CO group from the $(M-CH_3)^+$ ion suggests the presence of a hydroxyl group at the C_3 position, not at the C_2 position. The relatively high peak at m/z 131 also suggests 3-hydroxyvaleric acid. The compound was finally identified as 3-hydroxyvaleric acid by comparison with its published mass spectrum [16]. 3-Hydroxyvaleric acid was detected in the urine of three out of four diabetic patients with ketosis, but was not detected in the patients' sera. 3-Hydroxyvaleric acid was not detected in the urine and serum of the other non-ketotic diabetic patients or healthy subjects.

The diabetic ketotic patients showed a large urinary excretion of lactic acid, 2-hydroxybutyric acid, 2-hydroxyisovaleric acid, 3-hydroxyisovaleric acid, adipic acid and 2,3-dideoxypentonic acid compared with the normal adults. The excretion of 2,3-dideoxypentonic acid in four diabetic ketotic patients was 1.4 ± 0.75 [peak-height ratio with respect to the internal standard, 50 μg of *p*-(*n*-amyl)benzoic acid per mg of creatinine] compared with 0.23 ± 0.38 in five healthy adults. The serum concentration of 2,3-dideoxypentonic acid in the patients, i.e., 7.0 ± 13 [peak-height ratio with respect to the internal standard,

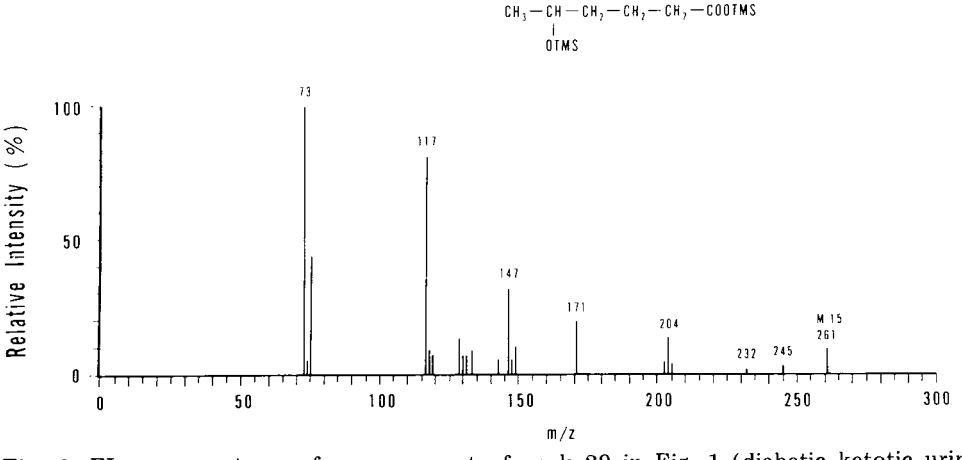

Fig. 2. EI mass spectrum of a component of peak 29 in Fig. 1 (diabetic ketotic urine). Ionizing energy, 70 eV; ionization current, 300 μA.

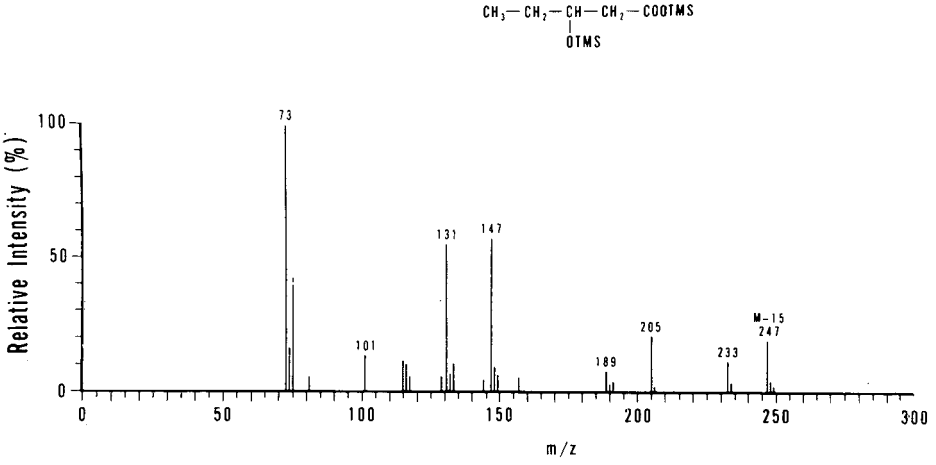

Fig. 3. EI mass spectrum of peak 17 in Fig. 1 (diabetic ketotic urine). Ionizing energy, 70 eV; ionization current, 300 μA.

10 μg of *p*-(*n*-amyl)benzoic acid] was markedly increased compared with that of the healthy subjects (0.084 ± 0.04). The diabetic ketotic patients showed only a slight increase in urinary excretion of 2-methyl-3-hydroxybutyric acid compared with the healthy controls.

DISCUSSION

5-Hydroxyhexanoic acid was first reported to be detectable in the urine of twins presenting an unusual Reye's-like syndrome characterized by severe hypoglycaemia, and the acid was considered to be a metabolite of hex-4-enoic acid [15]. Mamer et al. [17] also detected 5-hydroxyhexanoic acid in the urine of an infant with episodic hypoglycaemia together with a homologous series of hydroxy acids. In the present study 5-hydroxyhexanoic acid was also detectable in the urine of the diabetic patients with ketoacidosis.

The source of 5-hydroxyhexanoic acid may be $\omega-1$ oxidation of the accumulated medium chain length fatty acids (particularly hexanoic acid). In diabetic ketoacidosis, liberation of free fatty acids from the peripheral fat deposits is increased. Moreover, ω-oxidation of the fatty acids is greater, causing increased excretion of medium chain length dicarboxylic acids. The increased urinary excretion of adipic acid in the present diabetic ketoacidosis cases is in agreement with published findings [2].

3-Hydroxyvaleric acid has not previously been reported to be present in the urine of diabetic patients with ketoacidosis, but has been found in the serum of patients with severe propionic and methylmalonic acidaemia [18]. Stokke et al. [18] considered that 3-hydroxyvaleric acid could be formed by condensation of propionyl-CoA and acetyl-CoA. Truscott et al. [19] reported 3-hydroxyvaleric acid in a case of propionic acidaemia during ketosis, but in the non-ketotic state 3-hydroxyvaleric acid could be detected only in trace amounts. In non-ketotic propionic acidaemia, self-condensation of propionyl-CoA is the major metabolic pathway. In diabetic patients with ketosis, the accumulation

of propionyl CoA may occur because of the enhanced degradation of isoleucine and odd-chain fatty acids. It is reasonable to consider that the condensation of propionyl-CoA and acetyl-CoA to form 3-hydroxyvaleric acid also occurs during diabetic ketoacidosis.

The up to 14-fold increase in urinary excretion of 3-hydroxyisovaleric acid in our diabetic ketotic patients was in agreement with a previous report [3]. Contrary to the latter findings, however, we also observed up to a 100-fold increase in the serum concentration of 3-hydroxyisovaleric acid during ketosis. In normal serum the compound was detectable in trace amounts. The increased urinary excretion of 3-hydroxyisobutyric acid as reported by Landaas [4] was not confirmed by us, because the peak of 3-hydroxyisobutyric acid could not be separated from the large peak of 3-hydroxybutyric acid in our GC profiles. The urinary excretion of 2-methyl-3-hydroxybutyric acid was only slightly increased in our diabetic patients with ketosis.

Ketoacidosis is usually accompanied by enhanced protein catabolism. 3-Hydroxyisovaleric acid, 3-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid are known to be the intermediates of the metabolism of the branched-chain amino acids leucine, valine and isoleucine, respectively. The increase in the urinary excretion of these acids during ketoacidosis is considered to be due to the inhibition of their further metabolic breakdown by the accumulated 3-hydroxybutyric acid and acetoacetic acid [20].

Increased urinary excretion of 2-hydroxybutyric acid during ketoacidosis was also observed in our profiling analysis. The serum concentration of 2-hydroxybutyric acid in the diabetic ketotic patients, however, varied anywhere from a trace amount to a 20-fold increase in relation to the normal concentration. Pettersen et al. [5] suggested that the excretion of the compound is correlated only with lactic acidosis and not ketosis. All of the diabetic ketotic patients in the present study showed 3—50 times the control urinary excretion of lactic acid. 2-Hydroxybutyric acid is derived from 2-ketobutyric acid by action of lactate dehydrogenase. 2-Ketobutyric acid is an intermediate of the metabolism of several amino acids. An increased $NADH_2/NAD$ ratio, which is often present in lactic acidosis, is considered to be the most important factor for the accumulation of 2-hydroxybutyric acid [6].

Our diabetic patients with ketosis also showed increased urinary excretion of 2-hydroxyisovaleric acid. This finding agrees well with a previous report [7]. The urinary excretion and the serum concentration of 2,3-dideoxypentonic acid were increased during diabetic ketoacidosis. The metabolic origin of the compound, however, is not yet known.

The profiling analysis of the organic acids in 1 ml of serum ultrafiltrate became feasible by use of GC—MS with a glass capillary column and a splitless injector. As this method yielded better separation with greater sensitivity than a packed column, it may prove useful for routine clinical examinations.

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CHROMBIO. 915

IMPROVED METHOD FOR SELENIUM DETERMINATION IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY

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(First received December 31st, 1980; revised manuscript received March 31st, 1981)

SUMMARY

A gas chromatographic assay employing electron-capture detection for the determination of selenium in biological samples is reported. A calibration curve of 4-nitro-*o*-phenylenediamine derivative of selenium as a function of peak area was linear from 5—1000 pg. The limit of detection for the electron-capture detector was approximately 0.5 pg. Recoveries of selenium added to various biological materials ranged from 95—105%. This procedure reduces the number of transfers thereby reducing errors associated with losses or contamination. One advantage of the present method is that interfering compounds occurring in previously employed chromatographic methods are eliminated. This procedure can be used for routine microanalysis of selenium. Samples containing less than 2 ng selenium in 200 μl of biological fluid can be routinely analyzed using this method.

INTRODUCTION

The essentiality of selenium for higher animals, including man, has been clearly established [1—3]. A dietary deficiency or excess of this element has been associated with numerous disorders in mammals [2—6]. Furthermore, recent epidemiological data have suggested that selenium may also have a role in the etiology of cancer. Schrauzer et al. [7, 8] have shown an inverse relationship between selenium consumption and cancer mortality both nationally and internationally. These observations plus increasing experimental data suggest that selenium may have a preventative [9—11] and therapeutic [12—15] role in the etiology of cancer.

Selenium is often considered one of the most toxic elements in nature. Clearly excessive intakes of selenium lead to pronounced toxic symptoms [16—20]. Because of the biological and toxicological implications of selenium consumption, there is need for an analytic method that can be applied to various biological tissues.

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Recently, electron-capture gas chromatography (GC) has evolved as a sensitive and reliable method for selenium analysis. Various *o*-diamines derivatives have been used to form piazselenoles which after extraction are analyzed by GC. Some of the *o*-diamines that have been used include 2,3-diaminonaphthalene, and the 4-chloro, 4,5-dichloro, 4-nitro and 3,5-dibromo derivatives of 1,2-diaminobenzene [21—29]. The various reagents employed for fluorometric and electron-capture analysis of selenium were reviewed by Shimoishi [26].

A variety of instruments and conditions have been used for selenium analyses. Most methods of selenium analysis require prolonged sample digestion, extensive extraction and purification steps. This paper describes a GC method for selenium analysis applicable to numerous biological samples. This method minimizes the errors associated with sample transfers, reduces the laboratory glassware needed, and eliminates interferences which have been reported in previous procedures.

MATERIALS AND METHODS

Apparatus

A Hewlett-Packard Model 5830 gas chromatograph equipped with a pulsed current ^{63}Ni electron-capture detector was used for selenium analysis. Instrument operating conditions were as follows: temperatures, detector, 350°C; injector, 225°C; column oven, 200°C. The carrier gas was argon—methane (95:5) at a flow-rate of 30 ml/min. A 1.8 m × xx mm I.D. silanized glass column packed with 10% OV-225 on 120—140 mesh Gas-Chrom Q purchased from Applied Science Labs. (State College, PA, U.S.A.) was used for separation of the *o*-diamine derivative of selenium.

Reagents

All chemicals used were analytical reagent grade. Metallic selenium from J.D. Mackay (New York, NY, U.S.A.) and selenium dioxide were used for preparation of standard selenium solutions and the purified 5-nitropiazselenol reference material, respectively. Glass distilled toluene from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) was used for all extractions. A reference standard of 5-nitropiazselenol prepared as described by Poole et al. [28] was used in the initial calibration.

Formation of 5-nitropiazselenol

The complex reagent 4-nitro-*o*-phenylenediamine (4NPD) from Aldrich (Milwaukee, WI, U.S.A.) was converted to the chloride salt by dissolving in hot 2 *M* hydrochloric acid. The solution was then filtered and chilled in an ice bath. The resulting crystals were collected by suction filtration, rinsed with concentrated hydrochloric acid and stored frozen in a sealed glass container for subsequent use. The complexing solution was prepared by dissolving the chloride salt of 4NPD in 1 *N* hydrochloric acid (0.5%, w/v). The complexing solution was extracted with toluene prior to use. The complexing solution was found to be stable for at least two weeks when kept in a dark bottle and refrigerated.

Biological materials

Heparinized blood samples were collected from humans, cows, pigs, and sheep by venipuncture. Blood samples were obtained from experimental mice by heart puncture. Feed samples were collected from local grown sources. The selenium content of liver homogenates obtained from mice receiving supplemental selenium in their drinking water was also determined. Mice received water supplemented with 0, 1, 3, or 5 ppm selenium as sodium selenite. Liver homogenate (10%) was prepared in Krebs Ringers phosphate buffer, pH 7.4 before analysis.

Recoveries of selenium in various biological samples were also determined by the addition of known quantities of selenium to the sample before digestion. A comparison was made between the present GC method and the fluorometric method of Spallholz et al. [30]. Fluorometric measurements were made with the excitation and emission wavelengths set at 363 and 525 nm, respectively, using an Aminco-Bowman spectrophotofluorometer.

Sample preparation

Biological fluids. Approximately 5—200 μl of blood, plasma or tissue homogenate were added to 7-ml glass stoppered weighing vials equipped with ground glass stoppers. Following the addition of 1.5 ml of concentrated nitric acid containing 30 g $Mg(NO_3)_2$ per 100 ml acid (w/v) (50°C), the vials were placed in a heating block for 60 min at 105°C, followed by 30 min at 115°C. The vials were then removed from the heating block and placed on a hot plate turned to the highest setting, 450°C. All vials were removed from the hot plate when fumes of nitrogen oxide ceased. Nitric acid is used to oxidize the lower oxidation states of selenium to selenium(IV). The digestion was completed by placing the vials in a muffle furnace at 500°C for 60 min. The conversion of selenium(VI) into selenium (IV) is achieved by the addition of hydrochloric acid. After cooling, 1.5 ml of concentrated hydrochlorid acid was added to the vials. The vials were capped and placed in a heating block at 90°C for 15 min and then allowed to cool to room temperature. A 2.5-ml solution containing 1% hydroxylamine sulfate, from Alfa Division, Ventron Corp. (Danvers, MA, U.S.A.), 1% EDTA, 15% urea (w/v) in water was added to each vial, mixed and allowed to stand for 10 min. A 100-μl aliquot of the complexing solution was added and mixed. The vials were then placed in a heating block at 45°C for 30 min. After addition of 1 ml of toluene, the vials were mixed for 5 sec. A portion of the toluene layer was removed and placed in a screw cap tube for subsequent GC analysis.

Feeds. To ensure adequate sampling feed samples had to be prepared in the following manner. Samples (1.0 g or less) ground to pass a 20-mesh screen were added to beakers containing 4 g magnesium nitrate and 10 ml concentrated nitric acid. These samples were digested at a solution temperature of 100°C for 60—90 min. The digest was then heated at the highest hot plate temperature until fumes of nitrogen oxide ceased. The beakers were then placed in a muffle furnace of 500°C for 60 min. After cooling samples were removed from the furnace and 10 ml of concentrated hydrochloric acid added. The samples were then placed on a hot plate and the solution temperature maintained at 90°C for 15 min. An aliquot of the hydrochloric acid was

removed and placed in a weighing vial. The procedure was then the same as for fluids with the additions of the hydroxylamine sulfate, EDTA and urea solution.

RESULTS AND DISCUSSION

The most common procedures used for selenium digestion for GC analysis employ wet digestion with nitric acid or combinations of nitric acid with sulfuric and perchloric acids. These procedures generally require longer digestion times and require greater technical care to prevent selenium loss due to charring than the present method. Although some procedures do employ a shorter digestion period, greater time was required for sample clean-up [29]. Solvent partitioning or liquid column chromatography or combinations have often been used to remove interfering components present with such digestion procedures.

The present digestion procedure was a modification of the original procedure developed by Holkak [31] and later modified by Poole et al. [28]. This procedure was found to result in chromatograms with minimal interferences compared to assays using wet digestion with nitric, sulfuric, or perchloric acids in combination with sample clean-up.

Initial investigations of selenium digestion in the presence of magnesium

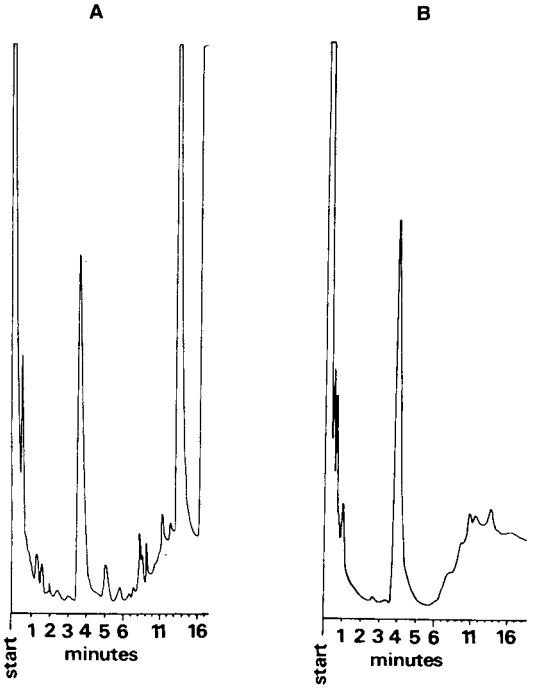

Fig. 1. Selenium analysis of a bovine blood sample containing 12 ng per 200 μl (A) without or (B) with hydroxylamine and EDTA addition before formation of the *o*-diamine complex. Note two interfering peaks occurring at 14 and 17 min during temperature program analysis in Fig. 2B.

nitrate, nitric acid and urea revealed that two interfering peaks occurred in the chromatograms. Fig. 1 shows these interfering peaks occurring at 14 and 17 min post injection when a temperature program was run. These peaks occurred after approximately 25—30 min without temperature programming. These interferences could reduce the number of repetitive samples that can be analyzed. The addition of hydroxylamine sulfate and EDTA in combination with urea was found to eliminate the two interfering peaks occurring during the analysis of selenium in standards and biological materials. The elimination of these peaks allows for repetitive analysis with minimal interferences. Typical chromatograms of a human blood and bovine plasma with the present procedure are shown in Fig. 2.

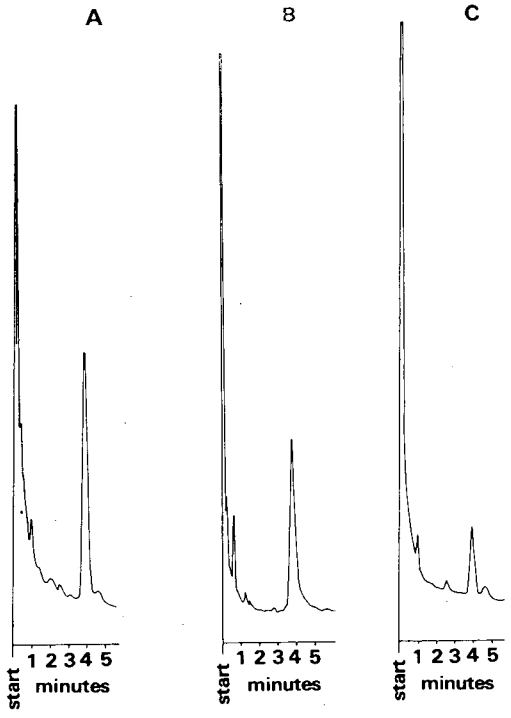

Fig. 2. GC analysis of selenium in (A) human blood (29.0 ng per 200 μl analyzed); (B) 20 ng selenium standard; and (C) bovine plasma (6.5 ng per 200 μl analyzed). Selenium peak occurs at 4 min.

The selenium content of biological materials can be readily detected by this GC procedure. A calibration curve of peak area versus selenium content in the range of 5—1000 pg per injection is linear. The correlation coefficient of selenium content against peak area was in the range of 0.999. The precision of repeated analyses of 4 μl of 30 ng/ml selenium was within 0.2%. The precision of repeated analyses of human blood selenium concentrations was similar. The present procedure can detect as little as 2 ng selenium in 200 μl of fluid. To obtain this sensitivity digestion blanks must be maintained at a level below 0.5 ppb. Hydrochloric acid was found to contribute most to

the content of selenium reagent blanks. Furthermore, large variations in the selenium content of reagent grade hydrochloric acid were observed. Acid specifically prepared for trace metal analysis was found to minimize the selenium background.

Initial calculations were based upon standard curves obtained by analysis of varying quantities of selenium carried completely through the analytical procedures. Recoveries of 5-nitropiazselenol were essentially quantitative as previously reported by Cappon and Smith [29]. Consequently, calculations were based on the standard selenium complex. Elemental analysis of 5-nitropiazselenol revealed 31.6% C, 1.3% H, 18.4% N, 14.0, and 34.7% Se. The theoretical content of C, H, N, and Se are 31.8, 1.2, 18.4, 14.7 and 33.9% respectively. The synthesized 5-nitropiazselenol had a maximum UV absorption at 349 nm and resulted in a single peak when analyzed by GC with flame ionization detection. The GC—mass spectrometric spectra gave two molecular ions corresponding to the two selenium isotopes.

The present procedure also eliminates the excessive use of glassware required in other methods. In addition to the time saved in glassware preparation, this procedure also reduces the errors associated with the losses during the transfer of the test solutions and errors resulting from contamination.

Excellent agreement of selenium content of reference material, as well as fluids and tissues from animals and humans occurred between this procedure and the fluorometric method of Spallholz et al. [30] (Table I). The determination of selenium by fluorescence with 2,3-diaminonaphthalene

TABLE I

DETERMINATION OF SELENIUM CONCENTRATIONS IN NORMAL ADULT BLOOD AND RAT LIVER BY GC AND FLUOROMETRIC ANALYSIS

	Method	
	GC*	Fluorometric**
Orchard leaf standard*** (ng/g)	84.8 ± 4.0	88.6 ± 2.7
Human blood (ng/ml)		
Mean	147.3	162.8
S.E.M.§	6.0	11.4
Range	106—175	106—233
Number of determinations	12	12
Rat liver§§ (μg/g)		
0	0.91 ± 0.1	1.11 ± 0.2
1	2.61 ± 0.4	2.28 ± 0.6
3	2.92 ± 0.8	2.51 ± 0.5

*See Materials and Methods for procedure.

**See ref. 25.

***National Bureau of Standards, Standard Reference Material 1571. Selenium content given as 80 ± 10 ng/g.

§Standard error of the mean.

§§Rats were given supplemental selenium in the drinking water at 0, 1, or 3 ppm as Na_2SeO_3 for 1 week before analysis. Means ± S.E.M. are for four mice per treatment. No significant differences in selenium content by the two analytical methods were detected.

has been well evaluated in biological materials. However, the fluorometric technique suffers from interference of co-extractants originating from the samples plus a low precision at low concentrations. When the selenium content is at trace concentrations or the supply of sample material is limited as may occur in blood samples obtained from infants and small experimental animals, the present method will be of considerable value.

Analysis of various biological samples has been performed using the described method (Table II). In all cases the reproducibility of the analysis

TABLE II

SELENIUM CONTENT OF BIOLOGICAL SAMPLES BY GC ANALYSIS

A. Physiological fluids*			B. Plant materials**	
	Whole blood (ng/ml)	Plasma (ng/ml)	Feed products	ng/g
Porcine	219 ± 11	192 ± 12	Corn	104 ± 1
Bovine	48 ± 3	28 ± 2	Hay Δ1	29 ± 1
Ovine	166 ± 13	57 ± 2	Hay Δ2	66 ± 2
			Silage	24 ± 0
			Grain mixtures	96 ± 3

*Values are means ± S.E.M. for 10 samples per species examined.
**Values are means ± S.E.M. for duplicate determinations of the same sample.

was within 3%. Table III lists typical recoveries of selenium that have been observed. Recovery of added selenium was always found to be in the range of 95—105%. This recovery was observed in the analysis of various samples including grasses, grains and animal tissues or fluids (Table III).

TABLE III

RECOVERY OF SELENIUM IN BIOLOGICAL SAMPLES

Values are means ± S.E.M. for duplicate determinations of two samples.

	Initial concentration (ppb)	Selenium addition	
		50 ppb	100 ppb
Corn	27 ± 1	78 ± 2	124 ± 3
Bovine liver	192 ± 8	248 ± 5	296 ± 4
		10 ppb	40 ppb
Bovine blood	48 ± 3	57 ± 2	90 ± 4

Experience with this GC method for over two years indicates that it is a suitable method for routine determination of selenium in terms of labor and precision. One individual can analyze without difficulty at least 60 samples, including GC and calculations in approximately 12 h.

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DETERMINATION OF (DICHLOROMETHYLENE)DIPHOSPHONATE IN PHYSIOLOGICAL FLUIDS BY ION-EXCHANGE CHROMATOGRAPHY WITH PHOSPHORUS-SELECTIVE DETECTION

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SUMMARY

An analytical method is presented for the determination of (dichloromethylene)disphosphonate (Cl_2MDP) in serum and urine. Cl_2MDP is isolated from biological samples by adsorption onto precipitated calcium phosphate. Orthophosphate is separated from Cl_2MDP by anion-exchange chromatography using AG 1-X8 resin. Detection is accomplished on-line using a flame photometric detector. Potentially interfering condensed phosphates are removed by acid hydrolysis. Sample handling losses are corrected by monitoring the recovery of a [^{14}C]Cl_2MDP spike added to the samples. Determinations of Cl_2MDP to concentrations as low as 2 μmol/l are possible. Extension of the method to determine other diphosphonates is discussed.

INTRODUCTION

Geminal diphosphonates are chemically similar to pyrophosphate, a substance found in urine and plasma which is able to inhibit the deposition of calcium phosphate salts both in vivo and in vitro [1]. Several calcium metabolic disorders have been linked to abnormally low levels of pyrophosphate in biological fluids [2]. Attempts to correct this imbalance with exogenous pyrophosphate have been unsuccessful presumably due to the rapid chemical and enzymatic hydrolysis of the P—O—P bond [3]. The geminal diphosphonates, possessing a P—C—P rather than a P—O—P linkage, are stable toward chemical and enzymatic hydrolysis and are being investigated as agents for treating certain calcium disorders [4].

The sodium salts of (1-hydroxyethylidene)diphosphonic acid (HEDP) and (dichloromethylene)diphosphonic acid (Cl_2MDP) are two of the more widely

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investigated diphosphonates. A method for the determination of HEDP in serum and urine has been reported which takes advantage of the photolytic sensitivity of HEDP [5]. Cl_2MDP is more resistant to photolysis, and therefore, a more generally applicable diphosphonate method has been developed.

An automated method for Cl_2MDP was sought for numerous serum and urine samples generated by animal studies and human clinical trials. Application of the method over a large concentration range was necessary. The Cl_2MDP concentration varied appreciably (2—25,000 μmol/l) because dosage, route of administration, species, sample type, sample volume, and sample collection time were among the variables.

Analysis of serum was the most challenging application because the concentration of Cl_2MDP following oral administration is low due to poor intestinal absorption, rapid uptake by the skeleton, and efficient clearance by the kidneys. The required detection limit for serum samples of 5 ml volume was 2 μmol/l. Pyrophosphate and orthophosphate are potential interferences that occur in appreciable concentrations in serum and urine. A selective procedure free from such interferences was needed. To meet these requirements, an automated ion-exchange chromatographic procedure for Cl_2MDP similar to a method for pyrophosphate [6] has been developed. Orthophosphate is separated from Cl_2MDP chromatographically and interference from pyrophosphate is eliminated by prior hydrolysis to yield orthophosphate.

Many diphosphonates, including HEDP and Cl_2MDP, lack the functional groups readily detected with conventional liquid chromatographic detectors. To allow automation of the chromatographic procedure, a (single-)flame photometric detector similar to that of Julin et al. [7] was built and used for dynamic detection of diphosphonate in the chromatographic effluent via light emission of HPO. In designing this detector the sources and types of noises leading to measurement uncertainties were considered and their influences minimized.

EXPERIMENTAL

Apparatus

The liquid chromatograph shown schematically in Fig. 1 was assembled from components available commercially. The use of hydrochloric acid as eluent restricted the choice of chromatographic equipment to components with glass, PTFE, Tefzel and Kel F eluent contact points. A Cheminert Metering Pump CMP-2VK (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used to deliver the 0.025 *M* HCl (Eluent A) at a constant flow-rate. The second, stronger eluent, 1.0 *M* HCl (Eluent B), was loaded into a 10-ml loop of an automatic slider valve No. SV 8031 (Laboratory Data Control) from a reservoir No. 1108PF with Tefzel valves and keys (Omnifit, Cedarhurst, NY, U.S.A.) pressurized with a Pressure Stat No. 050 PBS (Omnifit). Individual samples were loaded into a 20-loop rotary sample injection valve No. ROSV-1.0 controlled by a Valve Drive Unit VDU-20 (Laboratory Data Control). A cam programmer No. 324C-06-F2D-R1A-O1X with an 18-min cycle time (Automatic Timing and Controls, King of Prussia, PA, U.S.A.) indexed the valve drive for automatic injection of samples and controlled solenoids No. 902-00 (Altex Scientific,

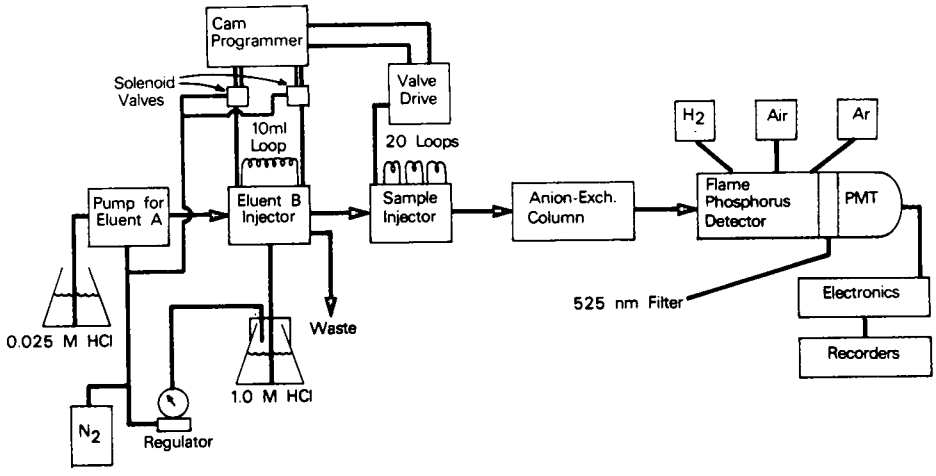

Fig. 1. Schematic diagram of the liquid chromatograph.

Berkeley, CA, U.S.A.). The solenoids were used to switch PA 875 pneumatic actuators (Laboratory Data Control) of the slider valve used for delivery of the second eluent. The chromatographic column No. 252-00-501 (Altex Scientific) was modified from the standard 250 mm length to approximately 100 mm. The column was packed with AG 1-X8 anion-exchange resin, 100—200 mesh (Bio-Rad Labs., Richmond, CA, U.S.A.).

The flame phosphorus detector is shown schematically in Fig. 2. Most of the components are available commercially. The total chromatographic effluent is introduced into a nebulizer No. 303-0352 and burner assembly No. 290-0358 (Perkin-Elmer, Norwalk, CT, U.S.A.) fitted with a burner head constructed by the Miami Valley Laboratories Machine Shop. The burner head design is similar to one described by Haraguchi and Winefordner [8] except that no capillaries are used. Instead, the flame is burned on the top of the inner cylinder. A hydrogen—argon—air entrained flame is used. Argon is applied to the nebulizer at 14 l/min, hydrogen is admitted through the fuel port at 3.6 l/min and air is applied to the sheath attachment at 7 l/min using standard flow controllers and rotameters (Brooks Instrument Division, Emerison Electric Co., Hatfield, PA, U.S.A.). The burner head is covered with a cylindrical sheath which also contains the photometer.

A 1 × 1 in. (2.5 cm) three-cavity interference filter with a center wavelength of 525.0 nm and a 4.0-nm bandpass (Ditric Optics, Marlboro, MA, U.S.A.) serves to isolate the HPO emission. A Model 83-021 Photomultiplier Housing (Jarrell-Ash Div., Fisher Scientific, Waltham, MA, U.S.A.) was used for either a type R106 photomultiplier tube (Hamamatsu Corp., Middlesex, NJ, U.S.A.) or a type 1P28A photomultiplier tube (Radio Corporation of America, Harrison, NJ, U.S.A.). The photomultiplier tube is connected to a Model 244 high-voltage supply and a Model 427 current amplifier (Keithley Instruments, Cleveland, OH, U.S.A.). A variable attenuator and a passive RC filter (time constant = 5 sec) constructed from standard electronic components are used to range and smooth the signal.

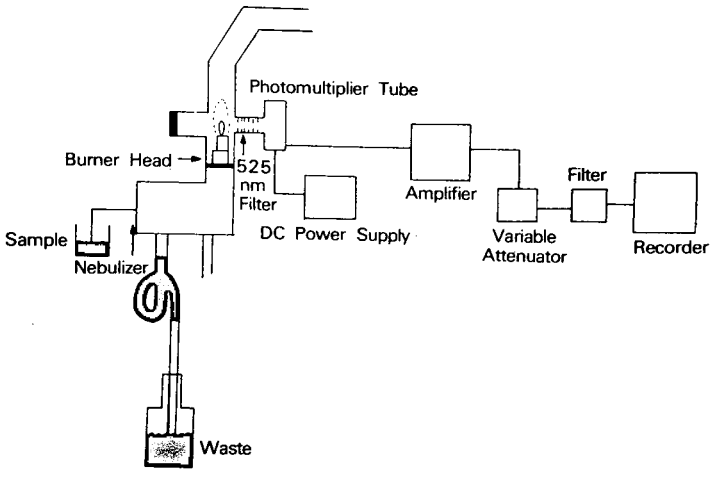

Fig. 2. Schematic diagram of the flame photometric detector.

Chromatograms are recorded using a Model 7132 strip chart recorder (Hewlett-Packard, Dayton, OH, U.S.A.).

Reagents and solutions

Test solutions of phosphoric acid and Cl_2MDP (acid form) were prepared to check detector performance. Test solutions of phosphoric acid were prepared by dilution of Baker (Phillipsburg, NJ, U.S.A.) reagent grade 85% phosphoric acid. Dichloromethane diphosphonate from Procter & Gamble (Cincinnati, OH, U.S.A.) was converted to the acid form by ion exchange on AG 50W-X8 resin (Bio-Rad Labs.) before preparation of less concentrated solutions of Cl_2MDP by dilution. Stock solutions of $[^{14}C]Na_2Cl_2MDP$ (5 μCi/ml) were provided by the Miami Valley Laboratories Radiochemistry Department for use as internal recovery standard. EDTA diluent was prepared by dilution of 1 part of a saturated solution of reagent grade disodium (ethylene-dinitrilo)-tetraacetic acid with 9 parts of water.

Eluent A (0.025 *M* hydrochloric acid) and Eluent B (1.0 *M* hydrochloric acid) were prepared by dilution of Baker reagent grade concentrated hydrochloric acid.

All other chemicals were reagent grade.

Sample preparation

The sample preparation procedure for Cl_2MDP determination is similar to that of Bisaz et al. [5] for HEDP in urine and plasma.

An internal standard of approximately 0.05 μCi (5 μg) of $[^{14}C]Cl_2MDP$ is added to each of the samples and allowed to equilibrate before preparation. Samples are then deproteinized with 25% trichloroacetic acid (4 ml added per 5 ml of serum or 1 ml for 5 ml of urine). Deproteinized serum and urine are then prepared similarly. To the protein-free filtrate, 100 μl of 0.5 *M* sodium dihydrogen phosphate and 50 μl of 2.5 *M* calcium chloride are added, and the pH is adjusted to 12.0—12.5 with 25% sodium hydroxide. The resulting precipitate is isolated by centrifugation, washed with deionized water, and redissolved

in 5 ml of 2 *M* hydrochloric acid. This solution is then heated for 30 min on a boiling water bath to hydrolyze any pyrophosphate present to orthophosphate. The pH is then adjusted to 7.0 using first 25% sodium hydroxide and finally 2.5% sodium hydroxide. Again a precipitate forms that is separated by centrifugation and is then dissolved in 2.0 ml of EDTA diluent. An aliquot of 100 μl is removed for determination of recovery of [^{14}C] Cl_2MDP by scintillation counting on a Model 2450 (Packard Instrument, Downers Grove, PA, U.S.A.). This is done to correct for losses occurring in the sample preparation. Aliquots are taken from the remainder for chromatographic analysis.

Standards are prepared by adding known quantities of Na_2Cl_2MDP and [^{14}C]-Na_2Cl_2MDP to blank urine or serum samples. These matrix standards are processed by the same procedure as used for samples. Quantitation of the eluted Cl_2MDP is accomplished by peak height measurement.

Chromatography

Samples, standards and controls are loaded into 1.0-ml loops attached to the 20-port valve. Samples are injected onto the column with Eluent A flowing. The Eluent B loop is bypassed for approximately 10 min, then is injected onto the column to elute the Cl_2MDP. The Eluent B loop is automatically loaded during the Eluent A cycle from a pressurized reservoir containing 1.0 *M* hydrochloric acid.

RESULTS

Detector performance

Aqueous standards of H_3PO_4 and Cl_2MDP (acid form) were directly aspirated into the detector to determine the dynamic range of the detector free of

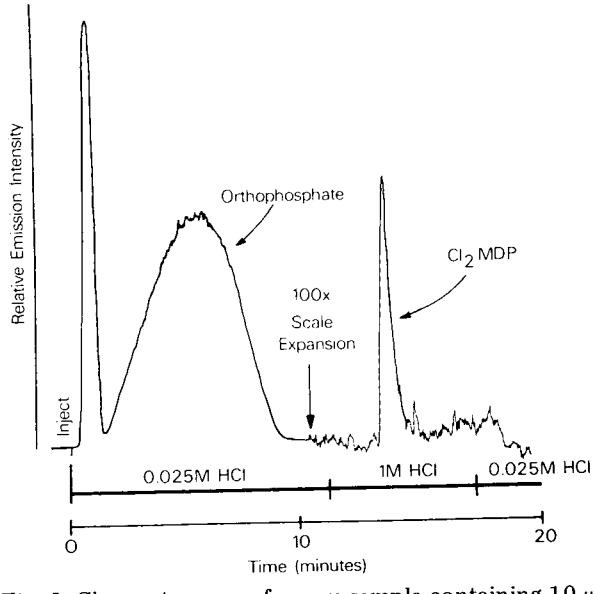

Fig. 3. Chromatogram of serum sample containing 10 μg of Na_2Cl_2MDP.

chromatographic constraints. The phosphorus detector behaved linearly for both compounds over the range of 0.1 μmol/l (0.003 mg phosphorus per liter, the detection limit) to approximately 3200 μmol/l (100 mg phosphorus per liter). Absolute response to phosphorus was approximately equal for these compounds. Response factors may vary for other analytes containing phosphorus, and, for chromatographic conditions, response factors will also depend on chromatographic variables.

Detector performance is severely degraded when solvents or chromatographic eluents other than deionized water or hydrochloric acid are used. However, hydrochloric acid is a suitable eluent for this chromatographic application. The small change in the baseline signal when changing from Eluent A to Eluent B is hardly noticeable in Fig. 3 and is quite acceptable for determination of Cl_2MDP. This small baseline change may reflect the difference in trace impurity level in the two eluents.

Validation of the method

Daily controls prepared by adding known amounts of Cl_2MDP to blank serum are analyzed with each group of samples. Validation data for these human serum controls are given in Table I to demonstrate the sustained accuracy and precision to be expected. Determinations are reliable (with relative standard deviations less than 10%) to concentrations as low as 2 μmol/l when 5 ml of serum are taken for analysis. For best accuracy, samples near this limit should be bracketed in concentration by standards. This limit is adequate for determination of serum profiles (0—10 h) following single oral doses of 3200 mg or 1600 mg Cl_2MDP. For oral doses less than 1600 mg of Cl_2MDP the resulting Cl_2MDP serum concentration is usually less than 2 μmol/l. Thus, the volume of serum required for reliable analysis becomes prohibitive (that is, greater than 5 ml).

A sample chromatogram is given in Fig. 3 showing the detector response for an injection of 10 μg (0.035 μmol) of Cl_2MDP.

Urinary determinations are especially valuable when lower oral doses are administered or when sufficient serum is unavailable. Validation data for

TABLE I

SUMMARY OF DAY-TO-DAY PRECISION AND ACCURACY FOR DETERMINATION OF Cl_2MDP IN HUMAN SERUM

Number of standards	Cl_2MDP (μmol/l)			S.D. (μmol/l)	R.S.D. (%)
	Added	Mean found	Difference		
7	3.46	4.74	1.28	0.26	5.5
5	10.4	9.62	−0.78	0.62	6.4
7	17.3	17.2	−0.1	0.69	4.0
7	24.2	26.4	2.2	2.1	8.0
3	69.2	67.8	−1.4	—	—
1	103.8	97.2	−6.6	—	—
1	138.4	133.2	−5.2	—	—

human urine are given in Table II. The precision for these blank samples spiked with Cl_2MDP adequately represents the agreement found for actual samples analyzed in duplicate. If chromatographic fractions are collected, wet-ashed with perchloric acid, and assayed colorimetrically for phosphorus [9], similar precision is obtained.

TABLE II

SUMMARY OF DAY-TO-DAY PRECISION AND ACCURACY FOR DETERMINATION OF Cl_2MDP IN HUMAN URINE

Number of standards	Cl_2MDP (μmol/l)			S.D. (μmol/l)	R.S.D. (%)
	Added	Mean found	Difference		
2	17.3	14.7	−2.6	—	—
2	34.6	34.1	−0.5	—	—
2	69.2	68.5	−0.7	—	—
2	103.8	110.0	6.2	—	—
1	115.2	109.0	−6.2	—	—
8	138.4	138.8	0.4	9.7	7.0
8	173.0	173.1	0.1	7.2	4.2
5	230.8	235.5	4.7	16	6.7

DISCUSSION

Detector design considerations

The most prevalent source of uncertainty (or noise) in this detector is the flickering of the flame. The photometric signal, which is due to the flame background light plus the light emission of HPO, changes as the flame moves around. Since flicker noise is directly proportional to its light flux carrier [10], collecting additional light with a mirror would increase both the signal and noise by the same factor. Since no net improvement in signal-to-noise ratio or relative measurement uncertainty would result, we omitted the light-gathering mirror used by other workers [7, 11].

The flame background is a near-continuum at 526 nm. Thus, a filter was chosen with a bandpass approximately equal to the width of the major peak in the HPO spectrum in order to maximize the signal-to-background ratio (and thus the signal-to-flicker noise ratio) [12]. This filter also had sufficient transmittance to keep photon noise and photomultiplier shot noise (which are proportional to the square root of the light flux and dark current, respectively) small compared to the flame background flicker noise.

The relatively inexpensive photomultiplier tube (RCA 1P28A) is perfectly adequate for this application since the limiting noise is carried on the flame background light. Switching to the photomultiplier tube (Hamamatsu R106) with lower relative dark current had no effect on the detector performance.

Chromatography

Small amounts of Cl_2MDP must be separated from much larger amounts of calcium phosphate salts and EDTA by the chromatographic procedure. The

instantaneous eluent upon injection of a 1.0-ml sample is not Eluent A but the sample diluent. The peak heights of aqueous Cl_2MDP standards do not adequately reflect the actual Cl_2MDP concentration due to broadening of the Cl_2MDP band on the column in the presence of this diluent. To eliminate low bias, we chose to standardize the method by the use of standards prepared in blank serum or urine matrix and processed by the same procedure as the samples. Matrix standard curves are prepared for each group of samples by plotting the mass of Cl_2MDP injected versus the peak height. The amount injected for each sample is calculated by a least squares linear regression of the matrix standards. The linear regression equation generally gives a negative intercept on the peak height axis indicating slight curvature in the calibration curve near the detection limit. The intercept is not eliminated by subtraction of the baseline signal change which accompanies the step gradient.

We have experience with chromatographic columns having smaller diameters but sufficient capacity to retain aqueous solutions of Cl_2MDP. The smallest detectable amount of Cl_2MDP was reduced from 7 nmol (2 μg) to 0.7 nmol (0.2 μg) injected, and a zero intercept was observed using these columns; however, the capacity was insufficient for actual samples. The recovery of Cl_2MDP in the chromatographic step is essentially quantitative for actual samples if the recommended column is used. Recovery of Cl_2MDP in the chromatographic step of the procedure was severely degraded when the smaller columns were tried for actual samples. A chromatographic peak of 7 nmol of Cl_2MDP represents the detection limit of the method because lesser amounts are indistinguishable from the background due to increased band spreading for the wider chromatographic column.

The use of more efficient chromatographic supports and stainless-steel columns is restricted by the use of hydrochloric acid eluents compatible with this flame phosphorus detector. A more versatile flame phosphorus detector for liquid chromatography has now been developed that can be used with many organic eluents and with ion-pairing reagents [13, 14].

We have successfully used this method for determination of Cl_2MDP in more than 3000 serum and urine specimens of animal and human origin. Generally 13—15 samples plus 4 or 5 standards are prepared the day prior to the chromatographic analysis. The operation of the chromatograph is completely unattended once the sample loops of the injector are loaded. The cycle time (18 min) and injector capacity (19 samples) are suitable for operation during a normal working day. We prefer to operate the flame phosphorus detector during normal working hours even though its operation has proven to be safe for the past two years. The capacity of the method can be more than doubled by using a single detector to dynamically monitor only the Cl_2MDP peaks from two chromatographs that are one-half cycle out of phase with one another. The orthophosphate from each column is then vented to waste using a slider valve to switch between column effluents.

The capacity of the method also can be increased by automated and unattended collection of the Cl_2MDP fractions during the evening. The Cl_2MDP fractions can then be loaded into an automatic sampler for direct aspiration into the detector the following morning. Oftentimes the range of Cl_2MDP concentrations is greater than the range of the standard curve. Dynamic detection

destroys the entire sample so those samples falling outside the range of the standard curve must be prepared again on a new dilution. This difficulty is circumvented by this alternative procedure because the entire sample need not be consumed: further dilutions of Cl_2MDP fractions can still be prepared. Another advantage is that the same group of standards can be used for more samples than is possible with dynamic detection.

Collection of Cl_2MDP fractions for a colorimetric total phosphorus determination after wet ashing with perchloric acid offers an alternative method for serum and urine samples when a flame phosphorus detector is unavailable.

Other diphosphonates

The method described here offers a general method for diphosphonates in physiological samples. Preliminary work has shown that the concentration of Eluent A should be decreased to 0.01 *M* hydrochloric acid for HEDP and (methylene)diphosphonate (MDP). An alternative diluent of 0.03 *M* hydrochloric acid will redissolve the final precipitate without introducing a reagent blank from EDTA that accompanies this change in the first eluent. The amount of resin must also be increased to prevent premature elution of some diphosphonates. With these modifications the application of the method to determine diphosphonates in physiological samples is straightforward. Determination of pyrophosphate in physiological samples is also a potential application with omission of the acid hydrolysis step. The cycle time must be increased to 40—45 min for HEDP and MDP determinations because the orthophosphate elution is extended with these modifications. Fraction collection with subsequent off-line detection is preferabie when many samples must be analyzed at longer cycle times.

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CHROMBIO. 921

DETERMINATION OF SEROTONIN, ITS PRECURSORS, METABOLITES AND [^{3}H]SEROTONIN IN LUNG BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A rapid, simple, sensitive method for the determination of serotonin, its precursors, metabolites and [^{3}H]serotonin in lung is described. Tissue preparation requires only homogenization in dilute perchloric acid and centrifugation prior to separation by high-performance liquid chromatography using a reversed-phase column. Detection is based on the native fluorescence of indole compounds and detection limits ranged between 30—90 pg injected. The method has been used to determine these compounds in mouse lung and plasma.

INTRODUCTION

The importance of the lungs in the control of circulating levels of vasoactive substances in health and disease has been established [1, 2]. Recent investigations [3—9] have demonstrated the pulmonary vasculature to be the major site controlling the arterial concentration of the vasoactive amine, serotonin (5-hydroxytryptamine, 5-HT). This control is accomplished by 5-HT uptake, storage, and metabolism and may be important in maintaining the fluidity of the pulmonary circulation. Drugs, and dietary and environmental toxicants which interfere with this pulmonary function may produce severe adverse effects. Furthermore, the control of circulating 5-HT levels by the lung and its function therein have been the subject of considerable speculation which has included such pathological states as anaphylaxis [10, 11], delayed-type hypersensitivity [12] and pulmonary hypertension [13].

Various analytical methods have been employed to measure 5-HT and its precursors and metabolites in tissues and fluids, including thin-layer chromato-

graphy [14], ultraviolet spectrometry [15], fluorescence spectrometry [16], gas chromatography [17], gas chromatography—mass spectrometry (GC—MS) [18] and radioimmunoassay (RIA) [19]. All of these analytical methods suffer from limitations of simplicity, selectivity and/or sensitivity. Recently these compounds have been analyzed in brain, cerebrospinal fluid, serum and urine by high-performance liquid chromatography (HPLC) with fluorescence [20—25] and electrochemical (EC) [26—31] detection.

In light of the importance of lung—serotonin interactions, we have developed a simple, sensitive method for the determination of tryptophan (TRP), 5-hydroxytryptophan (5-HTP), 5-HT, exogenous [^{3}H] 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxyindole-3-acetic acid O-sulfate (5-HIAA-O-sulfate), 5-hydroxytryptophol (5-HTOL), tryptamine, N-acetylserotonin and indole-3-acetic acid (IAA) in a single injection. This method has been used in the determination of these compounds in mouse lung and plasma.

EXPERIMENTAL

Reagents

Tryptophan, tryptamine hydrochloride, indole-3-acetic acid, 5-hydroxytryptophan, serotonin hydrogen oxalate, N-methylserotonin hydrogen oxalate, N-acetylserotonin, 5-hydroxyindole-3-acetic acid, and 5-hydroxytryptophol were purchased from Regis (Morton Grove, IL, U.S.A.). 5-Hydroxytryptamine binoxalate [1,2-^{3}H(N), specific activity 27.0 Ci/mmol] and 5-hydroxytryptamine binoxalate [2-^{14}C, specific activity 51.5 mCi/mmol] were purchased from New England Nuclear (Boston, MA, U.S.A.). The purity of all labeled compounds was determined by thin-layer chromatography on 250-μm silica gel G plates purchased from Analtech (Newark, DE, U.S.A.), using a solvent system composed of acetone—2-propanol—water—ammonium hydroxide (50:40:7:3). The thin-layer plates were scanned on a Packard 7200 radiochromatogram scanner (Packard Instrument Co., Downers Grove, IL, U.S.A.). Liquid scintillation fluid was prepared by dissolving 5 g of PPO—POPOP (98:2) purchased from Research Products International (Elk Grove Village, IL, U.S.A.) and 100 g of naphthalene in 1 l of *p*-dioxane. Type VII purified bacterial β-glucuronidase and Type H-1 partially purified β-glucuronidase—sulfatase from *Helix pomatia* were purchased from Sigma (St. Louis, MO, U.S.A.). Glass distilled methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and 0.01 *M* sodium acetate buffer, pH 4.7, was prepared from reagent grade sodium acetate. Solvents were filtered through a 0.2-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and vacuum deaereated. Standard solutions (1 m*M*) were prepared in 0.1 *M* perchloric acid and diluted to the desired concentration.

Apparatus

Liquid chromatography was performed using a Varian 5020 liquid chromatograph (Varian Assoc., Palo Alto, CA, U.S.A.) equipped with a universal loop injector, a 5-cm column guard packed with Vydac reversed-phase hydrocarbon (Separations Group, Hesperia, CA, U.S.A.) and a 30 cm × 3.9 mm I.D. μBondapak C_{18} reversed-phase column, particle size 10 μm (Waters Assoc.,

Milford, MA, U.S.A.). The mobile phase consisted of 12% methanol—0.01 *M* sodium acetate buffer (pH 4.7) and the flow-rate was 0.8 ml/min. Fluorescence detection was achieved using a Fluorichrom detector (Varian Assoc.) equipped with a deuterium arc source and using a 200 I excitation filter and a Corning 7-60 band filter (360 nm) for emission. Radioactive samples were counted in a Beckman LS-8000 liquid scintillation counter (Beckman Instruments, Fullterton, CA, U.S.A.) and programmed to count dual-labeled ($^3H/^{14}C$) samples.

Sample preparation

Male, Swiss-Webster mice weighing 20—25 g were purchased from Laboratory Supply Co., (Indianapolis, IN, U.S.A.). All animals were fed food and water ad libitum and were maintained on a 12:12 light/dark cycle for five days prior to use. 5-Hydroxytryptamine binoxalate [1,2-^{3}H(N)] was injected into the tail vein of mice at a dose of 0.2 μCi and 20 ng 5-HT binoxalate per g. The mice were loosely restrained under an inverted 100-ml blackened beaker with the tail protruding from the lip of the beaker. Fifteen min after administration of [^{3}H] 5-HT the animals were killed by ether asphyxiation/decapitation which involved placing the animal in a warmed (37°C), ether-saturated desiccator until all breathing ceased (1.1 min). The lungs were quickly removed, rinsed in ice-cold 0.9% saline, blotted dry and frozen at −80°C for analysis. Blood was collected from the decapitation site in glass tubes previously rinsed with EDTA anticoagulant solution [27]. Hematocrits were determined and compared to previously reported values [32]. Plasma samples were prepared by centrifuging the sample for 30 min at 600 *g* in a Beckman J-6B centrifuge cooled to 4°C. Aliquots of up to 300 μl of each plasma sample were transferred to 125 × 15 mm glass tubes and stored at −80°C for analysis.

Lungs were weighed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY, U.S.A.) at a setting of 6 for 0.5 min in 2.0 ml of ice-cold 0.1 *M* perchloric acid containing 100 μl of EDTA—ascorbic acid solution (200 mg ascorbic acid per ml of 10% EDTA solution, prepared fresh daily), 50 μl (0.4 μg) N-methylserotonin (as internal standard for endogenous indole determinations) and 50 μl (10 nCi) of [^{14}C] 5-HT as internal standard for [^{3}H] 5-HT determinations. Plasma samples were handled in the same manner. The homogenates were centrifuged for 10 min at 4°C and 2500 *g* and the supernatants transferred to screw-capped glass centrifuge tubes and 100-μl aliquots were injected onto the column. Samples were stored at −80°C for longer than two weeks without evidence of degradation.

Quantitation

The quantitation of the endogenous indoles was based on the calculated fluorescence intensity ratio of each indole to N-methylserotonin (internal standard), and the use of a calibration curve that was prepared by adding differing amounts of each indole compound to 0.4 μg of N-methylserotonin and analyzing the samples (see above).

The quantitation of [^{3}H] 5-HT was based on the [^{3}H] 5-HT to [^{14}C] 5-HT ratio determined by collecting the detector effluent associated with the 5-HT peak in 10 ml of liquid scintillation fluid and counting. A calibration curve was

prepared by adding known amounts of [^{3}H] 5-HT and 10 nCi of [^{14}C] 5-HT to blank lung tissue and analyzing the samples (see above).

Identification and quantitation of 5-HIAA-O-sulfate

The substance to be identified (Fig. 1A, peak 2) was repetitively collected from lung chromatographic analysis and evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 2.0 ml of water and divided equally. The divided samples were adjusted to pH 5.9 and pH 5.0, and treated

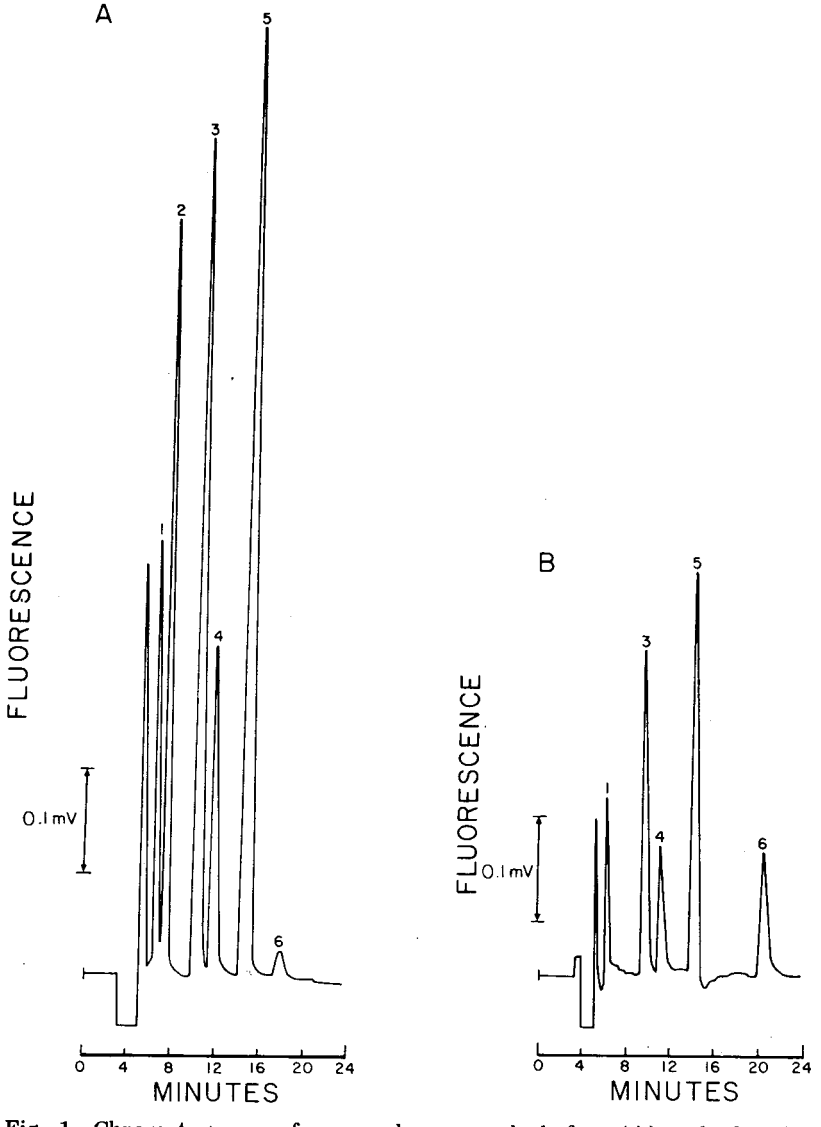

Fig. 1. Chromatograms of mouse lung sample before (A) and after (B) acid hydrolysis. Peaks: 1 = 5-HTP; 2 = 5-HIAA-O-sulfate; 3 = 5-HT; 4 = N-methylserotonin; 5 = TRP; 6 = 5-HIAA. See Experimental for chromatographic conditions. Samples were eluted with 12% methanol in 0.01 *M* sodium acetate buffer, pH 4.7 and a flow-rate of 0.8 ml/min.

with 9.3 mg (12,000 units) of Type VII purified β-glucuronidase and 10 mg (3000 units and 300 units) of partially purified Type H-1 β-glucuronidase—sulfatase, respectively. Each sample was incubated for 18 h at 37°C, after which it was adjusted to pH 1.0 with dilute perchloric acid, centrifuged, and analyzed.

A 0.5-ml aliquot of the lung supernate was treated with 100 μl of EDTA—ascorbic acid solution and then adjusted to a final concentration of 1.0 *M* perchloric acid by the addition of 90 μl of concentrated perchloric acid. The solution was heated for 10 min at 100°C, cooled, and neutralized with 180 μl of 2 *M* potassium acetate solution. The sample was centrifuged to remove the potassium perchlorate precipitate and a 100-μl aliquot was analyzed.

The quantitation of 5-HIAA-O-sulfate was based on the quantitative acid hydrolysis of the lung supernate, as described above. From the increased level of 5-HIAA following hydrolysis, the initial concentration of 5-HIAA-O-sulfate was readily calculated. Under the hydrolysis conditions 5-HTOL was not oxidized to 5-HIAA, the other indole derivatives were not converted to 5-HIAA, and both 5-HIAA and N-methylserotonin (internal standard) were stable. The peak height differences observed between Fig. 1A and B are due to dilution of the sample during the hydrolysis of the sample. All peak height ratios remained the same.

RESULTS AND DISCUSSION

The chromatographic charactersistics of the authentic indole compounds are depicted in Fig. 2 and the chromatographic and detectability data are presented in Table I. Under the experimental conditions, all of the indole compounds are readily separated and fluorescence detection provides detection limits which are comparable to GC—MS [18], RIA [19] and HPLC—EC [26—31].

The analysis of mouse lung indoles is presented in Fig. 1A. All of the compounds, except the one producing peak 2, were confirmed by both spiking the sample with authentic material and chromatographing the samples with differing percentages (5—15%) of methanol. In each instance the compound coeluted with the appropriate standard. The identification of 5-HIAA-O-sulfate as the compound chromatographing as peak 2 was based on its rapid hydrolysis to 5-HIAA, as shown by the marked increase in 5-HIAA in the hydrolyzed chromatogram (Fig. 1B). Under the hydrolysis conditions neither 5-HTOL nor the other indole compounds present in the lung sample were converted to 5-HIAA. In addition, both 5-HIAA and N-methylserotonin, the internal standard, were stable under these conditions, thereby providing an easy means of quantitating this metabolite. The identification of the metabolite as a sulfate conjugate was based on the facile hydrolysis of the concentrated metabolite by partially purified Type H-1 β-glucuronidase—sulfatase and the absence of hydrolysis with Type VII purified β-glucuronidase. Based on this differential hydrolysis the conjugate was identified as a sulfate.

The chromatographic analysis of mouse plasma is presented in Fig. 3A. The identification and quantitation of 5-HIAA-O-sulfate as the metabolite responsible for peak 2 in the chromatogram was based on the same criteria as

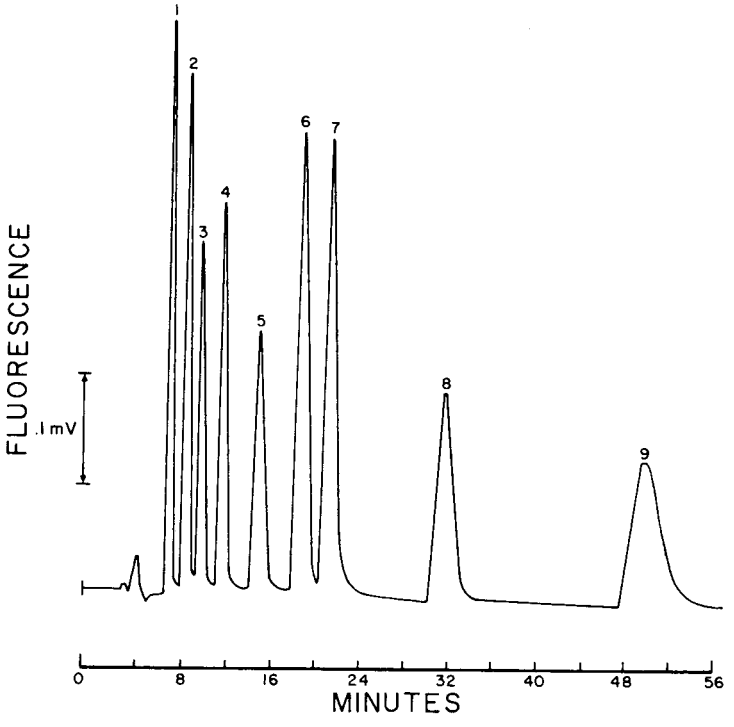

Fig. 2. Chromatogram of standard indole compounds. Peaks: 1 = 5-HTP; 2 = 5-HT; 3 = N-methylserotonin; 4 = TRP; 5 = 5-HIAA; 6 = 5-HTOL; 7 = tryptamine; 8 = N-acetylserotonin; 9 = IAA. See Experimental for chromatographic conditions. Elution conditions were the same as for Fig. 1.

TABLE I

CHROMATOGRAPHIC AND DETECTABILITY DATA

For chromatographic conditions see Experimental.

Compound	Retention time (min)	Detection limit (pg)*
5-Hydroxytryptophan	7.6	45
Serotonin	9.1	30
N-Methylserotonin	10.4	50
Tryptophan	12.4	50
5-Hydroxyindole-3-acetic acid	15.7	90
5-Hydroxytryptophol	19.6	70
Tryptamine	21.6	100
N-Acetylserotonin	31.9	300
Indole-3-acetic acid	50.0	210

*Injected quantity of authentic material giving a signal-to-noise ratio of 2.0.

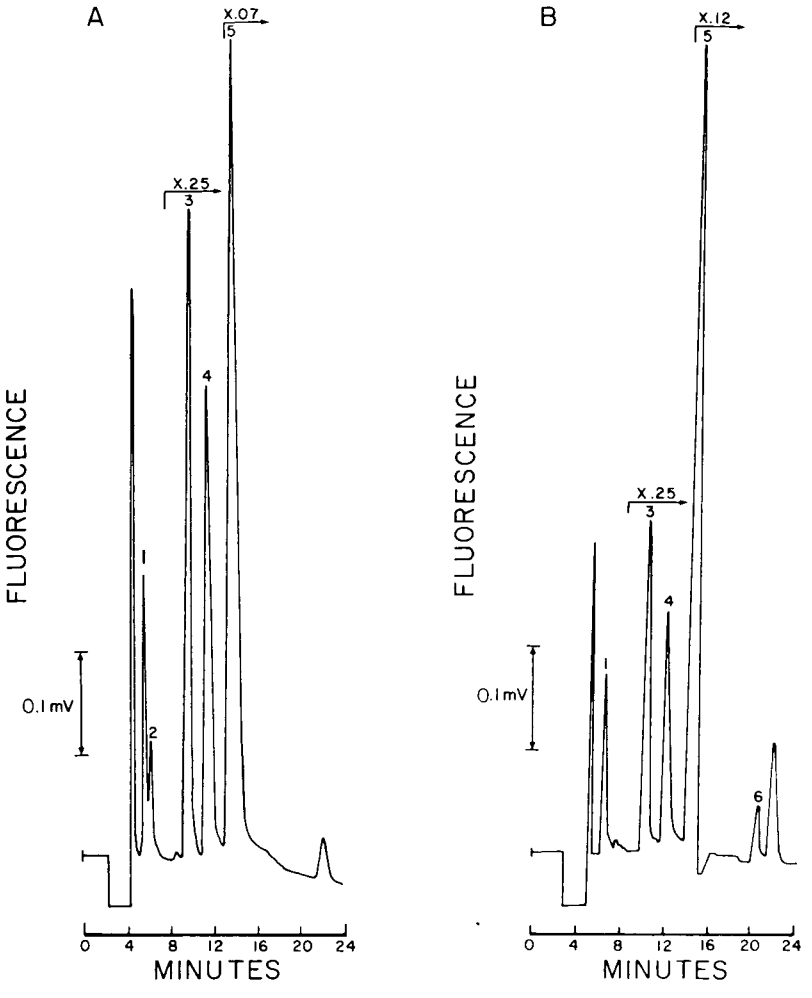

Fig. 3. Chromatogram of mouse plasma sample before (A) and after (B) acid hydrolysis. Peaks: 1 = 5-HTP; 2 = 5-HIAA-O-sulfate; 3 = 5-HT; 4 = N-methylserotonin; 5 = TRP; 6 = 5-HIAA. See Experimental for chromatographic conditions. Elution conditions were the same as for Fig. 1.

described above for lung samples and the corresponding hydrolyzed plasma sample is shown in Fig. 3B.

The determinations of TRP, 5-HTP, 5-HT, [^{3}H] 5-HT, 5-HIAA, and 5-HIAA-O-sulfate levels in mouse lung and plasma are shown in Table II. The lung 5-HT and 5-HIAA values are similar to those previously reported [33, 34], while the remaining indoles have not been previously reported in mouse lung. The plasma 5-HT levels are considerably lower than those reported [33] and may reflect differences in analytical method or differences in the method of sacrifice. The latter have been shown [35, 36] to markedly affect the lung and circulating levels of 5-HT.

In summary, we have described a simple, sensitive procedure for the rapid

TABLE II

LEVELS OF SEROTONIN, ITS PRECURSORS, METABOLITES AND [^{3}H]SEROTONIN IN MOUSE LUNG AND PLASMA

Values are expressed as μg/g or μg/ml and radioactivity is expressed as nCi/g or nCi/ml. All values are mean ± S.E.M.

Sample	TRP	5-HTP	5-HT	[^{3}H] 5-HT	5-HIAA	5-HIAA-O-sulfate
Lung	10.98 ± 0.89	0.45 ± 0.05	2.89 ± 0.26	1544 ± 138	0.12 ± 0.04	9.96 ± 0.39
Plasma	12.04 ± 1.34	0.15 ± 0.03	1.17 ± 0.16	586 ± 72	ND*	0.27 ± 0.01

*Not routinely detected.

determination of exogenous [^{3}H] 5-HT and endogenous 5-HT, its precursors and metabolites in lung. With a method capable of simultaneously measuring both endogenous 5-HT as well as exogenous [^{3}H] 5-HT, the dynamics of lung—serotonin interactions and their alteration by drugs, environmental toxicants, and disease can be more readily studied.

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CHROMBIO. 940

USE OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR SIMULTANEOUS DETERMINATION OF GLUTAMINE SYNTHETASE AND GLUTAMIC ACID DECARBOXYLASE IN CRUDE EXTRACTS

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SUMMARY

Glutamine and γ-aminobutyric acid (GABA), formed from glutamic acid in crude tissue extracts by glutamine synthetase and glutamic acid decarboxylase respectively, were separated by derivatization with dansyl chloride followed by reversed-phase high-performance liquid chromatography on an Altex Ultrasphere ODS-5 column. The mobile phase was a gradient of 100 m*M* potassium dihydrogen phosphate (pH 2.1) with 0—40% acetonitrile. The amounts of glutamine and GABA formed from glutamic acid were determined under different reaction conditions.

INTRODUCTION

Glutamine and γ-aminobutyric acid (GABA) are formed from glutamic acid by glutamine synthetase (GS) and glutamic acid decarboxylase (GAD) respectively. The presence of both enzymes has been demonstrated in neuronal as well as non-neuronal systems. GABA has been assumed to be a major inhibitory neurotransmitter in the central nervous system [1, 2]. Glutamine occupies a central position in nitrogen metabolism since it serves as a source of nitrogen for various metabolites, which in turn are used for the formation of proteins, carbohydrates and nucleic acids [3, 4]. The interest in our laboratory is aimed at differential metabolism of glutamic acid by retinal cells under various conditions. Recently, Pishak and Phillips [5] utilized a double column method to separate glutamine and GABA from glutamic acid. This was necessitated because under their assay conditions for GS, both glutamine and GABA were formed. To achieve resolution of the three components, this method requires two ion-exchange columns.

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Reversed-phase high-performance liquid chromatography (HPLC) offers advantages over conventional chromatographic techniques in both resolving power and time. We utilized this technique to resolve glutamic acid, GABA and glutamine from each other after derivatization with dansyl chloride. In addition, we present evidence that under proper assay condition, glutamine is the only product formed by the GS reaction and GABA is the only product formed by the GAD reaction.

MATERIALS AND METHODS

Reagents

Acetone, distilled in glass and acetonitrile, UV grade, distilled in glass, were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Glutamic acid, GABA, glutamine, dansylated derivatives of these amino acids, and ATP were obtained from Sigma (St. Louis, MO, U.S.A.). [3,4-^{3}H]Glutamic acid (40 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Potassium dihydrogen phosphate, analytical grade, was obtained from Gallard-Schlesinger Chemical Corp. (Carle Place, NY, U.S.A.). Concentrated phosphoric acid, AR, was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Distilled water was further purified by passing through a Milli-Q system (Millipore, Bedford, MA, U.S.A.) followed by glass distillation. Dansyl chloride for derivatization was prepared fresh daily by diluting the stock solution (10% in acetone obtained from Pierce (Rockford, IL, U.S.A.) with acetone to yield a final concentration of 0.25%. Sodium bicarbonate buffer (100 m*M*, pH 9.5) was passed through 0.45-μm filter (Millipore). All other chemicals were reagent grade and obtained from the usual sources.

Enzymes source

Crude enzyme preparations were obtained by homogenizing bovine retina and brain in three volumes of 25 m*M* imidazole buffer (pH 7.1), 10 m*M* mercaptoethanol and 0.1 m*M* EDTA. The homogenate was centrifuged at 1000 *g* to remove nuclei and cellular debris. The supernatant was withdrawn carefully and used as a source of GS and GAD. GS was also purified from retina by column chromatography and was judged to be homogeneous on the basis of electrophoretic techniques and immunodiffusion. The details of purification procedure and characterization will be described elsewhere [6].

Enzyme assays

The glutamine synthetase assay was carried out in a 50-μl aliquot (pH 7.4) containing 50 m*M* imidazole, 4 m*M* NH_4Cl, 10 m*M* ATP, 20 m*M* $MgCl_2$, 0.2 m*M* glutamic acid and 1–2 μCi of [^{3}H]glutamic acid. After addition of enzyme, the mixture was incubated at 37°C for 15 min and the reaction was stopped by rapidly cooling in an ice bath. A 20-μl aliquot was withdrawn and vortexed with 20 μl of 1 *N* acetic acid. The insoluble material was removed by centrifugation in a microfuge for 5 min. A 20-μl aliquot of the supernatant was withdrawn into a PTFE-lined capped sample vial, lyophilized and used for dansylation.

The buffer used for the GAD assay was 100 m*M* potassium phosphate (pH

6.8), 0.5 m*M* pyridoxal phosphate, 1 m*M* EDTA. The reaction was initiated by the addition of crude enzyme extract to 100 μl of buffer, 2 μl glutamic acid (5 m*M*) and 1—2 μCi of [^{3}H]glutamic acid. After incubation at 37°C, the reaction was stopped by rapidly cooling in ice. A 50-μl aliquot was withdrawn and the pH adjusted between 9 and 9.5 by the addition of 0.1 *N* sodium hydroxide. The insoluble material was removed by centrifugation in a microfuge for 5 min. A 50-μl aliquot was withdrawn into a PTFE-lined capped sample vial, lyophilized and used for dansylation.

Dansylation

Dansylation was carried out by a modification of the method of Gray and Hartley [7]. The lyophilized material in the sample vial was mixed with 50 μl of the bicarbonate buffer and 100 μl of the working dansyl chloride solution. The vial was capped and the contents were mixed thoroughly. After incubation at 70°C for 15 min in a water bath, the vial was removed, cooled in ice for about 5 min and 20 μl were injected into the HPLC column.

Chromatography

A Beckman Model 332 gradient liquid chromatograph equipped with a Beckman Model 210 sample injection valve with a 20-μl loop and an LKB UV monitor operating at 206 nm was used. An Altex Ultrasphere reversed-phase ODS-5 column (250 × 4.6 mm) was used. The mobile phase was a gradient of 100 m*M* potassium dihydrogen phosphate pH 2.1 with 0—40% acetonitrile. The flow-rate was 1 ml/min at ambient temperature. Fractions (0.5 ml) were counted in 2 ml of ACS scintillation fluid.

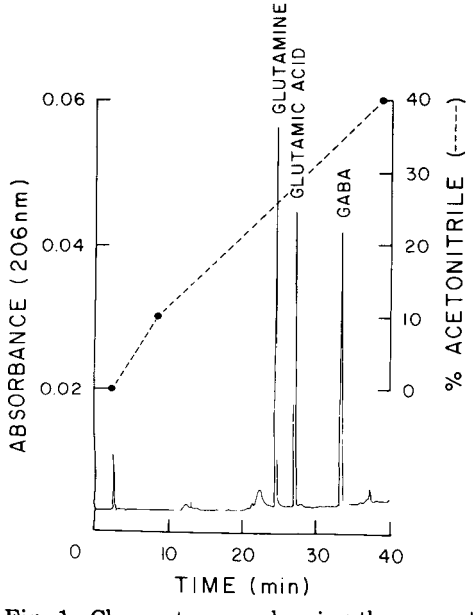

Fig. 1. Chromatogram showing the separation of 0.5 μg of dansylated glutamine, glutamic acid and GABA. The dashed line indicates the mobile phase gradient (% acetonitrile) used.

Protein determination

Protein was measured in all samples by the dye binding method [8] using bovine serum albumin as a standard.

RESULTS

The chromatogram showing the separation of dansylated glutamic acid, GABA and glutamine standards along with the gradient used can be seen in Fig. 1. The separation of these three compounds was reproducible, elution times were within ± 1%. As can be seen from the chromatogram well resolved peaks were obtained. Glutamine elutes at 25 min (flow-rate 1 ml/min), glutamic acid elutes at approximately 27.5 min, and GABA elutes at 33.5 min. Undansylated glutamic acid, glutamine and GABA are not separated from each other significantly and elute early. Prior dansylation also increases the sensitivity of detection of the amino acids by UV absorption and fluorescence. It is possible to detect as little as 100 ng of each amino acid by UV absorption at 206 nm and even less can be observed with a fluorescence detector.

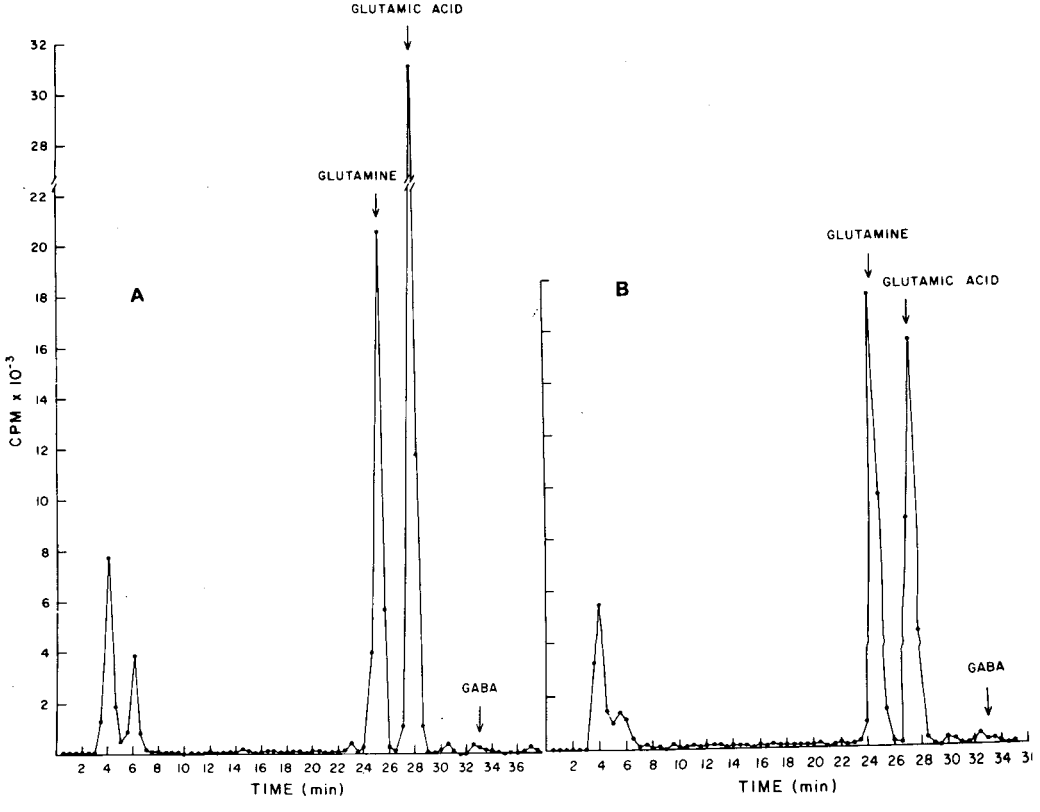

Fig. 2. Chromatograms of the reaction mixture using (A) purified retinal glutamine synthetase, 0.28 μg of purified enzyme protein plus assay mixture was incubated for 45 min; (B) 0.24 mg protein of crude retinal extract under GS assay conditions. For further details see Materials and Methods.

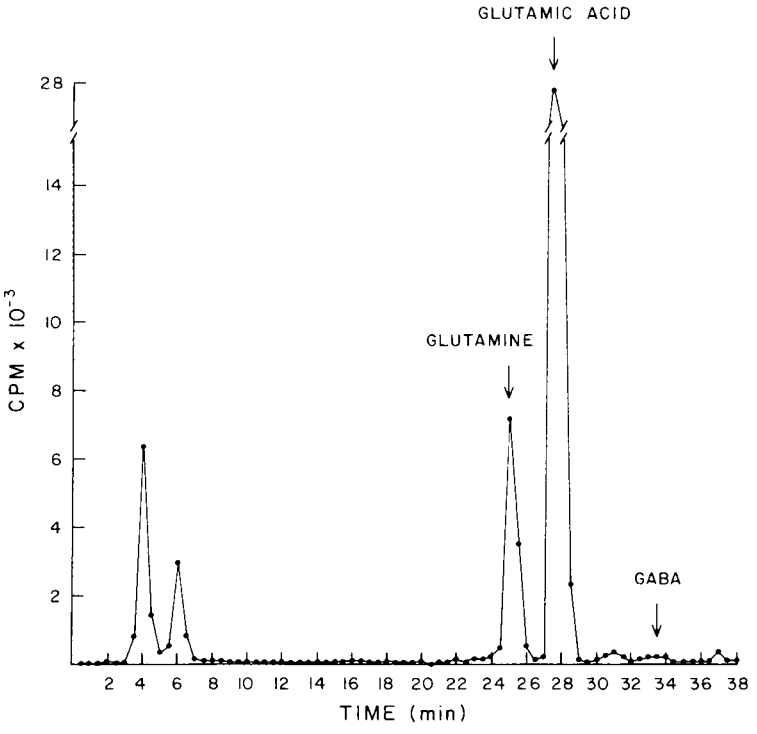

Fig. 3. Chromatogram of the reaction mixture using 0.32 mg of crude brain extract under GS assay conditions. For further details see Materials and Methods.

Two major peaks corresponding to glutamine and glutamic acid are observed for the reaction catalized by purified retinal GS and crude retinal homogenate (Fig. 2A and B). The two early peaks correspond to undansylated material. The control run of [^{3}H]glutamic acid after dansylation also revealed the presence of two early peaks besides a major peak for glutamic acid. The second early peak (6 min) was found to be a contaminant (2—4%) in our tritiated glutamic acid which would not dansylate. At the present time the identity of the peak is not known; however, due to its low concentration it appears to have no effect upon the results. With this method, it is possible to detect enzyme activity in a small amount of biological material having only 2 μg of protein. The amount of GABA found in the glutamine synthetase assay was less than 1% and so for all practical purposes no GABA is formed. Similar results were obtained using crude brain extract (Fig. 3) under the same GS assay conditions. In this case, however, lower enzyme activity resulted in a smaller glutamine to glutamate ratio. Again, no GABA was seen. When the experiment with crude brain extract was carried out without the addition of ATP or Mg^{2+}, (Fig. 4), there was no significant formation of glutamine. If, however, the reaction with the crude brain or retinal extract was carried out with pyridoxal phosphate but no ATP or Mg^{2+} (GAD assay conditions), GABA was formed but there was no significant formation of glutamine (Fig. 5A and B). With this method, it is possible to detect GAD in biological material having only 10 μg of protein. The percent dansylation from nine separate experiments was found to

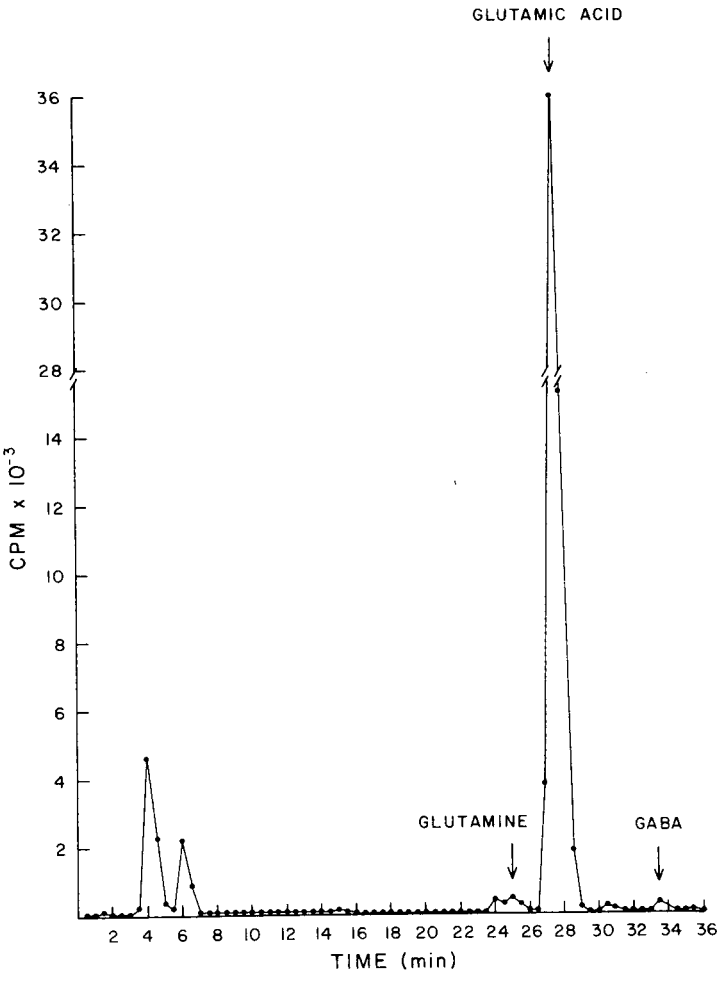

Fig. 4. Chromatogram of the reaction mixture of crude brain extract under GS assay conditions without ATP and Mg^{2+}. Other conditions were the same as in Fig. 3.

be 85.64 ± 3.52%. Recovery of amino acids from the column was 100% and no amino acid was detected after final wash with acetonitrile. Table I summarises the results of glutamine and GABA formation under various conditions by crude retinal and brain extracts.

DISCUSSION

Reversed-phase HPLC has been shown to be a powerful tool for studying the separation of amino acids [9] and therefore should be equally valuable in studying the metabolism of amino acids and their metabolites in cultured cells under different conditions. We have been interested in the metabolites, glutamine and GABA, formed from glutamic acid. At present the only method of studying these metabolites is by the use of two separate anion-exchange columns [5]. Therefore we developed a reversed-phase HPLC method for the

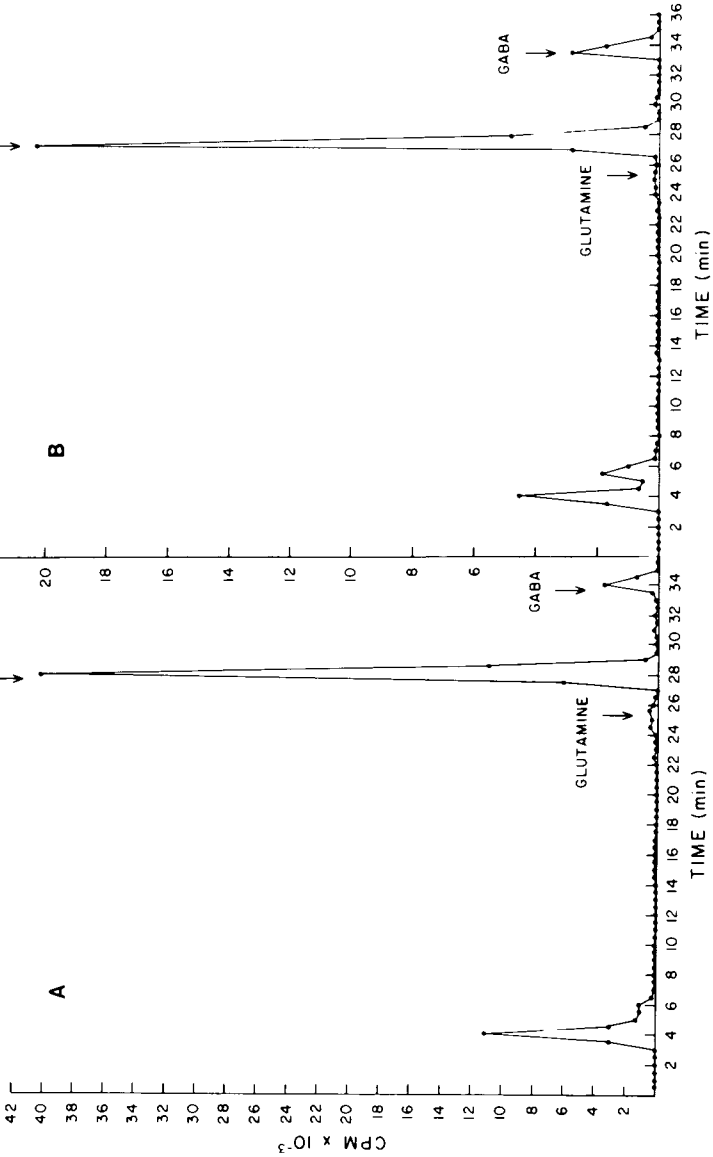

Fig. 5. Chromatograms of the reaction mixture of (A) crude brain extract under GAD assay conditions, 0.17 mg of crude enzyme protein plus assay mixture were incubated for 1 h; (B) crude retinal extract under GAD assay conditions, 0.30 mg of crude enzyme protein plus assay mixture were incubated for 2 h. For further details see Materials and Methods.

TABLE I

PERCENTAGE CONVERSION OF GLUTAMIC ACID BY RETINA AND BRAIN UNDER GS AND GAD ASSAY CONDITIONS

Tissue	Assay conditions	GABA (%)	Glutamine (%)
Retina*	GS assay	<1	50
Brain**	GS assay	<1	17
Brain***	GS assay, −ATP, $-Mg^{2+}$	<1	1.4
Retina§	GAD assay	15	<1
Brain§	GAD assay	8.3	<1

*Same conditions as Fig. 2B.
**Same conditions as Fig. 3.
***Same conditions as Fig. 4.
§ Same conditions as Fig. 5.

separation of these three compounds. This method can be utilized to determine the products formed by both GS and GAD. As seen in Table I, using crude enzyme preparations under proper assay conditions, either glutamine or GABA is the principal metabolite of glutamic acid. As shown here and by others [10, 11], ATP and Mg^{2+} are required as cofactors for glutamine synthetase and virtually no reaction takes place in their absence. On the other hand, ATP and Mg^{2+} are inhibitory for GAD [12, 13]. Thus, by simply varying the assay conditions one can detect either GABA or glutamine as the product from glutamic acid while using whole cell preparations where both reactions might occur. We are utilizing this method at present to study different metabolic routes of glutamic acid in retinal cells.

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RADIOIMMUNOASSAY OF PROSTAGLANDIN $F_{2\alpha}$ USING SEPHADEX G-75 GEL CHROMATOGRAPHY

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SUMMARY

The development of a "bound—free" separation technique and its application to the radioimmunoassay of prostaglandin $F_{2\alpha}$ is described. The method is simple, rapid, free of non-specific binding and could be performed either at 4°C or at room temperature. A total of 100 tubes could be subjected to "bound—free" separation in 30 min at 4°C. The bound fraction is collected directly into scintillation vials. The total column length was 9.5 cm, of which the bed volume was 2.5 ml. The $PGF_{2\alpha}$ radioimmunoassay incubation volume of 0.3 ml when bedded in required 1.4 ml of elution buffer to elute the antibody-bound fraction. The free fraction was washed out with 4.0 ml of buffer and the columns were ready for further use. A standard curve of high sensitivity (5 pg) and good reproducibility (CV %: intra-assay = 6.54; inter-assay = 9.68) was obtained.

INTRODUCTION

In the radioimmunoassay systems for prostaglandins, prostaglandin metabolites, steroids, peptides and other substances, the antigen—antibody complex and the free antigen occur in soluble form. It is therefore necessary to separate the antibody-bound fraction from the free antigens. A variety of methods have been utilized for the bound—free separation in the radioimmunoassay of prostaglandin $F_{2\alpha}$. These include dextran-coated charcoal suspension [1], double-antibody [2, 3], ammonium sulphate [4], ammonium sulphate in conjunction with calcium sulphate suspension [3], polyethylene glycol [5], and nitrocellulose membranes [6, 7]. The use of the solid-phase method originally described for oestradiol-17β radioimmunoassay [8] has not been utilized in the radioimmunoassay of prostaglandins. The use of gel chromatography for the separation of protein-bound insulin from free insulin was described for study-

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ing insulin antibodies [9]. The use of gel chromatography with Sephadex G-75 was reported for insulin radioimmunoassay [10]. A modified gel chromatographic technique using Sephadex G-50 in conjunction with centrifugation has been reported for the bound—free separation in the radioimmunoassay of oestradiol [11].

Although the majority of the techniques described are easy to perform, the unstable equilibrium of the antigen—antibody complex is easily disrupted during the process of bound—free separation. The dextran-coated charcoal method, despite being quick and cheap, causes "stripping" of the antigen—antibody complex, which can be minimized by performing the assay in the cold [1]. Chemical precipitation techniques [3—5] have the disadvantage of precipitating free (unbound) antigen. The double-antibody technique is an expensive method if the second antibody is obtained commercially. Furthermore, it requires rigorous titration from batch to batch. It is also time-consuming, requiring in most instances 24 h to complete the second incubation [2].

The present report describes the development and the application of a bound—free separation technique, using Sephadex G-75 and gravitational flow, in the radioimmunoassay of prostaglandin $F_{2\alpha}$.

MATERIALS AND METHODS

[5,6,8,11,12,14,15(n)-^{3}H]$PGF_{2\alpha}$ ($^3HPGF_{2\alpha}$) of specific activity 150 Ci/mmol and radioactive concentration 0.1 mCi/ml was obtained from the Radiochemical Centre (Amersham, Great Britain). $PGF_{2\alpha}$-1—BSA antiserum was raised in rabbits [2]. Pure prostaglandin $F_{2\alpha}$ was donated by the Upjohn Company, Kalamazoo, MI, U.S.A.

Radioimmunoassay diluent (assay buffer) was a 0.01 *M* phosphate-buffered saline (pH 7.40) with 0.1% human gamma globulin (IgG; supplied by the Commonwealth Serum Laboratories, Parkville, Australia) and 0.1% sodium azide (NaN_3) as the bacteriostatic agent.

Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) was soaked in the assay buffer for a period of 12—24 h in a conical flask with a side-arm. The Sephadex columns consisted essentially of 2-ml disposable plastic syringes supplied by Pharma-Plast Australia (New South Wales, Australia). The base of each column was covered with a vyon porous disc of diameter equal to the internal diameter of the syringe barrel (Fig. 1).

The swollen Sephadex gel was deaerated by connecting the side-arm of the capped conical flask to an aspirator. The development of excessive negative pressure inside the flask is avoided, to prevent any rupture of swollen Sephadex beads. The removal of trapped air bubbles is necessary for the preparation of uniform Sephadex columns free of air bubbles. The Sephadex was layered in the column to a height of 4.0 cm. The top of the bed was covered with a vyon disc. To carry out the elution of bound and free fractions by gravitational flow, it was found necessary to increase the effective volume of the space above the Sephadex bed. This was achieved by attaching a plastic tube 5.5 cm long to the top of the 2-ml syringe. One hundred columns were prepared and mounted on a stand. The stand was constructed

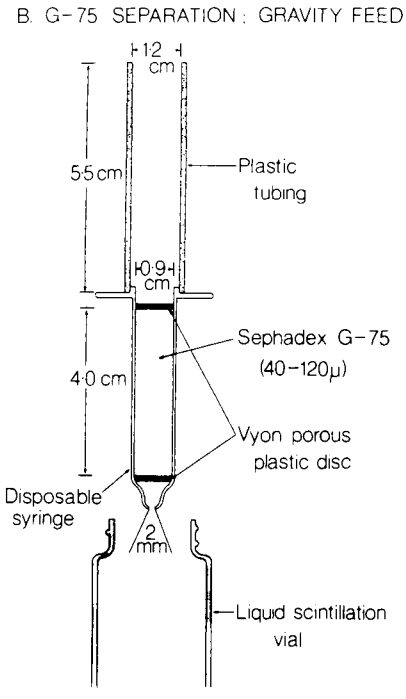


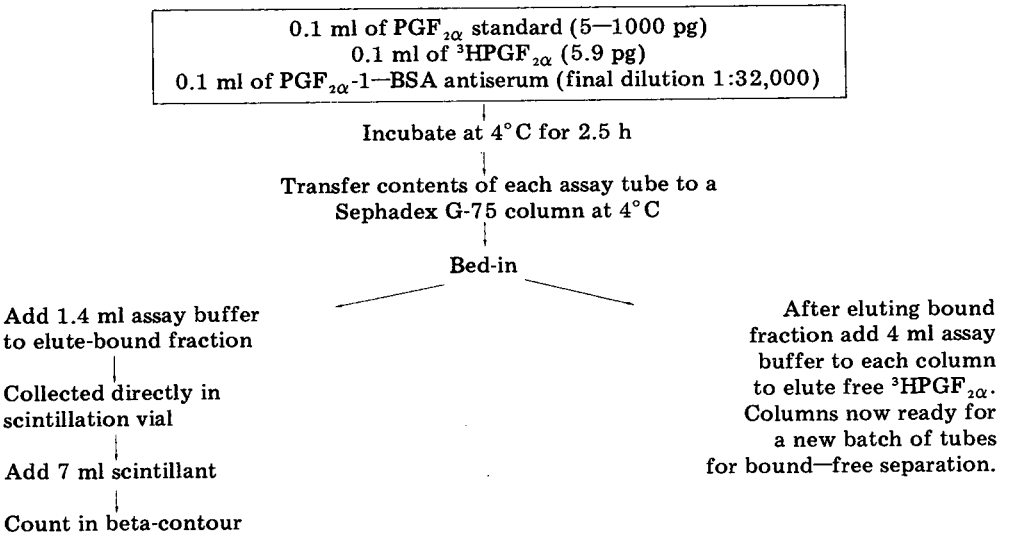
Fig. 1. Schematic diagram of a Sephadex G-75 minicolumn used in the "bound—free" separation of prostaglandin radioimmunoassay.

to the dimensions of a box of 100 scintillation vials purchased from the Radiochemical Centre (Australia) Ltd. This design allows the tip of the columns to point directly into the mouth of scintillation vials. When the columns were not in use for more than one week, they were individually placed in vials containing phosphate-buffered saline at 4°C. This prevents drying up the gel and the development of cracks in the bed. $^3HPGF_{2\alpha}$ solution in assay buffer was prepared by diluting 5 μl of the stock $^3HPGF_{2\alpha}$ in 25 ml of assay buffer. This solution, when used at 100 μl volume per assay tube, yielded 5.5 pg of $^3HPGF_{2\alpha}$. $PGF_{2\alpha}$-1—BSA antiserum was diluted in assay buffer and used in 100 μl volume per assay tube to yield a final dilution of 1 : 32,000 in a $PGF_{2\alpha}$ incubation volume of 300 μl.

Equal volumes of $^3HPGF_{2\alpha}$, diluted $PGF_{2\alpha}$-1—BSA antiserum and assay buffer were mixed in a glass vial. The reaction mixture was incubated at 4°C for a minimum period of 2.5 h. The time for $PGF_{2\alpha}$ radioimmunoassay to attain equilibrium had been previously established by a time-course study. A 300-μl volume of the reaction mixture was applied to the Sephadex G-75 column and allowed to bed-in. The void volume was collected into a scintillation vial. This was followed by the addition of 1.2 ml of assay buffer to the column and the void volumes were collected in fractions of four drops per vial. This procedure was repeated with increasing volume of eluting buffer. The elution pattern was studied in duplicate for each volume of eluting buffer studied. To the vials containing the eluted fractions, 7 ml of a toluene—

Triton X 100 (1.9:1) scintillant containing PPO and POPOP were added. The vials were counted in a beta-spectrometer (Searle Isocap/300, 6868 liquid scintillation system) after 1 h of dark equilibration.

Having established the appropriate volume of assay buffer which eluted only the antibody-bound fraction, standard curves for prostaglandin $F_{2\alpha}$ were established. $PGF_{2\alpha}$ standards (5, 10, 25, 50, 100, 300, 500, and 1000 pg per 100 μl per assay tube) were prepared in assay buffer. The preparation of standard curve tubes and the incubation procedure were carried out as illustrated in the flow diagram in Scheme 1.



Scheme 1.

RESULTS

The volume of buffer which eluted only the bound fraction completely, but left the free $^3HPGF_{2\alpha}$ held by Sephadex beads, was determined by studying the effect of sequential increase in elution volume on the elution pattern. Each fraction eluted from the column in this study was four drops per vial. Addition of 1.4 ml of buffer to the column resulted in the elution of only the bound fraction (Fig. 2). It is evident that any increase in elution volume above 1.4 ml resulted in the elution of "free" as well. This was demonstrated by the occurrence of a second wave of counts when 1.5, 1.6, 1.7, and 1.8 ml eluting volume was used (Fig. 2).

The fraction eluted in the first 1.4 ml was, in fact, antibody-bound and was confirmed by carrying out an elution profile in the presence and in the absence of $PGF_{2\alpha}$ antiserum (Fig. 3). The resolution of the elution profile was enhanced by collecting two drops per fraction per vial. It could be seen that in the absence of $PGF_{2\alpha}$ antiserum (open circles in Fig. 3) no $^3HPGF_{2\alpha}$ was eluted in the first 1.4 ml elution volume. However, the addition of 4 ml of buffer resulted in the elution of $^3HPGF_{2\alpha}$. The elution of this free fraction

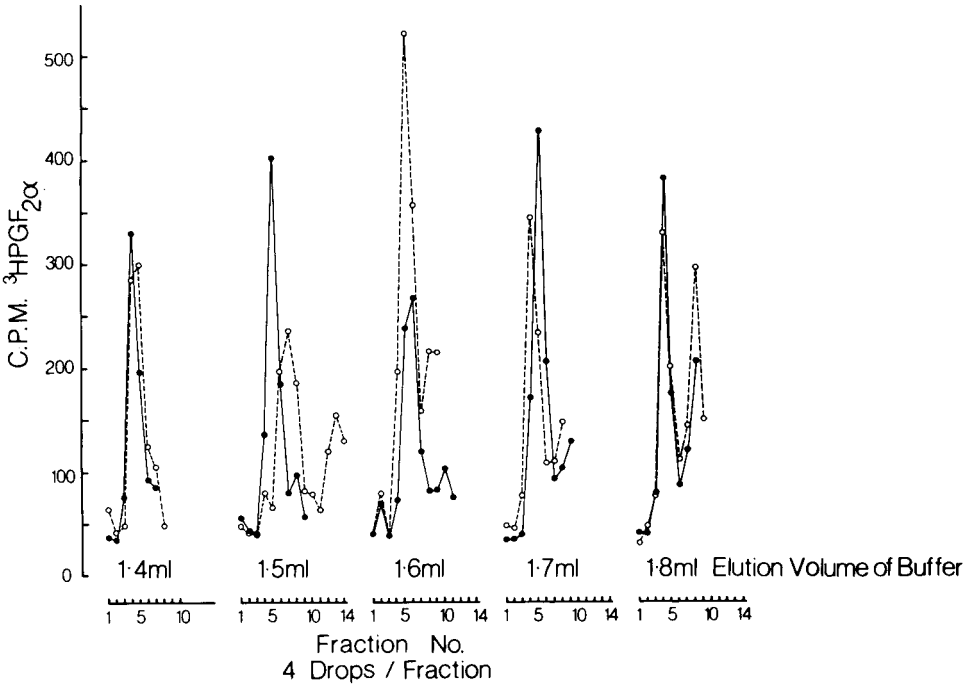


Fig. 2. Determination of the optimum volume of buffer for the elution of antibody-bound fraction in the radioimmunoassay of prostaglandin $F_{2\alpha}$. Each fraction collected represents four drops. Elution profiles at each elution volume were studied in duplicate. Closed circles represent column 1; open circles represent column 2. Note that the elution of bound fraction takes place within first 7 or 8 fractions.

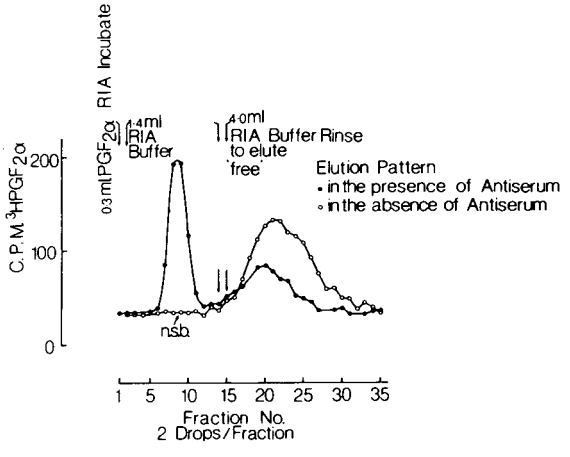
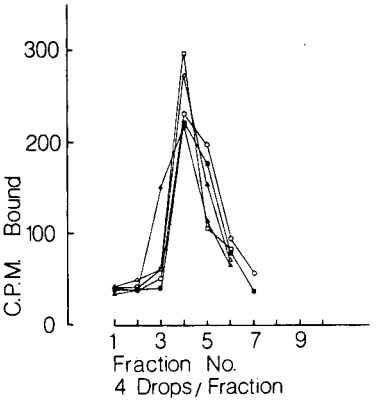


Fig. 3. Elution profile of antibody bound and free $^3HPGF_{2\alpha}$. Elutions were carried out both in the presence of $PGF_{2\alpha}$—BSA antibodies (closed circles) and in the absence of antibodies (open circles). Fractions were collected at the rate of two drops per vial.

was complete, indicating that the columns were ready for further use.

The reproducibility of the elution of antibody-bound fraction was studied from column to column. It can be seen from Fig. 4 that the elution of bound $^3HPGF_{2\alpha}$ was similar provided the column dimensions and Sephadex bed volume were identical.

Based on these observations, standard curves of $PGF_{2\alpha}$ radioimmunoassay were established. The content of each standard curve tube was transferred to a column using a pasteur pipette. One pasteur pipette per set of tubes was adequate to obtain reasonable within-standard variability. The bound fraction was eluted with 1.4 ml of assay buffer using an Oxford pipetter. Fig. 5 shows the standard curve of $PGF_{2\alpha}$ radioimmunoassay. Each point of the standard curve represents the mean of fifteen individual standard curves. The standard curve was of a high degree of reproducibility (intra-assay CV % = 6.45 ± 0.54, I.S.D.; inter-assay CV % = 9.68 ± 0.96, I.S.D.) and sensitivity (5.0 pg per tube per 0.3 ml reaction mixture was different from 0 tube at the 95% confidence limit) with a working range of 5—500 pg.



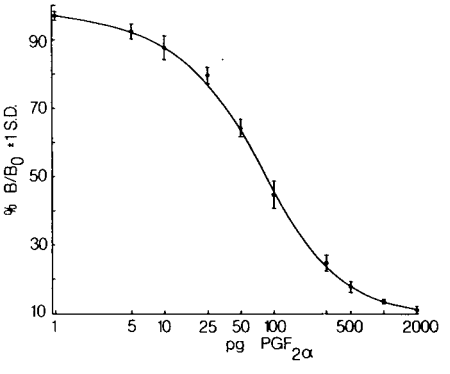


Fig. 4. Reproducibility of "bound—free" separation on Sephadex G-75 columns using $PGF_{2\alpha}$ radioimmunoassay system. Note that the elution of bound fraction using 0.3 ml incubation volume and 1.4 ml of eluting buffer is complete within 8 fractions.

Fig. 5. Standard curve for prostaglandin $F_{2\alpha}$ radioimmunoassay. Each point is the mean of fifteen individual standard curves. "Bound—free" separation was achieved by means of Sephadex G-75 gel chromatography at 4°C.

DISCUSSION

The method of gel chromatography by gravitational flow is a convenient technique for the separation of antibody-bound fraction from the free in the radioimmunoassay of prostaglandins. The use of gel chromatography in conjunction with centrifugation in the radioimmunoassay of oestradiol reported earlier [11] may not be a suitable technique because of the development of cracks in the Sephadex bed during centrifugation. In the method described in the present report no such problem was encountered and the columns were ready for use for another 100 tubes at the end of the wash with

4 ml of buffer to remove free radioactivity. Unlike the earlier report of Fránek and Hruška [11], who used 10 ml of elution buffer to remove free ligands at the end of the separation of antibody-bound fraction, in the technique reported in this paper a volume of buffer as low as 4 ml was found to be sufficient. This is a considerable saving on the quantity of buffer used, besides allowing repeated use of the columns.

The use of gel chromatography has the advantage of allowing the separation on the basis of molecular size alone [12]. The low molecular weight $PGF_{2\alpha}$ is taken up by the swollen Sephadex beads through the pores on its surface. The large molecular weight immunoglobulins with the attached (ionic bond) ligand are larger than the largest pores of the swollen Sephadex. Because the immunoglobulins are above the exclusion limit, they cannot penetrate the gel particles and therefore pass through the bed in the liquid phase between the Sephadex beads. This is the reason for the smaller volume of buffer which elutes the bound fraction and for the bound fraction to be eluted first. Smaller molecular weight $PGF_{2\alpha}$ retained within the gel particles requires a much larger volume to be eluted out of the column.

Bound—free separation techniques by chemical precipitation have yielded adequate standard curves [3—5]. However, these techniques are beset with the problem of non-specific precipitation of unbound $^{3}HPGF_{2\alpha}$ [3]. The double-antibody technique [2] requires rigorous titration of the second antibody for each assay and for each dilution of first antibody. This may prove costly unless the second antibody is raised in the same laboratory. Furthermore, this technique is time-consuming, requiring at least 24 h for the separation. The dextran-coated charcoal technique [1], although a cheap and commonly used method, has the distinct disadvantage of "stripping" the bound fraction.

The main advantages of the gel chromatographic technique presented in this paper over the existing techniques are that it is rapid and simple to operate. The columns can be used repeatedly over a considerable period of time. The bed volume can be maintained constant by adding freshly soaked Sephadex G-75 for any lost by leaching during the washing procedure.

Unlike previous methods, the gel chromatographic method offers the advantage of maintaining the composition of the reaction mixture throughout the separation procedure, which minimises dissociation of the antigen—antibody complex. The design of the stand on which columns were placed facilitates the collection of the bound fraction directly into the scintillation vials, which enables the measurement of the antibody-bound radioactivity. The stand was constructed to the measurements of a standard scintillation vial box supplied by Radiochemical Centre (Australia) Ltd. A total of 100 tubes could be subjected to bound-free separation in 30 min.

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POLYMORPHISM OF URINARY 4-HYDROXYPROLINE-CONTAINING POLYPEPTIDES

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SUMMARY

Using molecular sieve chromatography on Bio-Gel P-2 and then on Bio-Gel P-30, hydroxyproline-containing urinary polypeptides (molecular weight > 1500 daltons) were separated into eight fractions. The three main fractions were separated further on phosphocellulose giving seven, nineteen and twelve peaks, respectively, each containing 4-hydroxyproline. Hypotheses about the origin of certain polypeptides are proposed, which take into account the sequence of type I collagen. Among these 38 polypeptides only one shows a quantitative variation in Paget's bone disease and was thus purified. It consists of equal amounts of glycine, proline and 4-hydroxyproline. This particular polypeptide may originate from the N-terminal propeptide of type I collagen.

INTRODUCTION

4-Hydroxyproline (4-Hyp)-containing peptides derive mostly from collagen metabolism, except for the small amounts that are present in elastin, the C_1 q fraction of complement and acetylcholine esterase. General improvements in techniques have resulted in a deeper knowledge of peptides from human urine [1—12]. Important progress has been made in the study of collagen [13, 14] and at present five different types have been described: types I, II, III, IV and AB. Several of the major metabolic steps of collagen synthesis have been elucidated and the precursors preprocollagen and procollagen have been isolated. The amino acid sequence of the N-terminal type I collagen propeptide has been established, showing the presence of five residues of 4-Hyp [15]. These results led us to re-investigate the urinary polypeptides in order to reveal whether or not some of the peptide population

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originates from the pro-peptides and if they can be distinguished from the remainder derived from collagen metabolism.

We describe in this paper experiments showing the number of hydroxyproline-containing polypeptides and initial results concerning the structure and origin of one of them.

EXPERIMENTAL

All reagents were purchased from Merck (Darmstadt, G.F.R.), except for *p*-dimethylaminobenzaldehyde (Carlo Erba, Milan, Italy) and Chloramine T (Prolabo, Paris, France). Any other sources are mentioned later.

In order to obtain sufficient amount of peptides and to simplify the problems of separation we used urinary polypeptides pooled from five patients suffering from Paget's bone disease. The 4-Hyp urinary output consisted of between 3120 and 6350 μmol per 24 h. A 500-ml volume of urine was

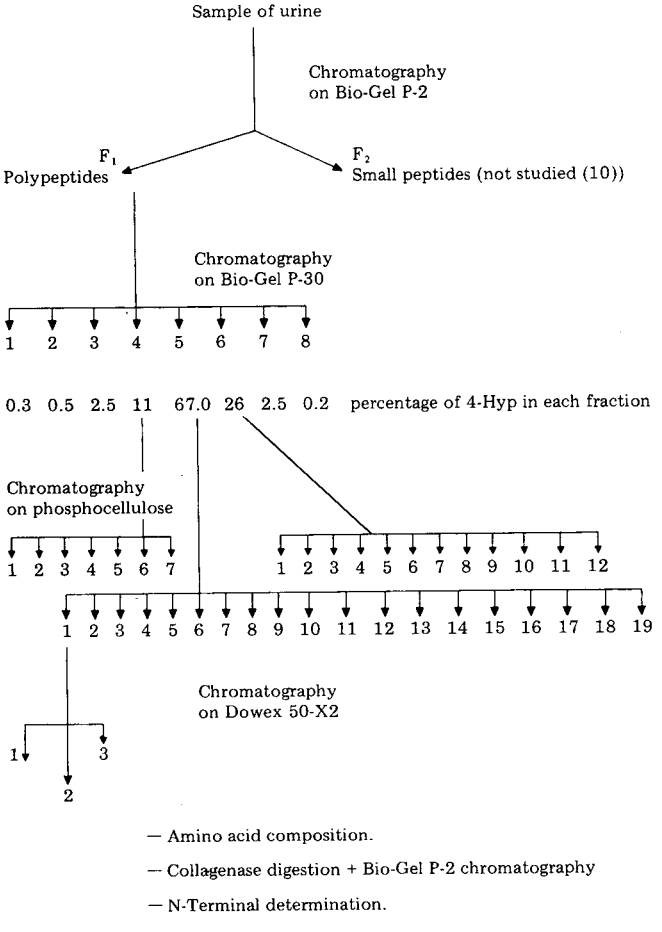

Fig. 1. Analytical procedure for the study of 4-hydroxyproline-containing urinary polypeptides.

used for the study. Previous work had shown a reproducible pattern obtained with different subjects [10, 16]. The experimental procedure is outlined in Fig. 1. The urine sample was lyophilized and dissolved in 50 ml of 0.1 mol/l acetic acid. Aliquots of 5 ml were layered on to a 90 × 2.6 cm column of Bio-Gel P-2 (50—100 mesh) (Bio-Rad Labs., Richmond, CA, U.S.A.) and eluted with the same solution at a flow-rate of 60 ml/h. The effluent was collected in 5-ml fractions with a Gilson automatic collector. 4-Hyp and all amino acids were measured in each tube after alkaline hydrolysis using an automatic system [16]. The material eluted in the void volume and recovered from the Bio-Gel column was lyophilized and dissolved in 10 ml of 0.1 mol/l acetic acid and layered on to a 90 × 2.6 cm column of Bio-Gel P-30 (100—200 mesh) (Bio-Rad Labs.). The analytical procedure was similar to that described above. The main fractions recovered were lyophilized and dissolved in an acetate buffer (0.01 mol/l, pH 3.8) and analysed on a 30 × 1.6 cm column of phospho-cellulose P11 (Whatman, Maidstone, Great Britain) [17]. The column was eluted with a solution of increasing ionic strength obtained with a four-chamber Varigrad (Boskamp, Strasbourg, France). The first three compartments contained buffered solutions of sodium acetate (0.01 mol/l, pH 3.8) and the last contained in addition 1 mol/l sodium chloride. The total volume of solution was 800 ml.

The peptides were eluted at a flow-rate of 60 ml/h and the eluate was collected with an automatic collector in 5-ml fractions. Some of the peptides were purified by chromatography on a 30 × 1.6 cm column of Dowex 50-X2 (200—325 mesh) (Bio-Rad Labs.) and eluted with 0.5 mol/l acetic acid. Clostridium collagenase (Worthington, Freehold, NJ, U.S.A.) was used under the conditions described by Krane et al. [2]. The reaction products were separated on Bio-Gel P-2 as described above.

The amino acid compositions were determined for peptides hydrolysed with 5.6 mol/l hydrochloric acid at 105°C for 24 h. The procedure involved the use of a Beckman Multichrom B analyser as described previously [18]. The determination of N-terminal amino acids were made according to the procedure of Gray and Hartlay [19] using silica gel thin-layer plates (Merck, Darmstadt, G.F.R.). 4-Hydroxyproline was also determined as described previously [20]. The percentage of 4-Hyp in each peak was deduced from the area calculated on the chromatogram.

RESULTS

The behaviour of urinary polypeptides on Bio-Gel P-2 is illustrated in Fig. 2. We term polypeptides the molecules eluted in the void volume on the Bio-Gel P-2 column corresponding to an apparent molecular weight of 1500 daltons. The separation of the polypeptides (fraction F1) on Bio-Gel P-30 is shown in Fig. 3. The peaks were numbered from P30-1 to P30-8. Most (95%) of the total 4-Hyp is present in the three peaks P30-4, -5 and -6. These fractions were submitted to complementary fractionation procedures.

Each of the three peaks were analysed by chromatography on phospho-cellulose and showed different patterns, as illustrated in Fig. 4a, b and c, respectively. The amount of hydroxyproline in each peak was calculated

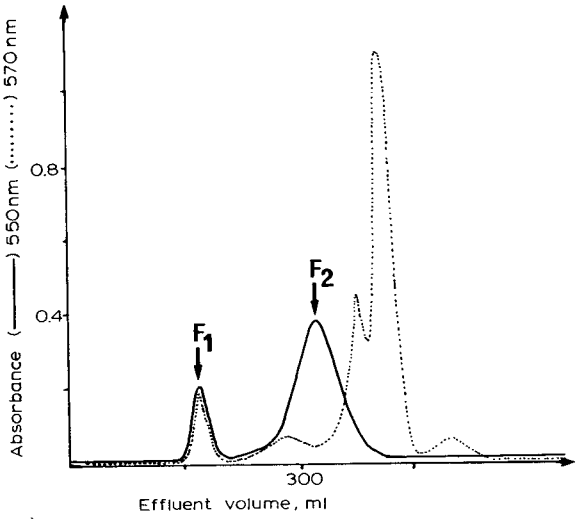

Fig. 2. Chromatography on Bio-Gel P-2 of one sample of urine. Solid line, automatic detection of 4-Hyp in each fraction; broken line, automatic detection of all peptidic molecules with ninhydrin. The first fraction, F_1, is then fractionated on Bio-Gel P-30.

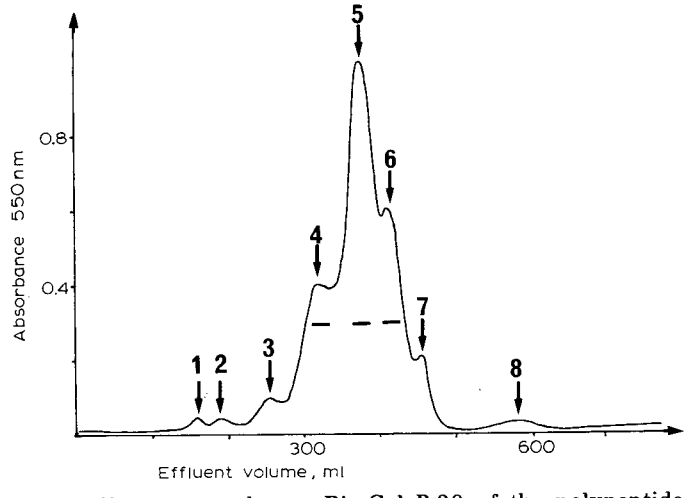

Fig. 3. Chromatography on Bio-Gel P-30 of the polypeptide recovered from the Bio-Gel P-2 column. 4-Hyp is detected in each fraction with an automatic device. Peaks 4, 5 and 6 are separated by chromatography on phosphocellulose.

as a percentage of total polypeptide 4-Hyp [10]. Between normal subjects and patients with Paget's bone disease, only one such fraction showed quantitative variations and was identified in this system as the fraction P30-5-1. This particular fraction contained high levels of glucosamine and galactosamine in addition to amino acids and was purified by chromatography on Dowex 50-X2 (Fig. 5). The purified peptide was digested with bacterial collagenase and its amino acid composition is given in Table II. The amino acid compositions of all peptides are given in Tables I—III.

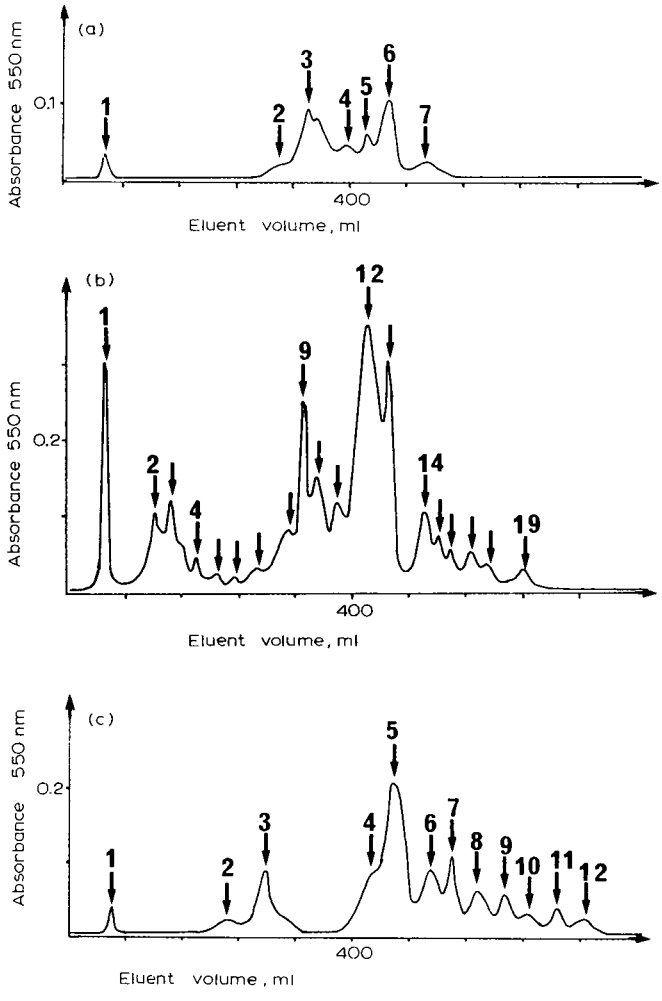

Fig. 4. (a) Elution on phosphocellulose of polypeptides contained in fraction P30-4. 4-Hyp is detected in each fraction. (b) Elution on phosphocelullose of polypeptides contained in fraction P30-5. (c) Elution on phosphocellulose of polypeptides contained in fraction P30-6.

DISCUSSION

About one fifth of the total urinary 4-Hyp is present in polypeptide sequences [21]. The technique we have used permits the separation of at least 38 fractions of large-sized polypeptides. In fact, we have not demonstrated the homogeneity of each fraction and it is possible that some of them may contain several different molecules. As a result of the large number of different fractions, each peptide represents only a small percentage (0.1—8%) of the total polypeptide 4-Hyp. These quantitative results may explain why previously only a small number of the polypeptidic fractions have been isolated, viz., ten by Krane et al. [2] and more recently only six by Dubovsky

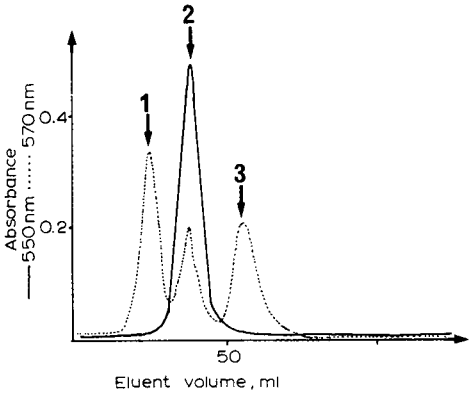

Fig. 5. Separation of the peak P30-5-1 on Dowex 50-X2. Solid line, specific detection of 4-Hyp; broken line, detection of all molecules with ninhydrin after alkaline hydrolysis. Peak 2 contains the polypeptide $(Gly—Pro—4\text{-}Hyp)_n$.

TABLE I

AMINO ACID COMPOSITIONS OF POLYPEPTIDES CONTAINED IN FRACTION P30-4 AND SEPARATED BY CHROMATOGRAPHY ON PHOSPHOCELLULOSE (FIG. 4a)

The fraction P30-4 was obtained during Bio-Gel P-30 chromatography (Fig. 3). Results are given as residues per 100 residues.

Amino acid	Peak						
	1	2	3	4	5	6	7
4-Hyp	2.6	9.9	8.5	7.8	7.8	8.8	5.0
Asp	14.2	9.3	7.0	5.9	6.4	8.7	7.5
Thr	11.9	7.3	3.7	4.1	3.5	4.0	4.4
Ser	8.3	5.7	3.9	3.7	4.1	5.9	6.5
Glu	9.1	8.9	7.7	8.9	9.3	10.1	12.0
Pro	14.1	9.8	12.0	10.0	9.8	7.7	8.1
Gly	10.2	21.9	27.7	29.6	30.1	28.3	27.4
Ala	8.6	8.7	12.2	13.3	11.9	9.3	7.5
Val	7.6	3.7	2.7	2.8	2.4	1.9	1.8
Met	—	—	—	—	—	—	—
Ile	2.2	1.7	1.1	0.8	0.6	0.6	0.4
Leu	5.8	3.1	1.9	1.9	1.6	1.5	1.9
Tyr	1.3	0.5	—	0.8	0.7	—	—
Phe	3.1	1.5	0.7	0.9	0.9	0.8	1.2
Hyl	—	0.4	0.6	—	—	0.7	0.6
Lys	—	4.5	6.9	4.6	5.5	4.2	6.7
His	0.5	1.4	0.9	1.2	0.9	1.8	1.8
Arg	0.8	1.6	2.2	3.7	4.7	5.8	7.4
Glc-NH_2	48.7	15.1	26	3.5	1.5	0.9	—
Gal-NH_2	16.1	2.5	—	—	—	—	—

TABLE II

AMINO ACID COMPOSITIONS OF POLYPEPTIDES CONTAINED IN FRACTION P30-5 AND SEPARATED BY CHROMATOGRAPHY ON PHOSPHOCELLULOSE (FIG. 4b)

The fraction P30-5 was obtained during Bio-Gel P-30 chromatography (Fig. 3). Results are given as residues per 100 residues.

Amino acid	Peak																			
	1	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
4-Hyp	13.3	32.4	13.3	19.6	17.2	11.1	9.7	7.8	9.2	10.3	10.6	11.7	9.3	11.0	9.5	9.0	8.0	9.4	8.5	8.8
Asp	11.6	1.0	10.7	4.8	5.5	6.2	7.6	8.6	6.9	10.8	11.4	9.1	9.8	9.5	7.7	7.5	6.9	8.7	8.5	8.8
Thr	7.0	0.6	4.4	3.5	5.4	9.1	7.3	5.8	4.2	2.5	3.0	3.1	2.1	3.2	3.1	2.9	3.0	3.0	3.5	3.8
Ser	6.3	1.2	9.1	6.5	5.1	4.5	5.5	5.1	4.5	3.5	3.6	3.3	3.1	4.7	5.0	4.8	4.7	4.3	4.8	4.2
Glu	6.6	—	5.0	5.0	5.4	7.1	9.2	9.2	9.0	6.1	6.7	8.4	8.8	10.1	10.5	10.5	10.6	10.8	11.1	11.4
Pro	21.3	31.2	10.7	27.1	21.6	20.3	14.4	12.8	12.4	4.9	8.1	9.8	10.1	8.4	8.0	9.0	9.6	6.7	5.1	4.8
Gly	18.3	33.6	26.2	26.8	28.9	24.0	24.4	24.4	26.3	31.1	29.9	29.4	31.6	29.9	31.3	29.9	29.1	29.1	27.7	25.7
Ala	3.8	—	8.6	2.1	5.8	6.6	8.0	9.9	10.9	18.2	9.6	12.5	14.2	8.6	8.6	9.2	9.8	6.9	6.4	2.5
Val	3.9	—	4.3	0.7	1.9	2.1	2.5	3.3	3.4	0.8	1.9	2.0	1.8	2.3	1.3	1.5	1.6	1.4	2.2	1.3
Met	0.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ile	0.8	—	1.2	0.2	0.4	0.6	0.8	1.1	1.0	0.5	0.6	0.4	0.6	0.6	0.5	0.5	0.4	0.6	0.9	1.0
Leu	2.5	—	2.7	1.2	1.9	4.0	3.2	3.1	3.0	1.7	1.6	0.9	0.6	1.2	0.9	0.8	0.9	1.5	2.1	1.9
Tyr	0.7	—	1.2	—	—	—	0.6	0.7	0.4	0.3	0.2	0.2	—	—	—	—	—	—	—	—
Phe	1.4	—	2.4	—	0.4	0.6	0.9	1.1	1.3	0.5	0.5	0.4	—	—	—	—	—	—	0.9	—
Hyl	—	—	—	—	—	—	0.6	—	0.4	0.5	0.7	0.6	0.6	0.8	0.9	1.2	1.5	2.1	2.2	1.1
Lys	1.3	—	—	2.3	0.4	3.0	3.3	4.2	4.9	5.8	9.4	5.1	6.9	5.5	5.7	5.7	5.7	6.7	6.8	12.3
His	0.2	—	—	—	—	—	0.6	0.8	0.6	0.4	0.6	0.6	0.6	0.8	1.1	1.1	1.1	1.9	2.4	3.6
Arg	0.3	—	—	—	0.4	0.8	1.1	2.0	1.6	2.0	1.5	2.4	0.7	3.3	5.9	6.4	7.2	6.9	6.8	8.8
Glc-NH_2	34		32																	
Gal-NH_2	9.4		—																	

*Amino acid composition of the fraction P30-5-1 purified on Dowex 50-X2.

TABLE III

AMINO ACID COMPOSITIONS OF POLYPEPTIDES CONTAINED IN FRACTION P30-6 AND SEPARATED BY CHROMATOGRAPHY ON PHOSPHOCELLULOSE (FIG. 4c)

The fraction P30-6 was obtained during Bio-Gel P-30 chromatography (Fig. 3). Results are given as residues per 100 residues.

Amino acid	Peak											
	1	2	3	4	5	6	7	8	9	10	11	12
4-Hyp	13.2	9.3	14.9	11.9	16.1	9.3	9.5	9.0	9.9	10.7	8.7	5.7
Asp	10.9	10.7	7.5	11.4	9.0	9.4	12.0	9.6	9.4	11.1	11.8	9.7
Thr	4.8	6.4	3.1	3.1	2.4	2.1	3.1	3.6	3.3	2.7	2.8	3.3
Ser	8.8	5.7	3.6	4.1	3.5	8.2	5.5	5.6	6.4	4.7	5.0	5.2
Glu	17.7	14.0	9.7	11.0	9.5	12.8	11.1	11.6	12.7	11.7	13.0	12.4
Pro	4.7	8.5	14.7	8.0	11.3	7.0	4.7	7.7	5.8	4.0	3.4	3.2
Gly	25.7	24.0	31.4	26.8	29.0	29.8	27.6	26.9	25.0	24.1	23.0	24.0
Ala	6.3	6.7	5.0	6.5	8.4	4.1	8.0	6.8	7.6	7.9	5.9	7.4
Val	1.4	3.4	1.2	2.7	1.0	1.1	2.0	1.5	1.4	2.2	1.7	1.8
Met	—	—	—	—	—	—	—	—	—	—	—	—
Ile	0.7	1.0	0.4	0.7	—	0.4	0.5	0.4	—	—	1.3	1.3
Leu	2.5	4.0	1.9	2.8	1.0	1.2	1.5	1.2	1.7	2.7	3.1	3.2
Tyr	1.2	—	—	1.0	—	—	—	—	—	—	—	—
Phe	0.7	1.0	—	0.5	—	—	—	—	—	—	—	—
Hyl	—	—	0.3	0.5	0.8	1.6	1.4	1.3	1.2	1.5	1.1	1.7
Lys	1.3	3.3	2.6	5.8	3.8	9.3	7.0	6.7	6.3	8.0	9.1	9.5
His	—	—	—	0.3	—	0.5	1.1	1.1	1.3	2.4	2.1	2.8
Arg	—	1.6	3.7	2.5	4.2	3.1	5.1	7.0	7.9	6.4	8.0	8.8
Glc-NH_2	3.5											
Gal-NH_2	3.5											

and Meyer [22]. None of them have been either purified or characterized. The techniques we used here are not adequate for the rapid or complete isolation of these components.

Amino acid analyses of fractions P30-5-3 and P30-5-4 showed high levels of 4-Hyp, Pro and Gly. Fractions P30-5-9 and P30-5-12 are characterized by their content of 4-Hyp, Pro, Gly and Ala. These results correspond to several amino acid sequences in types I and III collagen. Half of the fractions contain a small amount (less than 1.7%) of Hyl and none contain 3-Hyp. It is probably for this reason that basement membrane collagen metabolism results in very few polypeptides. The separation patterns we have obtained for the polypeptides from different pathological situations were identical [12], except for those from patients suffering from Paget's bone disease. In these instances we found that the fraction labelled P30-5-1 increased significantly. It contains 4.1 ± 0.2% (n = 12) of the polypeptidic 4-Hyp. In normal subjects (n = 15) this fraction contains only 2.3 ± 0.3% of the polypeptidic 4-Hyp. This fraction was purified by chromatography on Dowex 50-X2 and separated into three peaks as shown in Fig. 5. The second peak eluted contained essentially three amino acids, Gly, Pro and 4-Hyp, corresponding

to the probable sequence $(Gly—Pro—4\text{-}Hyp)_n$. This polypeptide was digested with bacterial collagenase. No N-terminal amino acid could be identified. The molecular weight and amino acid composition of this component plus the quantitative variation associated with Paget's bone disease supported the concept that it may originate from the amino terminal extension of procollagen.

Further studies using labelled proline and antibodies might provide further information about the metabolic pathway and the physiopathological regulation mechanism of the different types of collagen.

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DETECTION OF THE CHANGES IN PROTEIN DISTRIBUTION OF RAT PLASMA INDUCED BY CARBON TETRACHLORIDE ADMINISTRATION BY MEANS OF TWO-DIMENSIONAL ELECTROPHORESIS

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SUMMARY

The changes in rat plasma protein distribution after carbon tetrachloride administration were examined using two-dimensional electrophoresis, utilizing isoelectric focusing in polyacrylamide gel in the first dimension and pore gradient polyacrylamide gel electrophoresis in the second dimension. Drastic changes in amount of protein were observed at more than 20 spot positions including those of transferrin, Gc-globulin and low-density lipoprotein. The time course of the changes was examined, and the most drastic changes were observed at 2 days after carbon tetrachloride administration.

INTRODUCTION

The degeneration of rat liver induced by carbon tetrachloride has been studied biochemically to understand the function of liver and clinically as a model system for human medicinal poisoning. Studies on the mechanism of fatty accumulation and fall in lipoprotein formation have been reported [1—4]. However, as for the changes in plasma protein distribution which must accompany the liver damage, little is known except for a decrease in the albumin/globulin ratio and an increase in the activities of some intracellular enzymes, due to poor resolution of the analytical techniques.

Recently, we described a two-dimensional electrophoretic technique which did not employ denaturing agents, and showed that human plasma proteins

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could be resolved into about 250 spots [5, 6]. Since the technique does not employ sodium dodecyl sulfate or urea throughout the electrophoretic run, it is suited for the analysis of mixtures of soluble proteins maintaining their native physicochemical properties [7, 8] and their biological activities [9].

In the present report we show that drastic changes in the distribution of rat plasma proteins induced by carbon tetrachloride administration could be analyzed by means of the two-dimensional electrophoretic technique. The results showed that the degeneration of rat liver after carbon tetrachloride administration could be followed by examining the plasma protein distribution patterns.

MATERIALS

Reagents

Ampholines (pH 3.5—10 and pH 3.5—5) were obtained from LKB Produkter (Bromma, Sweden). Acrylamide, N,N'-methylenebisacrylamide (both special grade for electrophoresis), glycine, Tris base, and ammonium persulfate were from Wako Pure Chemical Industries (Tokyo, Japan). N,N,N',N'-Tetramethylethylenediamine and Coomassie brilliant blue R-250 (both special grade for electrophoresis) were from Nakarai Chemicals (Kyoto, Japan). Heparin was obtained from Kodama (Tokyo, Japan).

Plasma samples

Male rats (Wistar strain, 18 weeks) weighing approximately 350 g were used. Food was withdrawn 12 h before carbon tetrachloride feeding. Carbon tetrachloride, 20% (v/v) in liquid paraffin, was introduced into the stomach under light ether anesthesia by intubation at a dose of 0.5 ml per 100 g of body weight. At scheduled time intervals 5 ml of blood were taken from the descending aorta of each rat with a disposable syringe, the inside of which had been coated with heparin solution. The blood was centrifuged for 15 min at 3000 *g*. Sucrose was added to the supernatant plasma to give a concentration of 40% (w/v), and the plasma sample was stored at −20° C.

METHODS

Two-dimensional electrophoresis in the absence of denaturing agents

The technique of two-dimensional electrophoresis in the absence of denaturing agents was described previously [5, 6]. First-dimension isoelectric focusing was performed on gel columns 14 cm × 0.5 cm I.D. A 4% acrylamide (0.2% bisacrylamide) solution containing 2% Ampholine pH 3.5—10, 0.5% Ampholine pH 3.5—5, and 0.05% ammonium persulfate, was poured into a glass tube. Gelling occurred in about 20 min. The electrode solutions were 0.04 *M* NaOH (cathode) and 0.01 *M* phosphoric acid (anode). An overlay solution (2% Ampholine pH 3.5—10 and 10% sucrose, 50 μl) was layered on top of the gel columns and then plasma samples (50 μl) were applied under the overlay solution. Electrophoresis was run at 2 mA constant current for 40 min and then at 460 V constant voltage for 20 h at 3° C. After electrophoresis, a rubber bulb was placed on top of the tube and the gel was pushed out and

placed on top of the second-dimension slab gel without equilibration.

Second-dimension gradient polyacrylamide gel electrophoresis was performed with a slab gel apparatus which forms a slab gel of 12 cm long, 16 cm wide, and 0.4 cm thick. A 4—21% acrylamide linear gradient (0.2% bisacrylamide) containing a 0—10% sucrose gradient and a 0.05—0.025% ammonium persulfate gradient was poured in about 50 min at 4°C. Gelling occurred in about 2 h in a water-bath at 30°C. The gradient gel buffer was 0.14 *M* Tris·HCl (pH 8.9) and the electrode buffer was 0.05 *M* Tris—0.38 *M* glycine (pH 8.3). Electrophoresis was run at 36 mA constant current for 20 h.

Measurement of the pH gradient

The isoelectric focusing gel was duplicated for each sample; one was cut into 10-mm sections which were placed in individual vials containing 2 ml of distilled water. These vials were capped and left to stand for 2 h, then the pH was measured on a pH meter.

Staining and destaining

The gel was stained overnight in 0.025% Coomassie brilliant blue R-250—7% (v/v) acetic acid—50% (v/v) methanol, and destained in 7% acetic acid at 80°C for 4 h, then in two changes of 7% acetic acid at room temperature for two days.

Photography

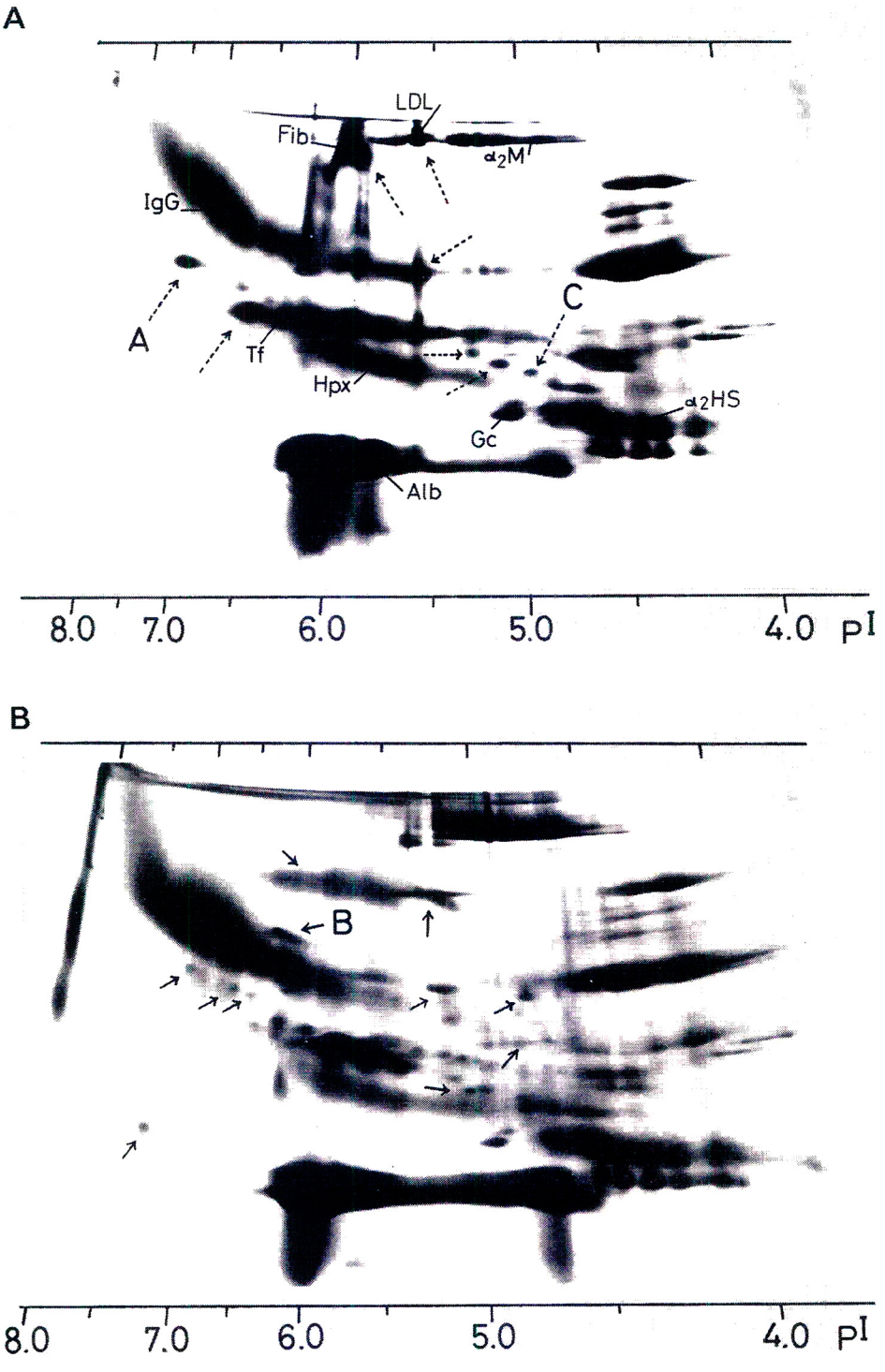
Photography was carried out by placing the gel on top of a viewing box positioned under a 35-mm reflex camera. A Toshiba Y-2 filter (Tokyo Shibaura Electric Co., Tokyo, Japan) was attached to the camera and Fuji minicopy II film (ASA 32, Fuji Photo Film Co., Tokyo, Japan) was used.

Densitometry

Densitometric quantitation of Coomassie blue-stained spots was carried out using a Shimadzu dual-wavelength thin-layer chromatographic (TLC) scanner CS-910 (Shimadzu Corp., Tokyo, Japan). Sample wavelength was set at 580 nm and reference wavelength was 750 nm. The densitometer was operated in "zig-zag scanning mode" and the protein amounts were quantitated by measuring the step height of the integrating signal.

RESULTS

Fig. 1A shows one of the two-dimensional electrophoretic patterns of normal rat plasma proteins, before administration of carbon tetrachloride. The protein distributions of several rat (Wistar strain) plasma samples were compared and the positions of the plasma proteins were quite reproducible. Major plasma proteins were located on the gel by comparing the patterns with those of human plasma proteins [6, 10]. Fig. 1B shows an example of the two-dimensional distribution of plasma proteins of a rat administered carbon tetrachloride (the plasma was taken 72 h after the administration). Drastic changes in protein distribution were observed. The locations of the proteins, the positions or spot areas of which apparently changed by the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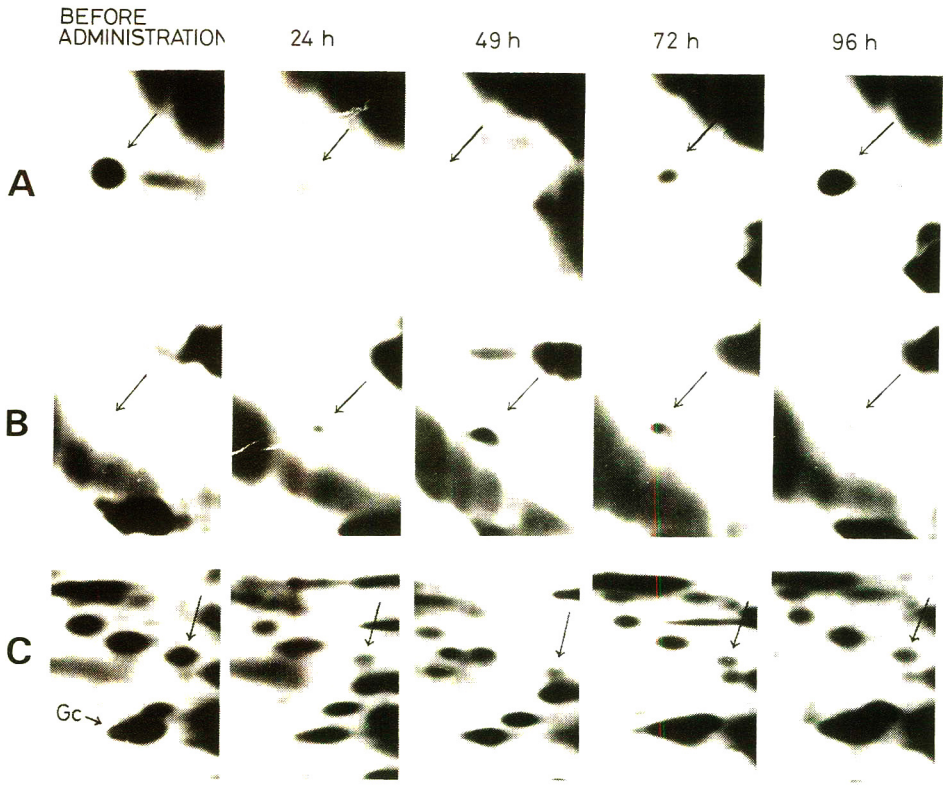

Fig. 2. Time course of the changes in the two-dimensional pattern of rat plasma proteins. A rat was administered carbon tetrachloride, and plasma samples at 0, 24, 49, 72, and 96 h after administration were analyzed by means of two-dimensional electrophoresis. Time-dependent changes at the gel sections around spot A (A), spot B (B) and spot C (C) are shown. Spot positions are indicated by arrows. Gc = Gc-globulin.

administration of carbon tetrachloride, are shown by arrows in the figure. The proteins that apparently decreased after administration are indicated by dotted arrows in Fig. 1A, and those that appeared are indicated by solid arrows in Fig. 1B. Some of the major plasma proteins, which were tentatively identified as IgG, albumin, hemopexin, and α_2HS-glycoprotein, were not affected by carbon tetrachloride administration. However, fibrinogen, transferrin, and Gc-globulin were apparently affected.

The time course of the changes in the two-dimensional pattern of plasma proteins was examined. A rat was administered carbon tetrachloride and plasma samples taken after 24, 49, 72, and 96 h were compared with plasma taken

Fig. 1. Two-dimensional electrophoresis of rat plasma proteins in the absence of denaturing agents. (A) Before administration of carbon tetrachloride, (B) 72 h after administration of carbon tetrachloride. Arrows with a dotted line in (A) indicate the spots that apparently decreased in area or disappeared after carbon tetrachloride administration. Arrows with a solid line in (B) indicate the spots that appeared after carbon tetrachloride administration. The positions of major plasma proteins were located on the gel by comparing the patterns of rat plasma proteins with those of human plasma proteins [10]. IgG = immunoglobulin G; Fib = fibrinogen; LDL = low density lipoprotein; α_2M = α_2-macroglobulin; Tf = transferrin; Hpx = hemopexin; Gc = Gc-globulin; α_2HS = α_2HS-glycoprotein; Alb = albumin.

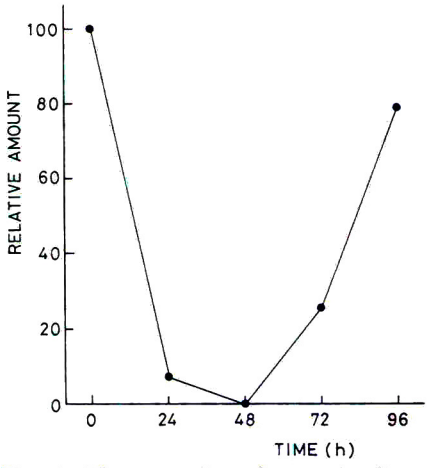

Fig. 3. The quantity of spot A after carbon tetrachloride administration as a function of time. Spots A on acrylamide slab gels were quantitated using a TLC scanner.

before administration. The time-dependent changes at the gel sections around spots A, B, and C (indicated by arrows in Fig. 1) are shown in Fig. 2, in which the positions of the three spots are indicated by arrows. The area of spot A which decreased after carbon tetrachloride administration, disappeared at 49 h after administration, then increased at 72 h, and almost recovered its original level at 96 h. In contrast, the protein of spot B appeared 24 h after administration and the spot area was maximum at 49 h, then it decreased with lapse of time. The area of spot C decreased at 24 h, but the spot did not disappear at 49 h, nor did it recover its original level at 96 h.

Densitometric quantitation of spot A was carried out with a Shimadzu CS-910 densitometer. Fig. 3 shows the time course of the quantity of spot A. A drastic fall in the amount of spot A at 49 h after carbon tetrachloride administration and its recovery at 96 h after administration were demonstrated.

DISCUSSION

One of the purposes of this study was to find out if the two-dimensional electrophoretic technique could contribute to diagnosing the progress of diseases. As an experimental disease, poisoning of rats with carbon tetrachloride was chosen since it has long been studied chemically and histologically [1—4]. As shown in Figs. 1—3 the technique can detect changes in plasma protein distribution caused by carbon tetrachloride. The time course of the increase or decrease of each protein spot also could be followed by means of the technique. The proteins affected by carbon tetrachloride administration can be divided into two types: type 1 includes proteins that disappeared (e.g. spot A in Fig. 2) or decreased in amount (e.g. spot C in Fig. 2) after carbon tetrachloride administration, and type 2 includes those that newly appeared (e.g. spot B in Fig. 2). These proteins specifically affected by carbon tetrachloride may be related to the mechanism of liver degeneration.

The electrophoretic technique employed isoelectric focusing in the first dimension and acrylamide pore gradient (4—21%) electrophoresis in the second

dimension. The technique uses no denaturing agent such as urea or sodium dodecyl sulfate throughout the run, thus equilibration of the first-dimension gel was not necessary. Further, when the isoelectric focusing gel was examined for the protein remaining after the second-dimension run, no Coomassie blue-stained band was observed. Therefore, comparison of spot areas was possible since there was no loss of proteins in the course of the electrophoretic run. Densitometric quantitation of isolated spots such as spot A was readily performed using a commercial TLC densitometer. The quantitation will help to determine the degree of poisoning.

The amounts of low-density lipoprotein, fibrinogen, transferrin and spot A decreased drastically as shown in Fig. 1A and B. Spot A showed a relatively basic p*I* (7.2) and its molecular weight was calculated to be about 110,000. From these values we suppose that spot A may be one of the complement components.

As for the spots that newly appeared after carbon tetrachloride administration (e.g. spot B), individual identification will not be easy since they can be either cellular proteins, or modified plasma proteins. Extraction of proteins from fixed, stained two-dimensional gels and amino acid micro-analysis of the extracted proteins have been performed for human plasma proteins [10]. These are promising techniques for further analysis of the proteins specifically affected by carbon tetrachloride.

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EXTRACTIVE ALKYLATION OF 6-MERCAPTOPURINE AND DETERMINATION IN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY*

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SUMMARY

An analytical procedure was developed for the determination of 6-mercaptopurine in plasma. Owing to the polar character and low plasma concentrations of the compound, extraction and derivatization was carried out directly from the plasma sample by extractive alkylation. Determination was made using gas chromatography—mass spectrometry with multiple-ion detection.

Conditions with respect to the rate of formation and the stability of the derivative formed in the extractive alkylation step were evaluated. The selectivity of the method to azathioprine and to metabolites was thoroughly investigated. No 6-mercaptopurine was formed from azathioprine added to water or plasma and run through the method. The method enables the detection of 2 ng of 6-mercaptopurine in a 1.0-ml plasma sample. Quantitative determinations were done down to 10 ng/ml 6-mercaptopurine in plasma.

INTRODUCTION

6-Mercaptopurine is an immunosuppresive cytostatic agent which is used in the treatment of leukemia in children. This compound is also the primary metabolite of azathioprine [6-(1-methyl-4-nitro-5-imidazolyl)thiopurine], a drug extensively used in autoimmune and other diseases and in organ transplantation [1]. Intracellularly, mercaptopurine is converted by hypoxanthine—guanine phosphoribosyltransferase to thiosinic acid, which is thought to

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be the main active metabolite, and to other thioanalogues of purine derivatives [1].

Until recently, available methods for the determination of 6-mercaptopurine [2—12] have not been sensitive or selective enough to enable studies on the pharmacokinetics of 6-mercaptopurine during immunosuppresive treatment with azathioprine or 6-mercaptopurine. A high sensitivity has been obtained after conversion of 6-mercaptopurine to purine-6-sulphonate and determination of the derivative by fluorimetry [13]. The lower limit of sensitivity was 10 ng/ml. Gas chromatographic analysis has been performed after flash methylation [8, 9] or extractive methylation [12]. However, these methods did not take into account that one of the metabolic pathways of 6-mercaptopurine is an S-methylation and this metabolite would be co-determined in the procedure. The poor stability of the derivative also enhanced the problems in quantitative determinations. Recently, two liquid chromatographic methods for the assay of azathioprine and 6-mercaptopurine with high sensitivity and selectivity were described [14, 15].

From the available pharmacokinetic data, it is obvious that very low plasma concentrations will be seen some hours after a single dose of 6-mercaptopurine. A quantitative extraction of 6-mercaptopurine is difficult to obtain owing to the hydrophilic character of the compound. Furthermore, a derivatization of the acidic groups is also mandatory before gas chromatographic analysis. The difficulties encountered above made it necessary to develop a method which performed extraction and derivatization in a one-step procedure and which used a very sensitive and selective detection device. Therefore, interest was focused on direct extractive alkylation of the plasma sample with pentafluorobenzyl bromide and determination by gas chromatography—mass spectrometry with multiple-ion detection.

EXPERIMENTAL

Gas chromatography and mass spectrometry

Studies on the conditions for extractive alkylation of 6-mercaptopurine (Wellcome, London, Great Britain) were performed in a Pye-Unicam series 204 gas chromatograph equipped with flame ionization detector. The glass column (150 × 0.18 cm) was packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh) and operated at 265°C in the analysis of pentafluorobenzylated mercaptopurine. Injector and detector temperatures were kept at 320°C. Nitrogen (30 ml/min) was used as carrier gas. Identification of the derivatives was done in an LKB 2091 mass spectrometer.

6-Mercaptopurine concentrations in biological samples were determined in a Finnigan 4000 gas chromatograph—mass spectrometer equipped with a multiple-ion monitoring device (Finnigan, Sunnyvale, CA, U.S.A.). The mass spectrometer was operated in an electron impact mode with an ionization energy of 70 eV. The injector of the gas chromatograph was of the Grob capillary type and operated at 275°C in splitless mode. The injector was equipped with valves which were programmed to vent the injector 60 sec after injection of sample. The glass capillary column (15 m × 0.2 mm inner diameter) contained SE-30 as stationary phase. A 20—25 kPa pressure of

helium was applied to the column, which was directly interfaced to the ion source. The temperature of the gas chromatographic oven was programmed from 240 to 280°C at a rate of 10 ml/min. Samples could be injected every 5—6 min.

For the injection, solid sample syringes (SGE, Ringwood, Australia) were used. They were cleaned in a Hamilton syringe cleaner between injections.

The molecular ions, m/z = 512 for S-9-dipentafluorobenzyl-6-mercaptopurine and m/z = 346 for the internal standard derivative (9-pentafluorobenzyl-S-methyl-6-mercaptopurine), were monitored.

Identity of the derivatives

The following mass spectrometric data were obtained from the derivatives after extractive alkylation with pentafluorobenzyl bromide. Derivative of 6-mercaptopurine: m/z (percentage relative abundance) = 119 (18); 163 (14); 181 (100); 331 (73); 332 (11) and 512 (M^+, 73). Derivative of S-methyl-6-mercaptopurine: m/z = 119 (14); 165 (100); 181 (48); 254 (15) and 346 (M^+, 62).

Reagents and chemicals

Tetrabutylammonium and tetrahexylammonium ion solutions were prepared by neutralization of the corresponding hydrogen sulphate (Labkemi, Stockholm, Sweden) and purified by washing with methylene chloride and heptane. Dilution to the desired concentration was made with buffer. Tetrapentylammonium ion solution was prepared by shaking the iodide salt (Eastman Kodak, Rochester, NY, U.S.A.) with an equivalent amount of silver oxide overnight.

Standard samples of 6-mercaptopurine were prepared by dissolving the compound in water and dilution to 100 ng/ml. Aliquots of 0.5, 1.0, 2.0 and 5.0 ml of this solution were diluted to 10 ml with blank plasma. A 1.0-ml aliquot was taken to analysis.

METHODS

Extractive alkylation of 6-mercaptopurine

One millilitre of an aqueous solution of 6-mercaptopurine (1 mg/ml) was mixed with quaternary ammonium ion solution and methylene chloride containing the alkylating reagent. Tetradocosane or triacontane (0.3 mg/ml) dissolved in the organic phase was used as internal marker. The mixture was shaken at room temperature or at 50°C. The alkylation reaction was quenched by the addition of 0.5 ml of 0.1 *M* hydrochloric acid, and after shaking for some minutes a few microlitres of the organic phase were taken for analysis by gas chromatography with flame ionization detection. The height ratio of the peak of the product to that of the internal marker was calculated.

Partition of 6-mercaptopurine as ion pair with tetrabutylammonium ion

The extraction constant, K_{EX}, of the 6-mercaptopurine—tetrabutylammonium ion-pair was determined by shaking equal volumes of aqueous phase

(pH 10) containing 2 mg/ml 6-mercaptopurine 0.005—0.05 *M* tetrabutylammonium ion, and an organic phase of methylene chloride containing *p*-chlorobenzophenone as internal standard. After equilibration for 30 min, the organic phase was separated, methyl iodide was added and the amount of 6-mercaptopurine extracted in the organic phase was determined in comparison to a sample with a known concentration of 6-mercaptopurine and methylated quantitatively by extractive alkylation.

Determination of 6-mercaptopurine in plasma

To a 1.0-ml plasma sample of 6-mercaptopurine, 0.1 ml of internal standard solution (500 ng/ml of S-methyl-6-mercaptopurine), 0.1 ml of 0.1 *M* tetrabutylammonium ion solution and 0.5 ml of 1 *M* phosphate buffer (pH 10) were added. This mixture was shaken for 30 min with 2 ml of methylene chloride containing 2% of pentafluorobenzyl bromide. After centrifugation (500 *g*, 15 min) the organic phase was transferred to another tube and evaporated to dryness at 60°C (Buchler Vortex Evaporator). The residue was dissolved in 20 μl of ethanol. Of this, 1—2 μl were then evaporated on the needle of the solid sample syringe.

RESULTS AND DISCUSSION

Extraction and alkylation conditions for 6-mercaptopurine

In recent studies 6-mercaptopurine was extracted from plasma with a fourfold excess of butanol [8] or with ethyl acetate—isopropanol after addition of a stabilizing agent [9]. It was anticipated that the extraction yield using these procedures was not quantitative, thus decreasing the precision of the analytical procedure. An improved extraction yield of polar organic compounds can be achieved after addition of complexation agents, such an approach being used with 6-mercaptopurine by the addition of phenylmercuric acid before toluene extraction [3].

Ionized compounds can also be extracted as ion-pairs, where the type and concentration of the counter-ion as well as the properties of the organic phase govern the degree of extraction. The extraction constant (K_{EX}) for 6-mercaptopurine with tetrabutylammonium ion was 4.6. Use of a more lipophilic quaternary ammonium ion in the extraction of 6-mercaptopurine would increase the extraction yield and a quantitative extraction should be possible. On the other hand, a more efficient extraction of interfering components from the biological sample would occur, which would decrease the selectivity of the method.

As the possibility for an efficient and selective extraction of 6-mercaptopurine was limited, interest was focused on simultaneous extraction and derivatization to enhance both yield and sensitivity of the analytical procedure.

Recently, 6-mercaptopurine was derivatized before gas chromatographic analysis using extractive alkylation conditions. Tetrahexylammonium ion was used as counter-ion and the pH of the aqueous phase was in the range 13—14 [12]. The yield of dimethyl derivative was reported to be low, most probably explained by a rapid degradation of the product with use of the

more lipophilic quaternary ammonium ions at high pH of the aqueous phase. The extraction of hydroxide ions as ion-pair with the tetrahexylammonium in the organic phase is not negligible. Extractive alkylation of 6-mercaptopurine using a less lipophilic counter-ion would thus be favourable both to the stability of the derivative formed and to selectivity in the analysis of biological samples.

Extractive alkylation with pentafluorobenzyl bromide

Extractive alkylation of metimazol with pentafluorobenzyl bromide and determination by gas chromatography—mass spectrometry with multiple-ion detection was recently demonstrated [16]. The use of pentafluorobenzyl bromide as alkylating reagent was justified by the higher detection selectivity obtained when a higher mass number of the derivative was monitored.

In the analysis of 6-mercaptopurine the use of pentafluorobenzyl bromide was not solely motivated by the increased detection selectivity, but also to avoid the co-determination of an S-methylated metabolite which can be formed in man [2]. From the general considerations above on the extractive alkylation of 6-mercaptopurine with alkyl iodides the counter-ion and pH were chosen. The rapid hydrolysis of pentafluorobenzyl bromide also supports the use of mild reaction conditions (cf. ref. 17). The high reactivity of the reagent made it possible to use only 2% of pentafluorobenzyl bromide in the reaction. A low concentration of tetrabutylammonium ion, 0.005 *M*, was also used to increase the selectivity of the method. Recently, it was emphasized that the use of low concentrations of reagent is necessary to achieve sufficient selectivity and sensitivity in the analytical procedure [18]. The time for complete extractive alkylation using the conditions of the method was 25 min and 30 min for 6-mercaptopurine and the internal standard, respectively. Temperature did not influence the reaction rate, as can be seen in Fig. 1. This observation is contrary to the extractive alkylation of some sulphonamide diuretics where an elevated temperature was essential both for rapid reaction and type of derivative [19, 20].

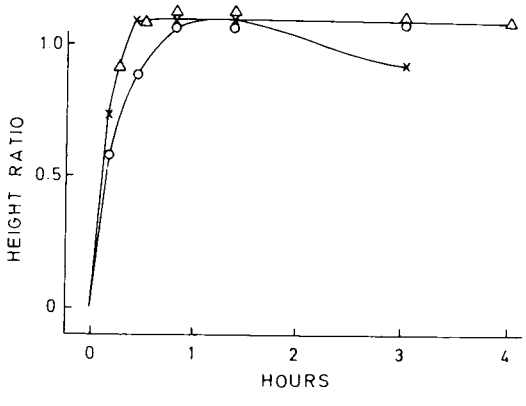

Fig. 1. Extractive alkylation of 6-mercaptopurine with pentafluorobenzyl bromide. Aqueous phase: 2 ml buffer solution (pH 10) containing 6-mercaptopurine (1 mg/ml) and 0.005 *M* tetrabutylammonium ion. Organic phase: methylene chloride (1 ml) with 2% pentafluorobenzyl bromide at 50°C (×) and at 25°C (△), or 1% pentafluorobenzyl bromide at 50°C (○).

An isomerization was observed of the product both with 6-mercaptopurine and with the internal standard when pentafluorobenzyl bromide was used as alkylating reagent. The isomers showed the same mass spectrometric fragmentation pattern as the derivatives from 6-mercaptopurine and the internal standard. The yields of the isomers were 7 and 11%, respectively. Furthermore, the isomers were formed in reproducible yield (± 7% of the main product, $n = 20$) and were completely resolved from other peaks in the gas chromatographic systems. This means that the extractive alkylation with pentafluorobenzyl bromide can be used in quantitative determinations with good precision. Isomerization in the extractive methylation of 6-mercaptopurine has also been reported [12]. This isomerization could not be observed in the above studies with any alkyl iodide. On the other hand, if methanol was added to the reaction mixture a much higher ratio of the two isomers of S-9-dipentafluorobenzyl-6-mercaptopurine was found.

Identity of pentafluorobenzyl derivatives

Extractive alkylation of 6-mercaptopurine with pentafluorobenzyl bromide resulted in a derivative with the pentafluorobenzyl groups in the 9-position and at the sulphur atom. The prominent peaks in the mass spectrum correspond to: $m/z = 181$, pentafluorobenzyl group; $m/z = 331$, molecular ion minus one pentafluorobenzyl group; and $m/z = 512$, the molecular ion. Mass spectral analysis of the derivative from the internal standard revealed a monopentafluorobenzylated product.

Gas chromatographic and mass spectrometric conditions

After alkylation of 6-mercaptopurine, the derivative showed good gas chromatographic properties. Symmetric peaks with no indication of absorption losses were seen using capillary columns with the non-polar stationary phase SE-30. A symmetrical peak was also obtained using packed columns with OV-17 as stationary phase.

Selectivity of the determination in the presence of azathioprine

6-Mercaptopurine is the major metabolic product of azathioprine. The conversion of azathioprine to 6-mercaptopurine is rapid [14], but nevertheless it is stated that azathioprine exhibits some pharmacologic activity and contributes to the overall effect. It was therefore of importance to establish whether azathioprine was co-determined in the method. Addition of azathioprine to water samples and run through the method using buffer with pH 8, 10 and 13 did not give any 6-mercaptopurine, indicating that azathioprine was stable in the extractive alkylation step. Addition of azathioprine to fresh human blood, however, gave considerable amounts of 6-mercaptopurine, which means a rapid conversion in the presence of red blood cells. This is shown in Fig. 2, which demonstrates that at room temperature about 50% conversion has already occurred within 30 min. This observation is in agreement with previous findings [2, 14]. The conversion was quantitative, and could be used as an indirect method for the determination of azathioprine.

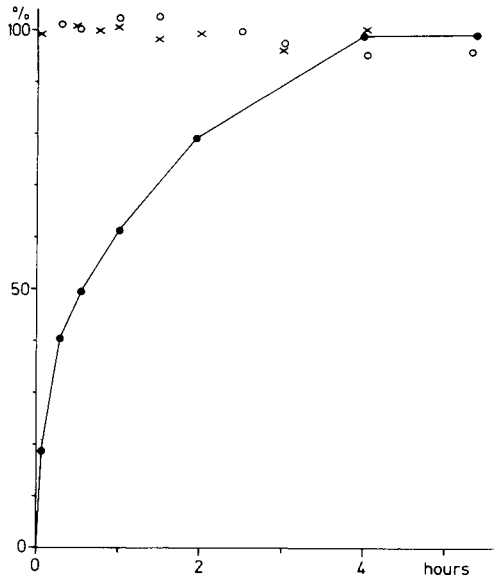

Fig. 2. Stability of 6-mercaptopurine in blood (○) and plasma (×). Yield of 6-mercaptopurine after addition of azathioprine to blood (●). Temperature 25°C.

Cooling the blood sample in an icebath immediately after drawing gave a very small conversion (< 10%) of azathioprine to 6-mercaptopurine.

It follows that this procedure is mandatory when studying the pharmacokinetics of azathioprine and 6-mercaptopurine after azathioprine administration. If this important measure were disregarded, the assay would give falsely high 6-mercaptopurine concentrations and correspondingly low azathioprine concentrations in the plasma.

Formation of 6-mercaptopurine from azathioprine was not observed in plasma. 6-Mercaptopurine was also found to be stable when added to plasma or human blood, as evidenced in Fig. 2.

Capability of the method

The limit of detection of the present method was 2 ng of 6-mercaptopurine per ml of plasma. Quantitative determinations could be done down to 10 ng/ml. The precision of the method at the 10 and 100 ng/ml level was 6 and 10%, respectively (n = 10). If 1,4-dithioerythritol (10 μl of a 1% solution) was added to plasma, a better precision of 7.1% at the 100 ng/ml level of 6-mercaptopurine was obtained (n = 10). 1,4-Dithioerythritol stabilizes 6-mercaptopurine [9], which improves the precision. However, 1,4-dithioerythritol rapidly degrades azathioprine and can not therefore be used for plasma samples from patients given azathioprine.

A typical chromatogram from the analysis of 6-mercaptopurine in plasma samples is shown in Fig. 3. No interfering peaks in the area of the peak of the derivative of 6-mercaptopurine could be seen. Also, standard curves for 6-mercaptopurine in the concentration range 10—100 ng/ml passed through the origin.

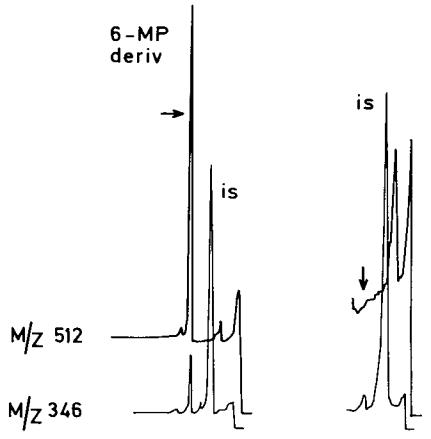

Fig. 3. Chromatogram from analysis of 6-mercaptopurine in plasma samples. *m/z* 512 = S-9-dipentafluorobenzyl-6-mercaptopurine; *m/z* 346 = derivative from internal standard. Right panel: blank plasma sample. Left panel: analysis of a patient plasma sample containing 30 ng/ml 6-mercaptopurine. is = internal standard.

Efforts were made to quantify the absolute recovery of 6-mercaptopurine through the analytical method. However, the derived compound could not be isolated in completely pure form after synthesis. Furthermore, the yield of derivative after addition of 6-mercaptopurine to plasma, 100 ng/ml, was virtually higher than that after preparation of the derivative at the mg/ml level and dilution to the desired concentration with solvent.

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CHROMBIO. 908

DETERMINATION OF THE ANTIOXIDANT 3-*TERT.*-BUTYL-4-HYDROXY-ANISOLE IN RAT PLASMA USING HIGH-RESOLUTION GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method is described for the determination of the antioxidant 3-*tert.*-butyl-4-hydroxyanisole in rat plasma using high-resolution capillary gas chromatography—mass spectrometry with selective ion monitoring. Following the addition of the isomer 2-*tert.*-butyl-4-hydroxyanisole, used as an internal standard, extraction was made with *n*-hexane and the extract derivatized with heptafluorobutyric anhydride.

The gas chromatographic separation was carried out on a SE-52 fused silica capillary column and the derivatized 3-*tert.*-butyl-4-hydroxyanisole and its isomer detected by recording the intensities of their common fragment ion at *m/e* 361. The sensitivity of the method allowed the antioxidant to be measured in 0.1-ml rat plasma samples down to a level of 10 ng/ml with a high degree of specificity and accuracy. The method has been applied to a preliminary pharmacokinetic study in rats after oral dosage.

INTRODUCTION

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used as antioxidant food additives.

BHA and BHT have been shown to be powerful perturbing agents for biomembranes in vitro [1]. BHA exhibits a very low toxicity in mammals when given orally, while its toxicity in the rat is greatly enhanced after intraperitoneal administration [2]. The observation that BHA is a substrate for mam-

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malian intestinal peroxidase would suggest that peroxidative oxidation at the intestinal wall may represent a contribution to the inactivation of BHA and other phenol derivatives potentially toxic to mammals [3].

In spite of lacking evidence of hazard from BHA at current use levels, the Federation of American Societies for Experimental Biology committee on GRAS (generally recognised as safe) substances has suggested the necessity to give an "interim status" to BHA until additional studies on the toxicity and metabolism of this compound in various animal species resolve the existing uncertainties [4].

As a contribution to a better knowledge of the toxicity and metabolism of BHA, a study on the kinetic behaviour of this compound in the rat has been started.

For the purpose of this study an assay procedure was required which would allow BHA to be measured in small volumes (0.1 ml) of rat plasma with a high degree of specificity and sensitivity. A number of methods using gas chromatography (GC) have previously been developed for detecting and quantifying this antioxidant in food products (references to these methods are given in a recent review article [5]). A spectrophotometric method suitable for aqueous samples has also recently been reported [6]. This assay procedure however requires 4 ml of aqueous sample and, as admitted by the authors, does not have the necessary specificity required for low-level determinations in biological samples. El-Rashidy and Niazi [7] have described a GC method using a packed column with flame ionization detection for determining BHA in human plasma and urine. The detection limit was reported as less than 100 ng/ml sample but the method required 1 ml plasma or 4 ml urine. In order to lower the detection limit and improve assay specificity we have developed a method for determining BHA in rat plasma based on the use of high-resolution capillary GC combined with mass spectrometry (MS).

MATERIALS AND METHODS

3-*tert.*-Butyl-4-hydroxyanisole (3-BHA) was a Fluka Chemical obtained from Prokeme (Florence, Italy). 2-*tert.*-Butyl-4-hydroxyanisole (2-BHA) was prepared from a commercial sample of 3-BHA from Sigma (London) (Poole, Great Britain) which contained approximately 7% of the 2-isomer. The separation of the isomers was carried out on a Sephadex LH-20 column using the solvent mixture chloroform—cyclohexane (1:1, v/v) according to the procedure described by Kauffman [8]. The purity of the isolated 2-BHA was confirmed by thin-layer chromatography, GC and GC—MS.

Heptafluorobutyric anhydride supplied by Pierce and Wariner (Chester, Great Britain) was stored at 0°C in dark bottles. *n*-Hexane and pyridine obtained from BDH (Poole, Great Britain) were of AnalaR grade and were used without further purification. Ethyl acetate (Fisons, Loughborough, Great Britain) of Distol Reagent grade was dried by adsorptive filtration through Alumina Woelm act. 1 (Koch-Light, Colnbrook, Great Britain).

Animal studies

Male LAC:Porton rats (200—230 g) were anaesthetised with diethyl ether

and a PE50 cannula inserted into the ventral tail artery. The animals were put into restraining cages and allowed to recover for 1 h before oral administration of 1 ml/kg of a 200 mg/ml solution of 3-BHA in dimethyl sulphoxide. Blood samples (200—300 μl) were withdrawn from the cannula into heparinised tapered tubes at 10, 20, 30 min and 1, 2, 3, 4, 5 and 24 h after administration of 3-BHA. Samples were immediately centrifuged at 2000 *g* for 5 min and the plasma fractions stored frozen until required for analysis.

Extraction and derivatization

All glassware was cleaned and silanized as described previously [9]. A 0.1-ml aliquot of rat plasma was diluted to 0.5 ml with distilled water in a 30-ml glass stoppered centrifuge tube and 20 μl of the internal standard in ethyl acetate containing either 40 ng or 4 ng 2-BHA were added. The sample was extracted with 3 ml *n*-hexane by Vortex mixing for 30 sec followed by centrifugation at 1500 *g* for 2 min. The organic layer was transferred to an 8-ml glass tube fitted with a Teflon®-lined screw cap. The plasma was extracted with a further 1 ml *n*-hexane and the combined extracts evaporated to dryness under nitrogen at 0°C. The dried extract was derivatized by adding 500 μl hexane, 20 μl pyridine and 25 μl heptafluorobutyric anhydride and reacting for 30 min at 60°C. After cooling 2.0 ml *n*-hexane and 1.0 ml 0.1 *M* phosphate buffer (pH 6.9) were added and the mixture shaken on the Vortex mixer for 30 sec and then centrifuged at 1500 *g* for 2 min. The hexane layer was transferred to a 3-ml Reacti-Vial (Pierce and Warriner) and the extract evaporated under nitrogen at 0°C before finally dissolving in ethyl acetate for GC—MS analysis.

Gas chromatography—mass spectrometry

The instrument used in this study was a 70-70F VG Micromass double focussing mass spectrometer linked with a VG 2035 Data System. The GC separations were made on a 25 m × 0.25 mm I.D. fused silica capillary column (Phase Separations, Queensferry, Great Britain) coated in the authors' laboratory with SE-52 stationary phase by the static coating procedure to give a film thickness of ca 0.3 μm. The capillary column was installed in a Pye-Unicam Series 204 gas chromatograph interfaced to the mass spectrometer with glass-lined stainless-steel tubing. The outlet end of the fused silica capillary was fed through the GC—MS interface up to the ion source entry tube. The capillary column connections in the GC oven were made using graphite ferrules. Samples were introduced into the capillary using either a falling needle solid injector or a solvent splitting device (10:1 split). Helium used as carrier gas was adjusted to give a column flow-rate of approximately 2 ml/min. The column was operated isothermally at 150°C for 2 min followed by a 10°C/min programme to 250°C. The temperature of the injection port, GC—MS interface and ion source were maintained at 250, 280 and 220°C, respectively. The mass spectrometer was operated in the electron impact mode with an ionization potential of 70 eV and a trap current of 200 μA. During selected ion monitoring the ions at *m/e* 361 were recorded using a Rikadenki Series DBE-6 Multi Pen recorder.

Calibration curve

For the analysis of samples with expected plasma 3-BHA levels of > 100 ng/ml the standard calibration curve was constructed with 0.1-ml blank rat plasma samples containing 5, 10, 20, 30, 40, 50 and 60 ng of 3-BHA and 40 ng 2-BHA. When the plasma 3-BHA levels were expected to be < 100 ng/ml one-tenth the above amounts of 3-BHA and internal standard were used for the calibration curve. Samples were extracted and derivatized by the method described above and subjected to GC—MS analysis. The peak height ratio of 3-BHA to 2-BHA was plotted against concentration of 3-BHA present.

RESULTS AND DISCUSSION

The heptafluorobutyryl derivatives were used in the assay procedure both to improve the GC properties of 3-BHA and to produce a suitable high mass ion for single ion detection. The electron impact mass spectrum of 3-BHA heptafluorobutyrate (Fig. 1a) exhibits both an intense molecular ion (*m/e* 376, 100%) and fragment ion at *m/e* 361 [$(M-CH_3)^+$, 93%]. The 2-BHA isomer which was selected as an internal standard for the assay showed a similar fragmentation pattern when derivatized with heptafluorobutyric anhydride (Fig. 1b). Quantitative analysis was based on recording in the electron impact mode, the ions at *m/e* 361 common to both derivatized isomers.

The GC separations were carried out on a fused silica capillary column coated with the non-polar silicone gum phase SE-52. Recent studies [10, 11] have shown that due to the very low metal oxide content of fused silica (less than 1 ppm) capillary columns fabricated from this type of glass are chromatographically very inert and exhibit good thermal stability. The flexibility and strength of the fused silica capillaries are advantages when handling and installing the columns in the GC oven. This flexibility will also allow the capillary to be inserted very close to the ion source of the mass spectrometer eliminating possible absorption or catalytic decomposition effects occurring in the GC—MS interface.

A typical GC—MS analysis with single ion detection of 3-BHA in a 0.1-ml plasma sample from a rat following an oral dosage of the antioxidant is illustrated in Fig. 2. The analyses of blank rat plasma samples gave very few background peaks at *m/e* 361 none of which interfered with the measurement of the peaks derived from 3-BHA or the internal standard. The level of detection with single ion monitoring was such that 100 pg of either of the derivatized compounds gave a signal-to-noise ratio of 10 : 1. Standard curves used for quantitation exhibited good linearity over the concentration ranges measured.

The accuracy of the method was determined from the analysis of 0.1-ml aliquots of blank plasma containing 10—100 ng of added authentic 3-BHA. The calculated recoveries over this range varied from 93.4—107.4% with a mean of 100.15% (S.D. ± 4.02). The absolute recovery of 3-BHA from plasma using 2-BHA as an external standard gave a mean recovery value of 80.3%. The precision of the assay was determined by performing replicate analyses at the concentration levels of 20, 100, 300 and 600 ng/ml plasma. The find-

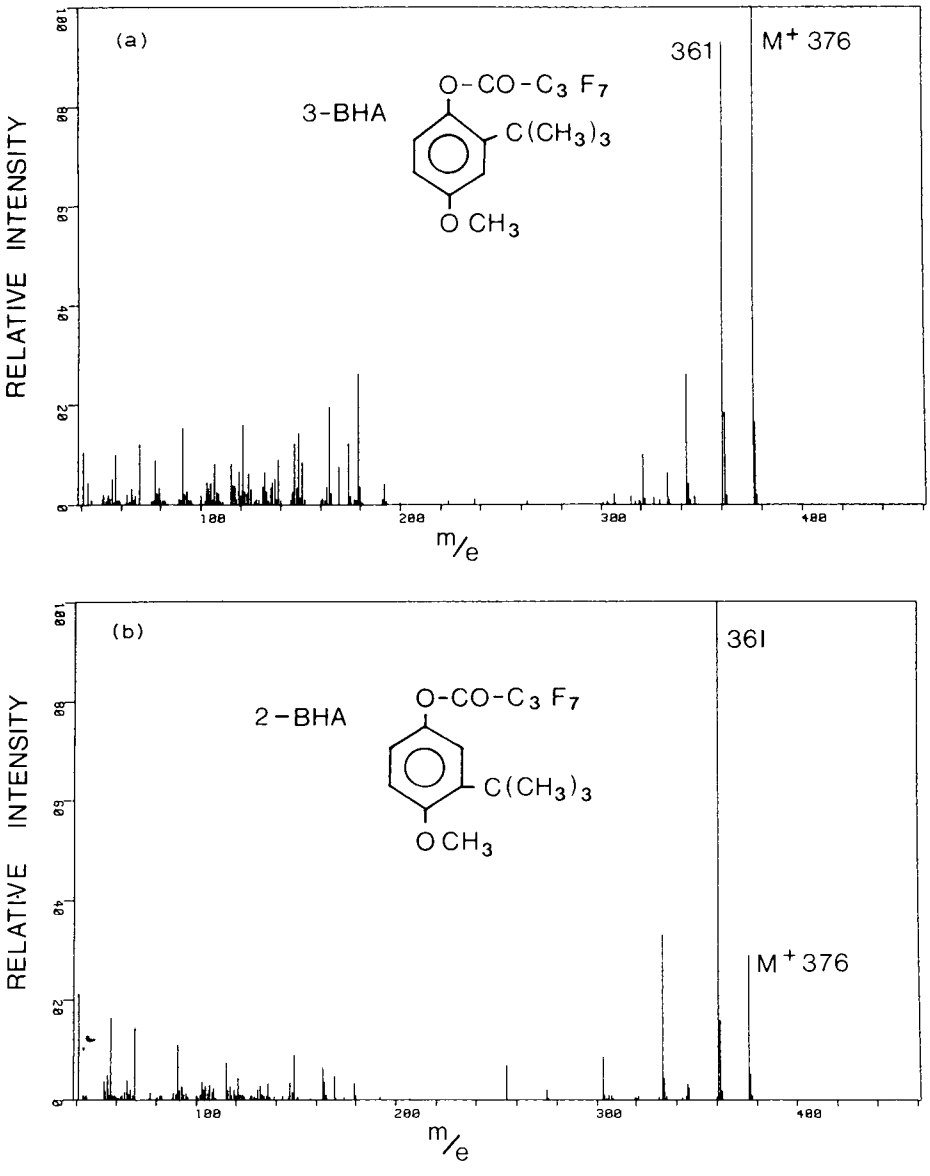
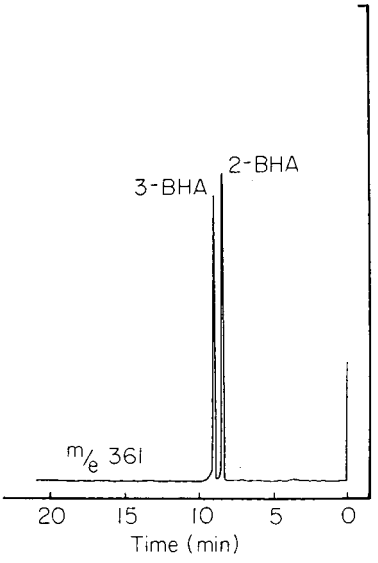


Fig. 1. Electron impact mass spectra of the heptafluorobutyryl derivative of 3-BHA (a) and 2-BHA (b).

ings are given in Table I. The specificity of the method was checked on a number of rat plasma samples by multiple ion monitoring, recording in addition to the *m/e* 361 fraction ion, the molecular ion at *m/e* 376. The peak height ratios of the two mass ions were similar to those recorded from the analysis of the standard derivatized compounds. The application of the method to a preliminary study of the plasma concentration profiles in three rats following a 200 mg/kg dose of the antioxidant is shown in Fig. 3. Following a rapid



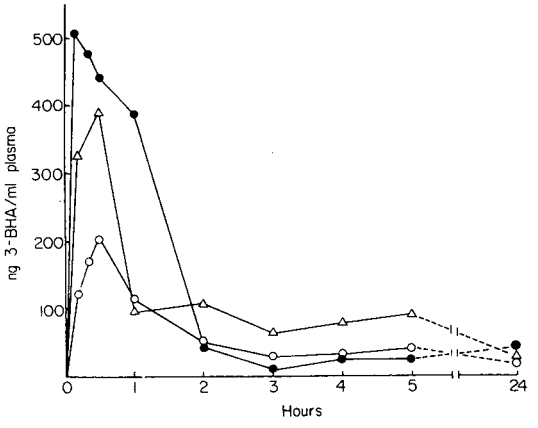


Fig. 2. Single ion monitor trace *m/e* 361 from the analysis of a plasma sample taken from a rat 2 h after a dosage of 200 mg/kg 3-BHA. The calculated concentration of 3-BHA in this sample was 65.4 ng/ml.

Fig. 3. Plasma concentration—time profiles in three rats following oral administration of 200 mg/kg of the antioxidant.

TABLE I

PRECISION OF THE METHOD FROM THE REPLICATE ANALYSES OF BLANK PLASMA SAMPLES WITH KNOWN AMOUNTS OF 3-BHA ADDED

$n = 5$.

Concentration added (ng/ml)	Mean concentration found (ng/ml)	S.D.	C.V. (%)
20	19.1	1.82	9.53
100	100.84	4.17	4.13
300	297.18	9.24	3.11
600	600.58	32.48	5.41

rise in plasma concentration, peak levels were reached at between 10 and 30 min after administration of the compound. After 2 h the levels fell below 10 ng/ml but measurable amounts were still present in the plasma 24 h after dosage.

In conclusion the method described in this paper allows the precise and accurate determination of 3-BHA at low levels in small plasma samples. The method should also be applicable to quantitating the antioxidant in urine and tissue samples.

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CHROMBIO. 911

QUANTITATIVE DETERMINATION OF 1-HEXYLCARBAMOYL-5-FLUOROURACIL AND ITS METABOLITES IN MAN*

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the quantitative determination of 1-hexylcarbamoyl-5-fluorouracil (HCFU) and its metabolites using μBondapak C_{18} and μPorasil has been developed. Two mobile phases containing PIC-B7 (consisting of acetic acid and 1-heptanesulphonic acid) were used for the separation, and good separations were obtained. With methanol—water (56:44) as the mobile phase, the separation of HCFU and its three metabolites was achieved within 4 min. With methanol—water (32:68) a new metabolite, 1-ω-carboxymethylcarbamoyl-5-fluorouracil, was revealed in human plasma. The recovery of each substance was 80% or greater and the sensitivity was at the nanograms per millilitre level. The coefficient of variation was less than 3.6% for each component.

INTRODUCTION

The antineoplastic activity of 1-hexylcarbamoyl-5-fluorouracil (HCFU) against various kinds of experimental tumours has been thoroughly examined by Kobari et al. [1] and Hoshi et al. [2], who found that HCFU is active against rapidly growing tumours and early or advanced slowly growing tumors. The metabolic pathway of HCFU in animals has been examined with ^{14}C-labelled HCFU [1]. However, the metabolic pathway of this drug in man has not been elucidated because the isolation and quantitative determination of HCFU and its metabolites have not been established. This paper describes a high-performance liquid chromatographic (HPLC) method for the quantitative determination of HCFU and its four metabolites in human plasma.

*A preliminary report was submitted to the Annual Meeting of the Committee on Clinical Evaluation of the Effectiveness of Newly Synthesized Antineoplastic Drugs (Tokyo, July, 1978) and at the 16th Annual Meeting of the Japanese Society of Cancer Chemotheraphy (Nagoya, September, 1978).

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EXPERIMENTAL AND RESULTS

Blood samples were obtained from patients who had been administered HCFU orally. Procedures for the extraction of HCFU and its metabolites are summarized in Fig. 1. The HPLC apparatus was obtained from Waters Assoc. (Milford, MA, U.S.A.). A stainless-steel column (300 × 3.9 mm I.D.) was filled with μBondapak C_{18} (8—10 μm) (Waters) and a pre-column (30 × 3.9 mm I.D.) filled with μPorasil (8—10 μm) (Waters) was used. The elution pattern was detected at 254 nm.

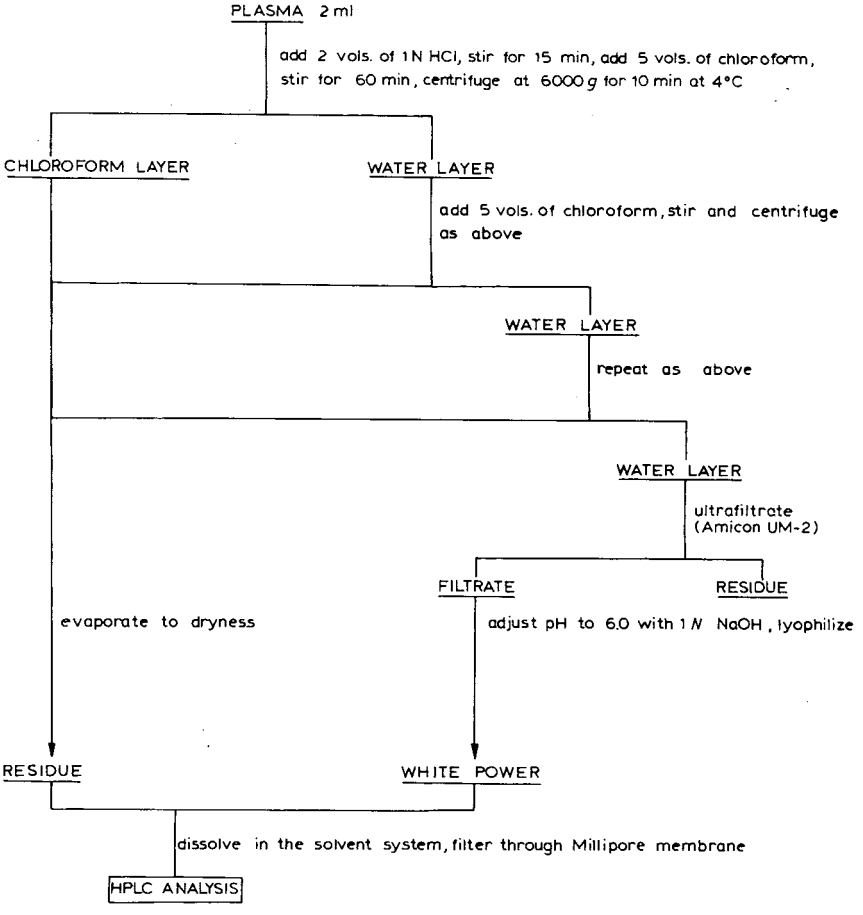

Fig. 1. Procedure for extraction of HCFU and its metabolites from human plasma.

All solutions used contained PIC-B7 (Waters), which consisted of acetic acid and 1-heptanesulphonic acid. One ampoule of PIC-B7 was dissolved in 1 l of water or methanol to give concentrations of 0.005 *M* acetic acid and 0.005 *M* 1-heptanesulphonic acid.

Authentic samples of HCFU, 1-ω-carboxymethylcarbamoyl-5-fluorouracil (CMEFU), 1-ω-carboxypropylcarbamoyl-5-fluorouracil (CPRFU), 1-ω-carboxy-

pentyl carbamoyl-5-fluorouracil (CPEFU) and 5-fluorouracil (5-FU) were kindly donated by Mitsui Pharmaceutical (Tokyo, Japan). The water used was distilled and deionized just before use with a Milli-R/Q water purifier. Methanol of chromatographic grade was obtained from Wako (Osaka, Japan).

Separation of authentic samples

Authentic samples were dissolved in methanol at a concentration of 0.12 μg/ml. A 20-μl aliquot of the solution was injected through the universal injector of the apparatus. When elution was performed with water—methanol (44:56), 5-FU, CMEFU, CRPFU, CPEFU and HCFU were separated and eluted with elution times of 1.6, 1.7, 1.8, 2.6 and 4.0 min, respectively. However, the separation of 5-FU and CMEFU was not satisfactory. The mobile phase was changed to methanol—water (32:68), and then the separation of 5-FU and CMEFU was adequate but the elution time was prolonged, as shown in Fig. 2, with HCFU being eluted after 17 min and CPEFU, CPRFU, CMEFU and 5-FU at 7.5, 3.2, 1.8 and 1.6 min, respectively.

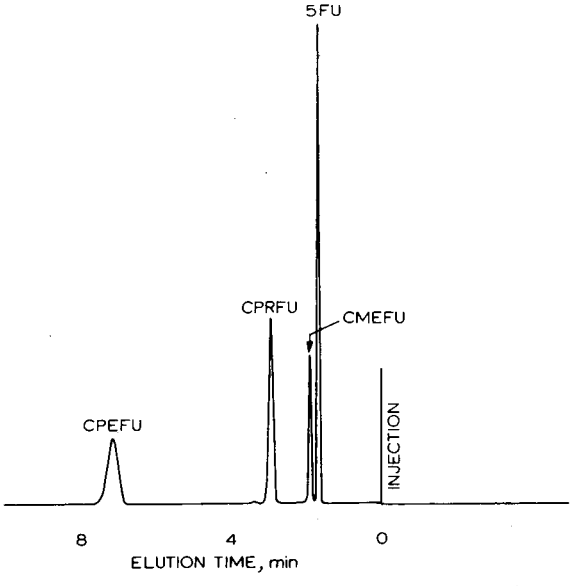

Fig. 2. Chromatogram of authentic HCFU and its metabolites. Mobile phase: methanol—water (32:68).

Calibration graphs for HCFU and its four metabolites

Calibration graphs for the determination of HCFU and its metabolites CPEFU, CPRFU, CMEFU and 5-FU were constructed from the results for authentic samples. The calibration graphs for the determination of HCFU, CPEFU, CPRFU, CMEFU and 5-FU can be expressed by the equations $y=1.958x-0.011$, $y=8.129x-0.284$, $y=4.112x-0.118$, $y=7.891x-0.654$ and $y=22.514x+0.140$, respectively. In addition, the r values for these equations were calculated to be 0.9985, 0.9962, 0.9990, 0.9882 and 0.9998, respectively.

Recovery of authentic samples from pooled plasma

The five authentic samples were dissolved in 2 ml of pooled plasma to give a final concentration of 0.12 μg/ml and then the samples were extracted as outlined in Fig. 1. The elution pattern obtained with methanol—water (32:68) as the mobile phase is shown in Fig. 3. The elution pattern of human pooled

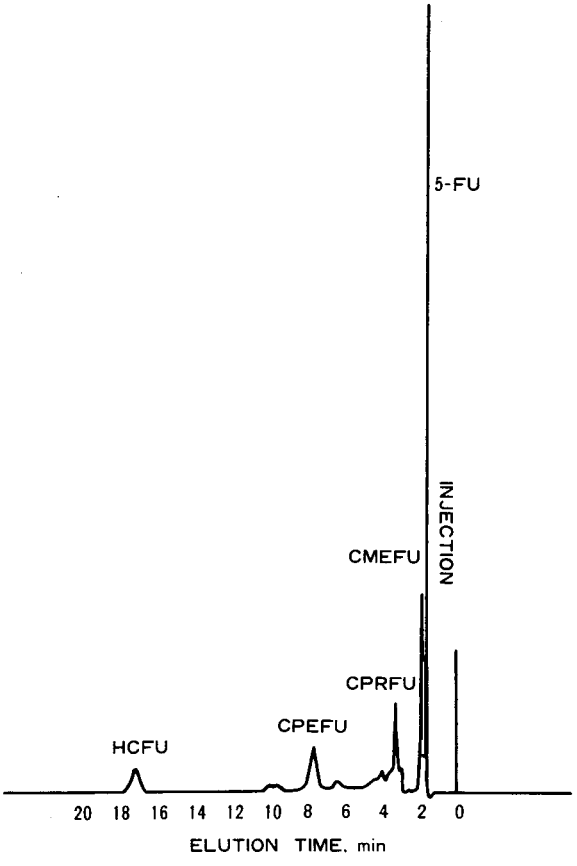

Fig. 3. Chromatogram of authentic samples of HCFU and its four metabolites extracted from human plasma. HCFU and the metabolites were dissolved in 2 ml of pooled plasma and stirred well, then extracted as shown in Fig. 1.

plasma used as a control did not exhibit any interfering peaks (Fig. 4). The recovery was 82 ± 1.0% for HCFU and 85 ± 1.0% for other metabolites. When different concentrations, ranging from 0 to 50 ng/ml, of the authentic compounds were injected into the HPLC apparatus the graphs of peak area against concentration showed good linearity up to 50 ng/ml in each instance. The coefficients of variation (n = 5) for HCFU, CPEFU, CPRFU, CMEFU and 5-FU were 3.2, 3.4, 3.4, 3.3 and 3.6%, respectively. In addition, the sensitivity for each component was 1 ng/ml.

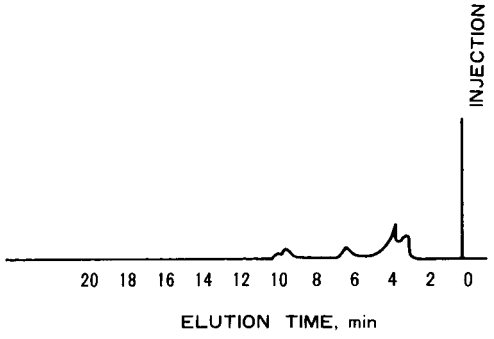

Fig. 4. Chromatogram of human plasma as control: 2 ml of pooled plasma were extracted as shown in Fig. 1 and the residues obtained were dissolved in methanol—water (56:44).

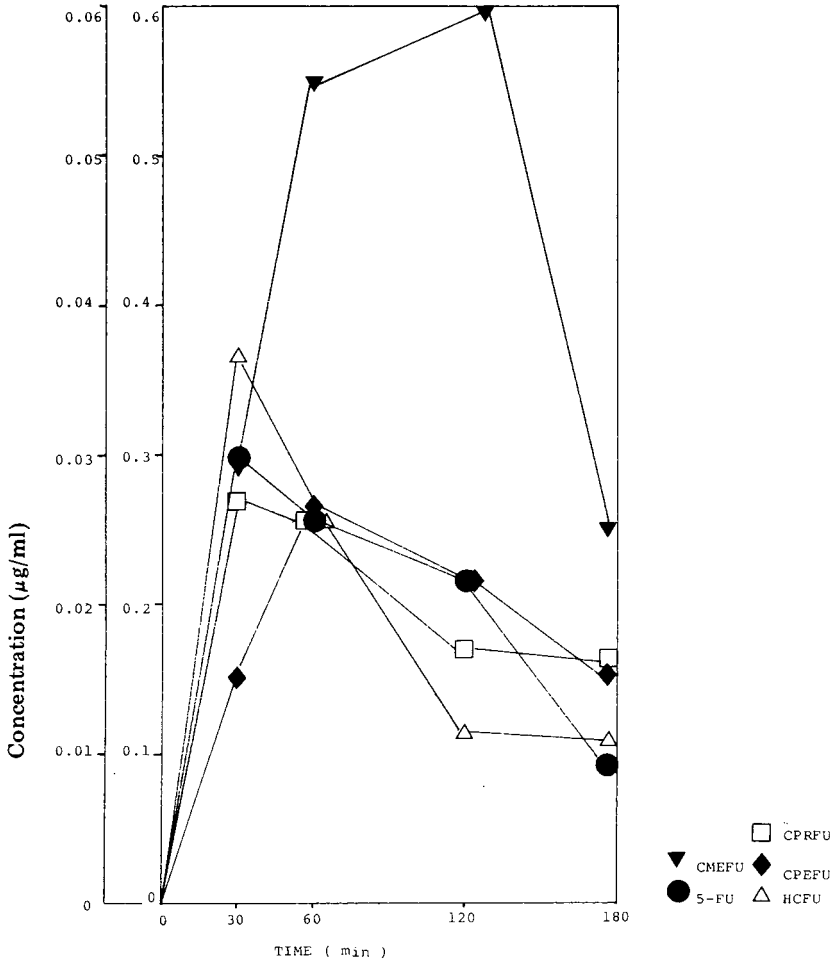

Fig. 5. Time course of HCFU and its four metabolites in a human patient. HCFU (300 mg) has been administered to a patient orally. Blood samples were taken from the cubital vein and the plasma was separated and extracted as shown in Fig. 1.

Concentration of HCFU and its metabolites in plasma

A patient was administered 300 mg of HCFU granules orally and then 5-ml blood samples were collected from the cubital vein after different times with 0.1 ml of heparin as shown in Fig. 5. The time course of HCFU showed a maximal value after 30 min. CPRFU and 5-FU also reached maximal concentrations after 30 min. However, CPEFU and CMEFU exhibited maximal concentrations 120 min after oral administration of HCFU.

DISCUSSION

Recently, many masked compounds of 5-FU have been synthesized, but there have been few papers [3] concerned with the pharmacokinetics of these drugs because of the difficulty of assaying them. We therefore tried to determine the concentrations of HCFU and its metabolites in human plasma.

The results on the experiments using authentic samples showed that μBondapak C_{18} is suitable for separating these compounds and exhibited good recovery and accuracy. Two mobile phases were examined for HPLC. With methanol—water (56:44) the separation of 5-FU and CMEFU was unsatisfactory but HCFU and its metabolites were eluted within 4 min. On the other hand, when methanol—water (32:68) was used, the separation of 5-FU and CMEFU was satisfactory, but the elution time of HCFU was prolonged to 17 min. Kono et al. [4] found that HCFU was eluted after approximately 15 min when water—acetonitrile (70:30) was used as the mobile phase. However, with tetrahydrofuran—water used as the mobile phase a longer retention time was found [4]. During the present experiments, PIC-B7 was added to the methanol—water [5, 6], which led to good separations of HCFU and its metabolites, as shown in Figs. 2 and 3.

In rats, HCFU is metabolized into 5-FU through two or three intermediates [1]. However, CMEFU has not been detected in the plasma of rat and it was therefore assumed that CMEFU is not an intermediate metabolite in the metabolic pathway of HCFU in humans also. However, in three out of five cases, CMEFU was detected in high-performance liquid chromatograms [7].

The time course of HCFU, CPEFU, CPRFU, CMEFU and 5-FU is shown in Fig. 5. The coefficient of variation of the determination of the concentration of each component was calculated from five experiments, and ranged between 3.2 and 3.6%, i.e., the accuracy was good.

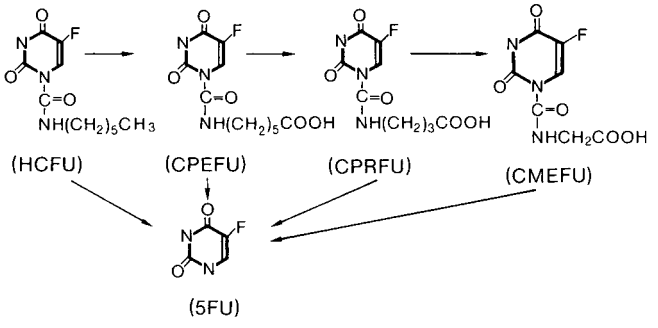

Fig. 6. Proposed metabolic pathway of HCFU in humans.

In conclusion, this system appears to be satisfactory for the quantitative determination of HCFU and its four metabolites in humans. Because it exhibits good recovery and has sufficient sensitivity it can be used to study the pharmacokinetics of HCFU. In addition, a new metabolite, CMEFU, has been detected using methanol—water (32:68) as the mobile phase, and a metabolic pathway of HCFU in humans is proposed in Fig. 6.

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CHROMBIO. 913

DETERMINATION OF CYTARABINE AND URACIL ARABINOSIDE IN HUMAN PLASMA AND CEREBROSPINAL FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of the antineoplastic agent cytarabine and its main metabolite uracil arabinoside in human plasma and cerebrospinal fluid is described. Complete separation from endogenous constituents was achieved by isocratic reversed-phase chromatography using phosphate buffer (0.05 *M*, pH 7.0) as the eluent. The limit of detection was 50 ng/ml. Day-to-day coefficients of variation were below 10%. The applicability of this rapid, simple and specific method for pharmacokinetic studies and monitoring of therapy was demonstrated.

INTRODUCTION

The pyrimidine analogue cytarabine (cytosine arabinoside; 1-β-D-arabinofuranosylcytosine; ara-C) is an antimetabolite that selectively inhibits DNA synthesis and is therapeutically effective in the treatment of acute myeloblastic leukaemia and other haematological malignancies [1—5].

Ara-C is rapidly deaminated in humans to uracil arabinoside (ara-U), a non-toxic compound [6, 7]. Owing to considerable individual variations in the pharmacokinetics of ara-C [8—11], the measurement of its concentration in plasma and cerebrospinal fluid is important for optimal dose scheduling [12]. Recently, extremely high doses of ara-C were successfully used for the treat-

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ment of acute leukaemia, refractory to conventional chemotherapy [13—15]. However, high doses of ara-C produce the risk of dangerously acute toxicity. Therefore, we monitored plasma levels of ara-C and its metabolite ara-U during and after infusions of conventional and high doses of ara-C using a rapid and sensitive high-performance liquid chromatographic (HPLC) method developed in our laboratory.

Several methods involving microbiological [16—21], radioenzymatic [22], radioimmunological assays [23—25] and some bioassay techniques [26, 27] have been employed for the analysis of the pharmacokinetics of ara-C. Some of these techniques are very sensitive (20 ng/ml). However, the analysis times are too long for clinical routine and the assays are subject to interferences from endogenous substances. An ultraviolet spectroscopic method for the determination of ara-C and ara-U in plasma lacks the sensitivity necessary for monitoring therapeutic concentrations in man [28]. The application of gas chromatography coupled with chemical ionization mass spectrometry [29] or the use of gas—liquid chromatography with a nitrogen-sensitive flame ionization detector and a coupled mass spectrometer [30] requires time-consuming steps of extraction and derivatization. Determination by paper chromatography [8, 31—33] or thin-layer chromatography [34] involves radioactive tracer technology (tritiated ara-C), which requires specialized handling and special permission for use in human studies.

HPLC has particular advantages for the analysis of ara-C and ara-U in that compounds may be analysed without initial derivatization. Wan et al. [8] emphasized the usefulness of HPLC but provided few details of the methodology of their assay. Kreis et al. [35] gave insufficient data about the scope and sensitivity of their HPLC assay. Bury and Keary [36] used cation-exchange chromatography for the determination of ara-C in human plasma, with a limit of sensitivity of 20 ng/ml. However, despite deproteinization of plasma samples with trichloroacetic acid the peak from endogenous plasma constituents was broad, allowing the measurement of ara-C as the tailing peak only. Ara-U could not be determined by this method.

This paper describes a rapid and simple reversed-phase HPLC assay for ara-C and ara-U suitable for drug monitoring and pharmacokinetic studies in human plasma and cerebrospinal fluid.

EXPERIMENTAL

Materials

Ara-C, ara-U, ara-C-5′-monophosphate, ara-C-5′-triphosphate and tetrahydrouridine were kindly supplied by Mack (Illertissen, G.F.R.). Tests for interfering peaks were performed with allopurinol, oxipurinol, uric acid (Henning, Berlin, G.F.R.), xanthine, hypoxanthine (Merck, Darmstadt, G.F.R.), cytidine (Sigma, St. Louis, MO, U.S.A.), methotrexate, leucovorin (Cyanamid, Munich, G.F.R.), 6-mercaptopurine, 6-thioguanine, 6-thiouric acid (Burroughs Wellcome, London, Great Britain) and doxorubicin (Farmitalia, Freiburg, G.F.R.). All other substances and solvents were of analytical-reagent grade and were used without further purification.

Chromatography

The chromatographic studies were performed with a high-pressure liquid chromatograph equipped with a Gynkotek Model 600/200 HPLC pump (Gynkotek, Munich, G.F.R.), a modified ASI 45 automatic sample injector (Kontron, Eching, G.F.R.), an Uvikon 720 LC variable-wavelength ultraviolet detector (Kontron), an SP 4100 computing integrator (Spectra-Physics, Darmstadt, G.F.R.) and a BD9 two-channel electronic recorder (Kipp and Zonen, Kronberg/Ts., G.F.R.).

Chromatography was performed in the reversed-phase mode using as eluent a phosphate buffer (0.05 *M*, pH 7.0), which was prepared by mixing 1 l of 0.05 *M* disodium phosphate (8.99 g of $Na_2HPO_4 \cdot 2H_2O$ per litre of distilled water) with 704 ml of 0.05 *M* potassium phosphate (6.87 g of anhydrous KH_2PO_4 per litre of distilled water).

The chromatographic column was a stainless-steel tube (30 cm × 4 mm I.D.) filled with Spherisorb ODS, 5 μm (Latek, Heidelberg, G.F.R.). The column was packed by forcing a slurry of 4.2 g of the stationary phase material suspended in 25 ml of carbon tetrachloride into the tube under a pressure of 400 bar by means of a Type MS 80/8 high-pressure membrane pump (Orlita, Giessen, G.F.R.). In general, the efficiency of the column was about 10,000 theoretical plates for the ara-C peak. The column was discarded when the number of theoretical plates for ara-C fell to less than 1000.

The chromatograph was operated at a flow-rate of 1.6 ml/min and a pressure of 210 bar at room temperature. The eluent was monitored at 270 nm, the absorption maximum of ara-C in 0.05 *M* phosphate buffer at pH 7.0. The detector was set at 0.04 a.u.f.s. The areas under the peaks of interest were computed by the integrator.

Assay procedure

Samples of 1 ml of blood were collected in tubes containing 75 units of heparin (15 μl of Thrombophob; Nordmark, Hamburg, G.F.R.) and tetrahydrouridine (final concentration 10^{-3} *M*), a blood deaminase inhibitor. After centrifugation at 8000 *g* for 5 min (Microfuge 5412; Eppendorf, Hamburg, G.F.R.), plasma samples of 10—50 μl were immediately chromatographed or stored frozen at −16°C.

Samples of 1 ml of cerebrospinal fluid were collected in tubes containing tetrahydrouridine (final concentration 10^{-3} *M*).

As aliquots of plasma or cerebrospinal fluid were injected into the chromatographic system without further clean-up, the method of external standardization was used for quantitation. Every sample was analysed in duplicate, and a third analysis was performed if the peak areas of the compounds did not agree to within ± 5%. The calibration graphs (peak area against concentration) were straight lines for each compound up to 120 μg/ml. Therefore, calibration samples containing 1 μg of each substance per millilitre of plasma were analysed every ten chromatographic runs for external standardization.

Protein binding

For the determination of protein binding, ara-C and ara-U were measured in plasma and in the plasma ultrafiltrate of spiked samples of plasma. Ultrafiltration was performed using a Model MM 302 ultrafiltration system (Amicon, Düren, G.F.R.) and Type PM 10 Diaflo membranes. In the controls there was no adsorption of either substance to this membrane.

RESULTS AND DISCUSSION

Plasma extraction

Attempts to extract ara-C and ara-U from aqueous solutions at different pHs with organic solvents (diethyl ether, dichloromethane, isooctane, *n*-butanol, isopropanol) gave poor recoveries even if the aqueous solutions were saturated with ammonium sulphate. The resolution of ara-C and ara-U from interfering plasma peaks was not improved by a preceding purification step, e.g., protein precipitation by trichloroacetic acid or ethanol. Protein removal by ultrafiltration using membranes which exclude substances with molecular weight >10,000 did not improve the separation. Therefore, plasma samples were used directly for HPLC without prior clean-up.

Chromatography

The effect of pH of 0.05 *M* phosphate buffer on the resolution of ara-C and are-U from plasma peaks was investigated. Optimal resolution was obtained at

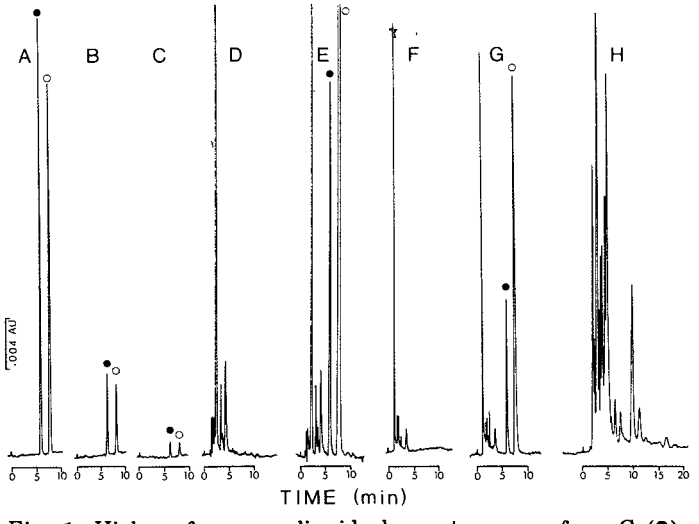

Fig. 1. High-performance liquid chromatograms of ara-C (●) and ara-U (○). Traces A—C; chromatograms of ara-C and ara-U dissolved in 0.05 *M* phosphate buffer (pH 7.0). Amounts chromatographed: (A) 5, (B) 1 and (C) 0.2 μg of each compound per millilitre. Traces D and E; chromatograms of plasma from a leukaemic patient, (D) before and (E) during infusion of ara-C. Traces F and G: chromatograms of cerebrospinal fluid from the same patient, (F) before and (G) at the end of intravenous infusion of ara-C. Trace H: chromatogram of drug-free urine, diluted 1:20 in distilled water. Stationary phase: Spherisorb ODS, 5 μm (30 cm × 0.4 cm I.D.). Mobile phase: 0.05 *M* phosphate buffer (pH 7.0). Sample volume: 20 μl. Flow-rate: 1.6 ml/min. Back-pressure: 210 bar. Room temperature. Detection at 270 nm.

pH 7.0. The use of pH 7 phosphate buffers at different molarities was investigated and it was found that the resolution was highest at values above 0.03 *M*. Of the several available reversed-phase materials, Spherisorb ODS, 5 μm, was the best stationary phase with respect to efficiency, permeability and stability. Therefore, chromatography was performed on a Spherisorb column (30 cm × 0.4 cm I.D.) with phosphate buffer (0.05 *M*, pH 7.0) as eluent. As shown in Fig. 1, the peaks of interest were sharp, symmetrical and well defined with respect to the baseline. Components of plasma (Fig. 1D and E) and cerebrospinal fluid (Fig. 1F and G) did not interfere in regions where ara-C and ara-U eluted. However, in urine samples the determination of both compounds was disturbed by endogenous components (Fig. 1H).

Specificity

No interfering peaks were found in plasma samples to which were added drugs that are usually coadministered for the treatment of acute leukaemia, e.g., methotrexate, 6-mercaptopurine, 6-thioguanine (6-thiouric acid), doxorubicin and allopurinol (oxipurinol, xanthine, hypoxanthine). Cytidine, tetrahydrouridine and the intracellularly occurring ara-C metabolites ara-C-5′-monophosphate and ara-C-5′-triphosphate were eluted together with components of plasma. Twenty-five drug-free blood samples obtained from healthy volunteers were processed and there was no evidence that normal components of plasma interfered with the determination of ara-C and ara-U.

Identification

Retention times were used for the identification of ara-C and ara-U. The variation of retention time was less than 5%, as demonstrated with spiked samples of plasma.

Quantitation

Unknown substances were evaluated by measuring their peak areas relative to that of the external standard. For ara-C and ara-U a linear relationship between peak area and concentration was obtained for the range 0.05—120 μg/ml. The correlation coefficient of each regression was better than 0.99.

Precision

Within-run precision was established in a drug-free plasma pool. Ara-C and ara-U were added at seventeen different concentrations within the range 0.05—120 μg/ml. For each concentration the within-run precision of ten consecutive runs was determined with a coefficient of variation of less than 5%. The day-to-day precisions as determined on ten consecutive days for frozen samples of plasma at levels of 0.2 and 1.0 μg/ml were found to be 9.3 and 7.0%, respectively.

Accuracy

The recoveries from blood and plasma were 93—104% by comparison with the peak areas obtained by direct injection of the pure compounds.

Sensitivity

The sensitivity of the assay allowed the quantitation of 50 ng/ml of ara-C and ara-U in a 1-ml blood sample with a precision of better than 5%.

Speed of analysis

The analysis is performed within 15 min: 5 min for centrifugation of blood and 10 min for chromatography.

Stability of standards

Ara-C and ara-U were found to be stable in human blood and plasma in the presence of tetrahydrouridine (10^{-3} *M*) and heparin during a 4-h incubation at 37°C. The same was true on storage for 8 h in 0.05 *M* phosphate buffer (pH 7.0) at room temperature. Frozen samples of plasma (0.2 and 1 μg/ml) were stable for at least 10 months.

Stability of the column

Baseline separations of ara-C and ara-U from plasma were obtained for up to 200 injections of 20-μl samples of plasma.

Protein binding

Plasma protein binding of ara-C was found to be 14.8 ± 4.7% (mean ± S.E.) for the concentration range 0.1—100 μg/ml. This value is close to that reported by Van Prooijen et al. [37], who obtained 13.3 ± 2.2% by ultrafiltration and ultracentrifugation. Protein binding of ara-U was 5.1 ± 4.9% (mean ± S.E.).

Applicability

The HPLC method described was used for the determination of ara-C and ara-U in plasma of leukaemic patients who were receiving conventional or high doses of ara-C. A representative plot of plasma concentration versus time for a patient following a 6-h infusion of 120 mg of ara-C (70 mg/m^2) is shown in Fig. 2A. Plasma pharmacokinetics of ara-C and ara-U for a patient who was treated with infusions of high doses of ara-C (2.6 g/m^2 · 2 h) are shown in Fig. 2B.

At the end of infusions the concentration of ara-C in cerebrospinal fluid was found to be 10—15% of simultaneous plasma concentrations.

The method described in this paper is rapid, sensitive and specific. In comparison with the method of Bury and Keary [36] our procedure requires no clean-up procedures before chromatography. By using reversed-phase chromatography instead of cation-exchange chromatography, baseline separations of ara-C and ara-U from plasma constituents are obtained. The analysis is very rapid and results are obtained within 15 min. The assay can be run using as little as 100 μl of plasma, making it appropriate for clinical use even with paediatric patients.

The method is sufficiently sensitive to monitor ara-C and its metabolite at the low concentrations usually found in blood when conventional doses of ara-C are applied to patients. Plasma levels of ara-C can be monitored during intravenous infusion so that a proper infusion rate can be used for establishment of cytotoxic concentrations of ara-C for leukaemic cells. In addition,

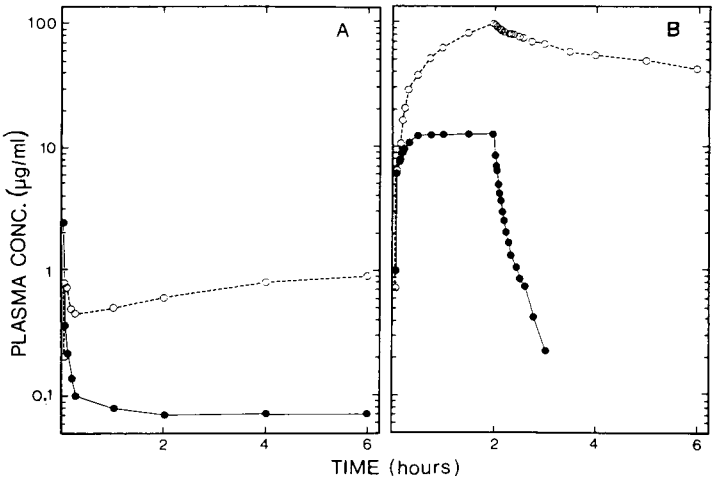

Fig. 2. Time course of plasma concentrations of ara-C (●) and ara-U (○) during and after infusions of ara-C. A, Continuous 6-h infusion of 120 mg of ara-C (70 mg/m²), which was preceded by an initial loading dose of 40 mg of ara-C (bolus injection). B, Continuous 2-h infusion of a high dose of ara-C (2.6 g/m²). Each point represents the mean value of three infusions performed at 12-h intervals.

rapid monitoring of ara-C in blood and cerebrospinal fluid is a valuable means for avoiding toxic concentrations during the infusion of high doses of ara-C.

Our assay has been in routine use for more than 1 year. It is simpler than the other methods available for the analysis of ara-C in clinical samples and can be applied to research, pharmacokinetic studies and monitoring of therapy.

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CHROMBIO. 918

DETERMINATION OF SOME PYRIDINIUM ALDOXIME COMPOUNDS BY MEANS OF ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATION IN BIOLOGICAL MATERIAL

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SUMMARY

Two reversed-phase high-performance liquid chromatographic systems are presented for the separation and assay of the pyridinium aldoximes benzyl-P2A, HI-6 and obidoxime in aqueous solutions and biological samples. The systems involve a 5-μm C_{18} silica gel stationary phase. The eluent consists of methanol, acetic acid buffer (pH 4.80), a counter ion (perchlorate or *n*-octanesulphonate) and a surfactant. The compounds were detected spectrophotometrically at 304 nm. In the concentration range used, linear plots of concentration versus extinction were obtained, both in blood and in water. Detection limits, even in blood, are satisfactory (0.5—1 μM).

Evidence is presented that, at least for HI-6, the addition of counter ions to the system does not lead to the formation of ion pairs to be retained by partition, but rather to a mechanism based on adsorption chromatography.

INTRODUCTION

Pyridinium aldoximes such as pralidoxime, 1-benzyl-2-hydroxyiminomethylpyridinium salts (benzyl-P2A) and so-called bispyridinium oximes, e.g., obidoxime and HI-6 (Fig. 1), are known to reactivate cholinesterases inhibited by organophosphates. Hence these compounds are therapeutically useful in the treatment of intoxications with organophosphates [1—3].

Until recently, the quantitative determination of these oximes, including in biological material, was usually performed by spectrophotometry under alkaline conditions (pH 10). A second method of analysis based on a trans-oximation reaction with *p*-nitrobenzaldehyde has also been described [4], but is less commonly used.

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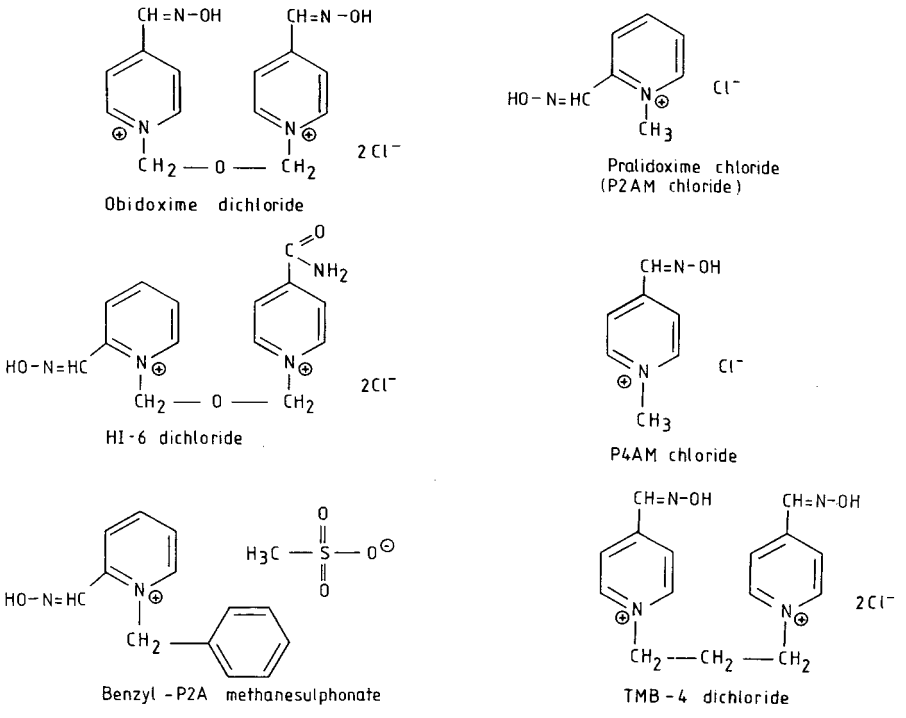

Fig. 1. Chemical structures of the pyridinium aldoximes.

As has been shown by Benschop et al. [5], the spectrophotometric method of analysis with biological materials may include the measurement of metabolites. This disadvantage can be overcome by chromatographic separation of the original product from other compounds present in the material to be investigated, and subsequent assay. This seems to be a preferable alternative. Because of the quaternary structure of the pyridinium aldoximes, ion-pair high-performance liquid chromatography (HPLC) is expected to be a suitable method of analysis for this type of compounds.

In ion-pair HPLC, an ion with a charge opposite to that of the ion to be assayed is added to the mobile phase in order to form a complex with increased lipophilic properties. The general aspects of this technique have been subject of various reviews, e.g., those by Johansson et al. [6], Gloor and Johnson [7] and Tomlinson et al. [8]. Perchlorate and *n*-alkanesulphonates are frequently used as counter anions for quaternary ammonium ions, including those in which the quaternary nitrogen atom is part of an aromatic system [8].

In order to decrease the tailing of peaks it has been suggested that compounds with detergent-like properties be added to the eluent, such as tetramethylammonium chloride, tetrapropylammonium chloride or dimethyloctylamine (DMOA) [6, 8].

Methods along these lines have proved useful, e.g., for the assay of paraquat in sunflower seeds [9]. Attempts have been made to determine oximes in aqueous solutions via analogous systems. Brown et al. [10] described ion-pair

HPLC with a mobile phase consisting of acetonitrile—aqueous buffer with *n*-heptanesulphonate as the counter anion. Their system yields only a partial separation of P4AM and TMB-4 (for chemical structures see Fig. 1).

We present here an ion-pair HPLC procedure for the determination of various quaternary oximes, which is suitable for analyses of blood. In addition, some experiments were carried out to study the nature of the chromatographic system used.

EXPERIMENTAL

Materials

The chromatographic system consisted of a Waters 6000A solvent delivery system, a Tracor 970A variable-wave length UV detector, a Rheodyne 7120 sample injector and an LDC 304-50 computing integrator coupled to a Kipp BD-41 recorder. The column was packed in our laboratory according to the procedure of Lindner et al. [11]. Column dimensions of 100 × 2.5 mm I.D. gave optimal sensitivity for the systems used. The plate number of the column for the compounds investigated in this ion-pair system was ca. 1500 per 10 cm.

HI-6 and benzyl-P2A methanesulphonate were synthesized in our laboratory. Obidoxime dichloride and the stationary phase RP-18 on 5-μm silica gel (LiChrosorb) were obtained from E. Merck (Darmstadt, G.F.R.). All other reagents were of analytical-reagent grade.

Procedures

HPLC procedure. A reversed-phase system was used to which counter ions and DMOA had been added. The stationary phase, RP-18 on 5-μm silica gel, was packed in a 100 × 2.5 mm I.D. column. The mobile phase for HI-6 and obidoxime consisted of methanol—0.1 *M* sodium acetate buffer (pH 4.80) (30:70) to which 4.6 m*M* sodium *n*-octanesulphonate and 0.3 m*M* DMOA had been added. The eluent used for benzyl-P2A did not contain methanol, but was a solution of 20 m*M* sodium perchlorate and of 0.3 m*M* DMOA in the same acetate buffer.

The flow-rate was kept constant at 0.2 ml/min, yielding a pre-column pressure of 10 bar. The injection volume was 25 μl. The detector was set at 304 nm, at which wavelength the molar absorptivity of the pyridinium aldoximes is maximal.

The stationary phase has to be well saturated with the eluent before the assay is started, because otherwise k' will decrease [9].

Work-up procedures. The procedure for preparing samples from blood was as follows. A 5-ml volume of whole blood was collected and deproteinated with 50% aqueous trichloroacetic acid (1.275 ml). After mixing on a Vortex mixer the sample was centrifuged at 28,000 *g* for 45 min. The supernatant can either be concentrated by freeze-drying before injection, or can be injected directly. The high speed of centrifugation is necessary in order to remove colloids which might cause obstruction of the HPLC circuit. In the same way other biological samples such as urine or homogenates of brains or other organs were assayed without difficulty.

Animal experiments. Oximes were given orally by oesophageal intubation to inbred rats of the WAG (small Wistar) strain. Blood was collected after decapitation.

RESULTS

The systems described provide a satisfactory method for the separation and determination of the oximes tested both in aqueous solutions and in blood*, as illustrated in Fig. 2. Detection limits and k' values are listed in Table I. Obidoxime can be used as the internal standard for HI-6, and vice versa.

Calibration graphs as obtained for the oximes in water and in blood are shown in Fig. 3. With increasing concentration the recovery from blood varies for HI-6 from 77% to 72%, for benzyl-P2A from 30% to 20% and for obidoxime from 60% to 35%. A blood level graph obtained after oral administration of benzyl-P2A methanesulphonate to the rat is shown as an example in Fig. 4.

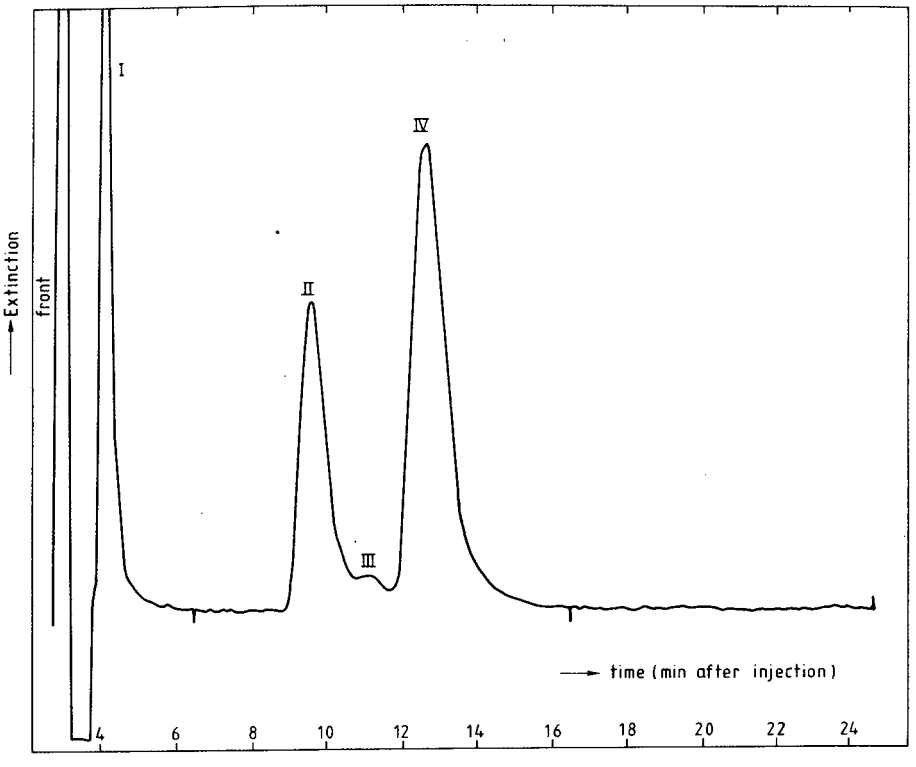

Fig. 2. Separation by ion-pair HPLC of some pyridinium aldoximes. I=benzyl-P2A; II=HI-6; III=decomposition product from the obidoxime bulk; IV=obidoxime. Eluent: methanol—0.1 *M* sodium acetate buffer (pH 4.80) (30:70), to which 4.6 m*M* sodium *n*-octanesulphonate and 0.3 m*M* dimethyloctylamine were added.

*The assay has proved to be equally reliable for homogenates of brains and other organs and for urine. We selected the assay in blood simply for description in this paper.

TABLE I

DETECTION LIMITS IN RAT BLOOD AND k' VALUES FOR ION-PAIR HPLC ANALYSIS OF PYRIDINIUM ALDOXIMES

Chromatographic conditions as in Experimental.

Compound	Detection limit (μM)*	k'
HI-6	1	2.75
Obidoxime	1	3.85
Benzyl-P2A	0.5	5.0

*Signal-to-noise ratio = 2:1.

The reproducibility of all assays was excellent over a period of at least 1 year.

Ion pairing

Two types of experiments were carried out to gain more insight into the nature of the chromatographic system used.

First, we tried to extract HI-6 by means of ion-pair extraction with *n*-octanesulphonate anion from the aqueous solution into an organic solvent. HI-6 dichloride was therefore dissolved at a concentration of 0.01 m*M* in 0.1 *M* acetate buffer (pH 4.80) containing 10 m*M* *n*-octanesulphonate anion, which was shaken for 30 min with an equal volume of an organic solvent (dimethyl ether, methyl isopropyl ketone or chloroform). A pH of 4.80 was chosen because at this pH the carboxamide group is not protonated, whereas the oxime function is undissociated. Concentrations were measured by HPLC as described above in both the aqueous and the organic phase. With none of these combinations could HI-6 be extracted to any extent into the organic phase as an ion pair with *n*-octanesulphonate anion.

Secondly, we measured k' as a function of the *n*-octanesulphonate anion concentration (1—18.5 m*M*) in the system described above. When log k' is plotted against log[anion], it appears that for our system log k' is proportional to log[anion] with a correlation coefficient of 0.999 (Fig. 5). This relationship is known as the Freundlich equation [12, 13].

DISCUSSION

The HPLC methods described here are very reliable for the assay of the oxime compounds investigated. Results for both chemical and biological purposes are satisfactory. Reasonable detection limits (0.5—1 μM) and good separations are obtained. Linearity and reproducibility are both excellent. Tailing of peaks is not completely absent, but this phenomenon is frequently met in so-called ion-pair chromatographic reversed-phase systems [9], and may also be due to the small diameter of the column.

All compounds are sufficiently stable in the trichloroacetic acid medium.

In a partition chromatographic system the retention of an ion pair depends on its lipophilic properties. Hence, k' should be inversely proportional to the concentration of the anion [12, 14]. When, on the other hand, the counter ion interferes with the adsorptive properties of the stationary phase, the system should follow the Freundlich equation [12, 13], in which case log k' should be proportional to log[anion].

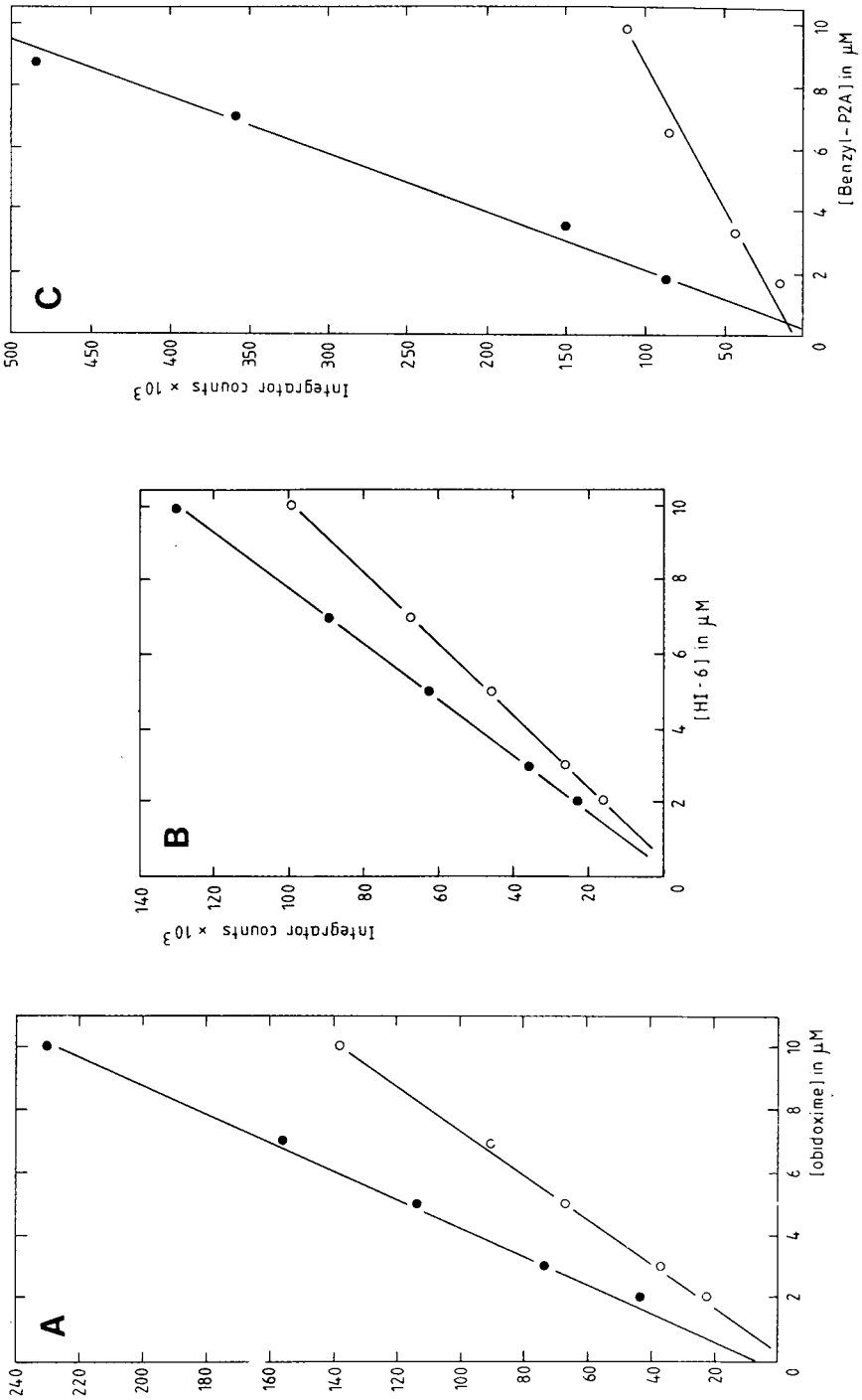

Fig. 3. Recoveries of various oximes in blood (○) compared to aqueous solution (●). A = HI-6; B = obidoxime; C = benzyl-P2A. Each point is the mean of at least five experiments; the standard deviations are too small to be represented. Blood samples were deproteinated with trichloroacetic acid. Eluent: for HI-6 and obidoxime, methanol—0.1 *M* sodium acetate buffer (pH 4.80) (30:70) containing 4.6 m*M* sodium *n*-octanesulphonate; for benzyl-P2A, aqueous sodium acetate buffer (pH 4.80) with 20 m*M* sodium perchlorate. Both eluents contained 0.3 m*M* dimethyloctylamine.

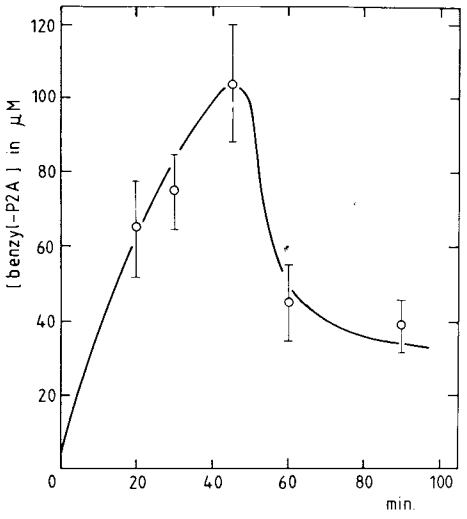

Fig. 4. Blood levels of benzyl-P2A after oral administration of 100 mg/kg to rats. Each point represents the average for 10 rats with the standard error of the mean. Eluent: 0.1 *M* acetate buffer (pH 4.80) to which 20 m*M* sodium perchlorate and 0.3 m*M* dimethyloctylamine had been added.

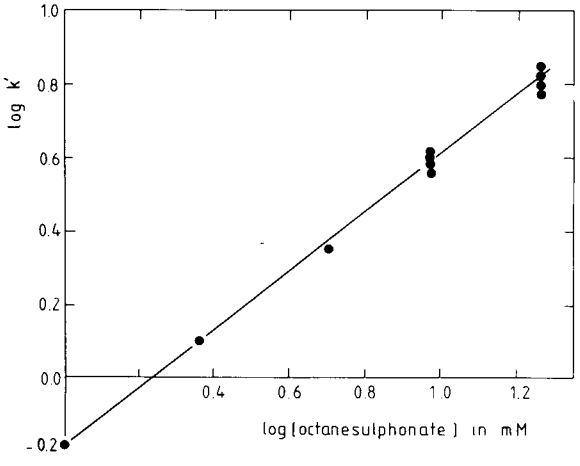

Fig. 5. Log [*n*-octanesulphonate] plotted against log *k'* for HI-6. Each value was determined from four measurements, which cannot be drawn separately at the lower levels of log [*n*-octanesulphonate]. Eluent: methanol—0.1 *M* sodium acetate buffer (pH 4.80) (30:70) containing 0.3 m*M* dimethyloctylamine. Slope, 0.79; standard deviation, 1%; correlation coefficient, 0.999.

The excellent correlation of our chromatographic system for HI-6 with the Freundlich equation, combined with the negative result of the attempt to extract HI-6 as an ion pair into an organic phase, suggests that this system does not work by ion-pair chromatography, but by adsorption chromatography. Evidently, the counter ion modifies primarily the adsorptive properties of the stationary phase, rather than forming a lipophilic ion pair with the pyridinium

cation. Others, such as Kraak et al. [15], Bidlingmeyer et al. [16] and Greving et al. [12], have already pointed out that similar systems do not obey the laws of partition chromatography.

Seen in the light of their and our results, it seems advisable that claims of performing ion-pair HPLC should be supported by clear experimental evidence.

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DETERMINATION OF THE α,β-ADRENOCEPTOR BLOCKER YM-09538 IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic method for the determination of the α,β-adrenoceptor blocker 5-{1-hydroxy-2-[2-(*o*-methoxyphenoxy)ethylamino]ethyl}-2-methylbenzenesulphonamide hydrochloride (YM-09538) in plasma, using 5-di-*n*-butylaminonaphthalene-1-sulphonyl chloride as a reagent for fluorescence labelling, is described. The detection limit is 20 ng/ml, which is sensitive enough to determine YM-09538 plasma levels after the oral administration of effective doses to dogs and humans.

INTRODUCTION

Imai et al. [1] have introduced various phenylethylamine derivatives as combined α- and β-adrenoceptor antagonists. Among these derivatives, 5-{1-hydroxy-2-[2-(*o*-methoxyphenoxy)ethylamino] ethyl}-2-methylbenzenesulphonamide hydrochloride (YM-09538) showed dose-dependent antihypertensive effects in rats and dogs without inducing tachycardia [2] which, as a homeostatic reflex mechanism [3], generally occurs after the administration of vasodilating drugs. For pharmacological and biopharmaceutical studies on YM-09538, a simple and sensitive method for assaying its concentration in plasma is required.

In order to increase the sensitivity of high-performance liquid chromatography (HPLC), derivatization to introduce a suitable fluorophore is necessary. Of various reagents for fluorescence labelling, 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dans-Cl) has been widely used and applied in HPLC [4, 5]. A structurally related reagent, 5-di-*n*-butylaminonaphthalene-1-sulphonyl chloride (Bans-Cl) [6] has been used in combination with thin-layer chromato-

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graphy (TLC) or mass spectrometry to determine amino acids or some other amines [7—9]. In the present paper an HPLC method for determining YM-09538 concentrations in plasma using Bans-Cl is described.

EXPERIMENTAL

Chemicals

YM-09538 (Fig. 1, I) and the structurally related compound (II), which was used as the internal standard (I.S.), were synthesized in our laboratory by the method of Imai et al. [1].

Bans-Cl (Fig. 1, III) and Dans-Cl were purchased from Tokyo Kasei (Tokyo, Japan). All other reagents and solvents used were of analytical-reagent grade.

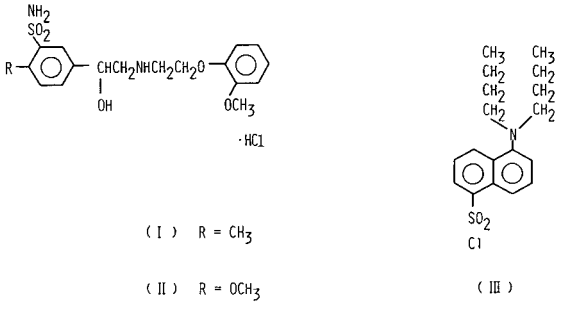

Fig. 1. Chemical structure of YM-09538 (I), I.S. (II) and Bans-Cl (III).

HPLC

The chromatograph used for HPLC consisted of a TWINCLE high-pressure pump (Jasco, Tokyo, Japan), a Model U6K injector (Waters Assoc., Milford, MA, U.S.A.) and a stainless-steel column (15 cm × 4 mm I.D.) packed with LiChrosorb SI-60 (particle size 5 μm) (Merck Japan, Tokyo, Japan). Fluorescence was measured at 500 nm with the 365-nm line of a medium-pressure mercury lamp, using a Model FP-110 spectrofluorimeter (Jasco). The mobile phase was benzene—methanol (50:1); the flow-rate was 2 ml/min. When many samples were analysed, a WISP 710A automatic sampler (Waters Assoc.) was employed for sample injections.

Assay procedure

To 1 ml of plasma were added 2 ml of an aqueous solution containing 1 μg of I.S. and then about 0.5 g solid $NaHCO_3$. The mixture was extracted with 4 ml of ethyl acetate, centrifuged and the ethyl acetate layer was evaporated to dryness under reduced pressure. To the residue were added 0.1 ml of an aqueous solution containing 300 μg sodium hydrogen carbonate and 0.2 ml of acetone containing 100 μg of Bans-Cl. After 90 min at 45°C, 4 ml diethyl ether were added, the mixture was washed with 3 ml of distilled water for 10 sec and the diethyl ether layer was evaporated to dryness at 40—50°C. The residue was dissolved in 0.1 ml of benzene and 5—10 μl of the solution were injected into the HPLC system.

Calibration was effected by applying the procedure to prepared plasma stan-

dards containing various concentrations of YM-09538 (20—1000 ng).

Physical properties and reaction conditions for the YM-09538 Bans derivative

YM-09538 (2 mg) was subjected to reaction with Bans-Cl as described above and the derivative was purified on a 100-mesh silica gel column (10 cm × 1 cm I.D.). Elution was performed with benzene—methanol (20:1).

Excitation and emission spectra of the derivative, dissolved in the HPLC mobile phase, were obtained on a Hitachi MPF-4 fluorescence spectrophotometer equipped with a xenon lamp. The slits were adjusted to 5.5 nm for both excitation and emission; the scanning speed was 20 nm/min. To confirm the structure of the fluorescent compound, we used a Hitachi RMU-6M mass spectrometer at an emission current of 40 μA, an electron energy of 20 eV, an ion source temperature of 170°C and an accelerating voltage of 1.3 kV.

The optimal derivatization time and temperature were evaluated as follows. Plasma extracts, each containing 500 ng of YM-09538, were dissolved in the reaction medium described under Assay procedure and incubated at 60°C, 45°C or room temperature (about 15°C). After appropriate intervals, each sample was admixed with 4 ml of diethyl ether, 0.5 ml of ethyl acetate containing 500 ng of the I.S. Bans derivative and 3 ml of distilled water, and then centrifuged. The organic layer was analysed by HPLC as described above and the peak-height ratios were plotted against incubation time.

Animal and human studies

After an overnight fast, four male beagles, weighing 11.5—12.5 kg, received 3 mg/kg of YM-09538 as a 0.3 mg/ml aqueous solution via a stomach tube. In the human study, three male volunteers, 24—35 years of age, received 50-mg YM-09538 tablets orally after overnight fasting. After appropriate intervals, blood samples were collected by venipuncture, using heparinized syringes, centrifuged and the plasma was stored frozen until taken for assay.

RESULTS AND DISCUSSION

Extraction of YM-09538 from plasma

The extraction of YM-09538 from plasma, using ethyl acetate, was pH dependent. At pH 7.5—8.5 the extraction was efficient, but there was a marked decrease in recovery at pH values below 7 (Fig. 2). Similar extraction curves were obtained with the other organic solvents examined. Maximal recoveries obtained at pH 8.0 with diethyl ether, chloroform and *n*-hexane were 55, 70 and 40%, respectively; almost 100% of the administered drug was extracted at pH 8.0 with ethyl acetate. To simplify the present assay procedure, we added solid sodium hydrogen carbonate to obtain an aqueous alkaline solution and achieved good results. Upon saturating a mixture consisting of 1 ml of plasma and 2 ml of aqueous I.S. solution with sodium hydrogen carbonate, the pH was about 8.5.

Studies on YM-09538 Bans derivative

Mass spectrometric analysis demonstrated that the fluorescence derivative was the mono-Bans derivative of YM-09538. It showed molecular ions at *m/z*

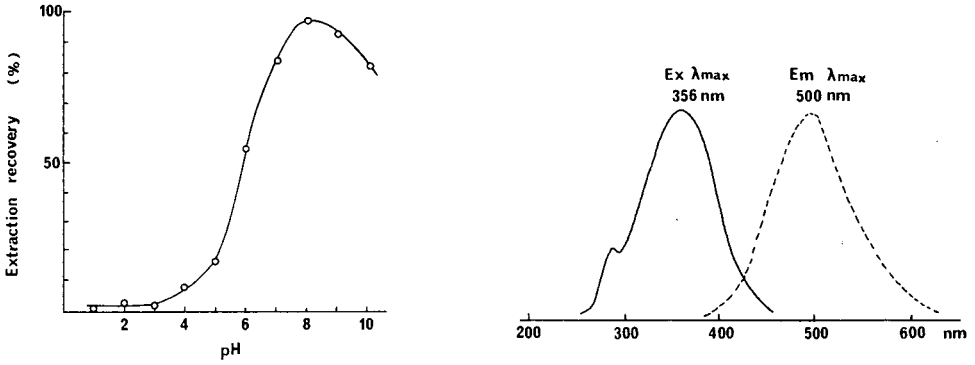

Fig. 2. Effect of pH on the extraction of YM-09538 from dog plasma, the pH of which was adjusted by adding 0.1—1.0 *N* HCl or 0.1—1.0 *N* NaOH solution. The YM-09538 extracted was quantitated by HPLC as described in the text.

Fig. 3. Excitation and emission spectra of the YM-09538 Bans derivative.

697, and the characteristic fragment ions of $(M-C_3H_7)^+$ at *m/z* 654, which had facilitated the discovery of the molecular ions in the spectra of Bans derivative mixtures of amino acids [6]. Mass fragmentation analysis (*m/z* = 213, 255, 373 and 497) confirmed that Bans was attached at the secondary amino group of YM-09538.

The excitation and emission spectra of the YM-09538 Bans derivative in the mobile phase are shown in Fig. 3. As the maximal excitation wavelength was 356 nm, the most intense emission lines of a mercury lamp at 365 nm could be used. When the maximal excitation wavelength of a compound of interest is close to one of the intense emission lines of a mercury lamp, for HPLC we advise the use of a fluorescence detector equipped with a mercury lamp rather than a xenon lamp, based on considerations related to the stability of the line and the size and cost of the instrument. In this study, fluorescence was measured at the maximal emission wavelength (500 nm).

At 45°C, the reaction of Bans-Cl with YM-09538 was quantitative within 60 min, whereas at room temperature, it was incomplete after incubation for 80 min (Fig. 4). When the mixture was incubated at 60°C, the reaction rate was higher than at 45°C, but, within 10 min the peak-height ratio reached a plateau, which was about 40% lower than at 45°C, and no further increase in the peak-height ratio was observed even on prolonging the incubation to 2 h. It appears that the reagent is decomposed rapidly at 60°C. Concomitantly, the yellow colour of the reagent disappeared on incubating the reaction medium for 10 min at 60°C. Therefore, in the analysis of plasma samples, the reaction mixtures were incubated at 45°C for 90 min to ensure complete derivatization.

HPLC

During derivative formation, Bans-Cl gives as many fluorescent side-products as Dans-Cl. With YM-09538, most of these side-products remained in the aqueous layer when the reaction mixture was shaken with diethyl ether and

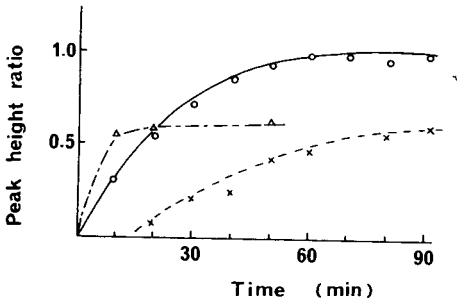

Fig. 4. Reaction of YM-09538 with Bans-Cl at 60°C (△ — · — △), at 45°C (○——○) and at room temperature (× — — — ×).

water. However, some fluorescent side-products were extracted into the organic layer and had to be separated from the YM-09538 and I.S. derivatives. HPLC proved very useful for this purpose.

The YM-09538 Bans derivative could be analysed by either normal-phase or reversed-phase chromatography. When the latter method was employed, the peak of interest was separated from interfering peaks on an RP-18 column (Merck Japan), using acetonitrile—water (1 : 1) as the eluent. However, the time required for separation exceeded 15 min and the sensitivity of this method was relatively low (limit of detection ca. 100 ng/ml).

The application of normal-phase chromatography was more favourable. The chromatograms obtained from human plasma samples are shown in Fig. 5. The Bans derivatives of YM-09538 and I.S. gave sharp peaks at retention times of 2.9 and 4.0 min, respectively; the drug-free control plasma gave no interfering peaks. When the peak-height ratios of YM-09538 and I.S. were plotted against the amount of YM-09538 added to the standard samples, a linear response was obtained over the concentration range 20—1000 ng/ml. Regression analysis of

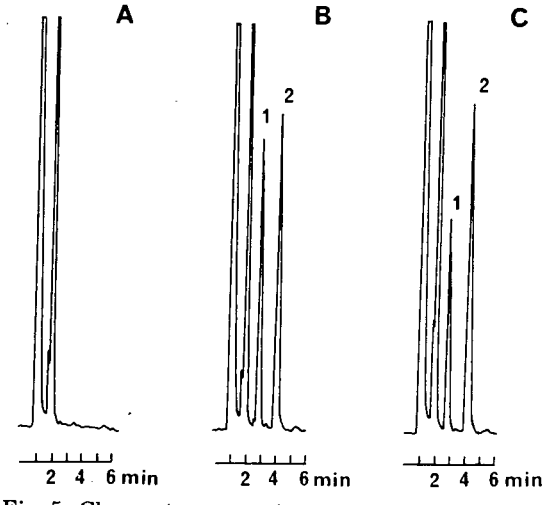

Fig. 5. Chromatograms of (A) control human plasma, (B) control plasma spiked with 0.5 μg of YM-09538 and 1 μg of I.S. and (C) plasma obtained from a volunteer 3 h after oral administration of 50 mg of YM-09538. Peaks: 1 = YM-09538; 2 = I.S.

these data gave slope 0.0018, intercept 0.023 and correlation coefficient 0.9987 (n = 18). The intra-assay coefficient of variation over the range 20–1000 ng/ml was less than 2.6% and the inter-assay coefficient of variation at 1000 ng/ml was 1.8% (n = 6).

Comparison of YM-09538 Bans and Dans derivatives

YM-09538 was subjected to reaction with Dans-Cl in a manner similar to that of YM-09538 with Bans-Cl and the natures of the two compounds were compared. The maximal excitation wavelength of the Dans derivative was 350 nm; that of the Bans derivative, at 356 nm, was closer to the line of the mercury lamp used (365 nm). This observation accords with the results of Seiler et al. [6], who pointed out the advantages of using Bans-Cl rather than Dans-Cl in the determination of various amines. The maximal emission wavelength of the Dans derivative was 505 nm.

The Dans derivative was more polar than the Bans derivative. Both compounds were subjected to HPLC and we found the Dans derivative to be eluted with a retention time of 4.1 min and the Bans derivative was eluted at 2.9 min.

Application of the method

The application of the method to determinations of plasma levels in dogs and humans is demonstrated in Fig. 6. After the oral administration of YM-09538, the plasma concentration in dogs and humans reached its maximum at 0.5 and 2 h, respectively; elimination half-lives were about 2.1 and 3.5 h, respectively. When the areas under the plasma concentration–time curves per dose were compared, the values in humans were 20–30 times higher than those in dogs.

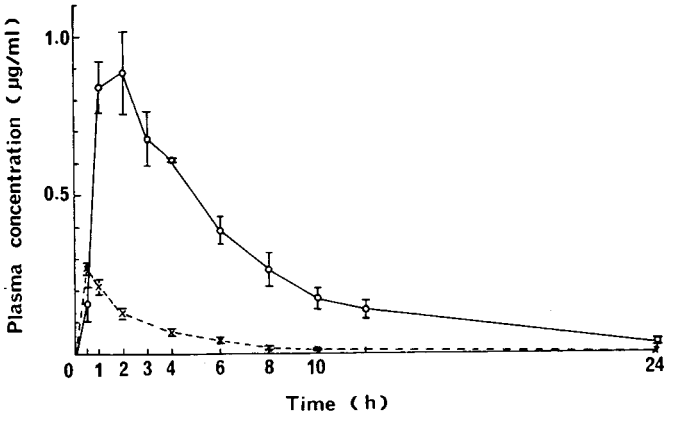

Fig. 6. Plasma concentration of YM-09538 in dogs after oral administration of 3 mg/kg (× – – – ×) and in humans after oral administration of 50 mg (o———o). Each point represents the mean ± S.E.M. of 3–4 experiments.

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MEASUREMENT OF SULINDAC AND ITS METABOLITES IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive high-performance liquid chromatography assay for simultaneous measurement of sulindac and its major metabolites was developed. The extraction methods provided greater than 89% recovery of sulindac and its sulfone and sulfide metabolites from both plasma and urine. Complete resolution and accurate detection of the three compounds was achieved with a reversed-phase column, UV detection at 254 nm and a methanol—acetate buffer mobile phase. Levels of sulindac and its metabolites were determined in plasma and urine from four volunteers after oral administration of 200 mg Clinoril. Glucuronide conjugates in urine were measured after alkaline hydrolysis.

INTRODUCTION

Sulindac is a nonsteroidal, anti-inflammatory agent efficacious in the acute and long-term treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and gouty arthritis [1—5]. When administered orally, sulindac is five to ten times more potent than aspirin in diminishing painful inflammation and, yet, causes significantly less gastrointestinal irritation and fecal blood loss than therapeutically equivalent doses of aspirin [6—9]. The sulfoxide moiety of sulindac (Fig. 1) can be reversibly reduced and irreversibly oxidized in vivo to form sulfide and sulfone metabolites, respectively [10, 11]. In animals, the sulfide metabolite is substantially more potent than sulindac as an anti-inflammatory agent, while the sulfone metabolite is pharmacologically inactive [12]. Sulindac theoretically functions as a prodrug in man and induces less gastrointestinal toxicity because its active metabolite is not present in large quantities in the intestinal lumen. In man, sulindac is eliminated primarily in the urine as intact sulindac, sulindac sulfone and

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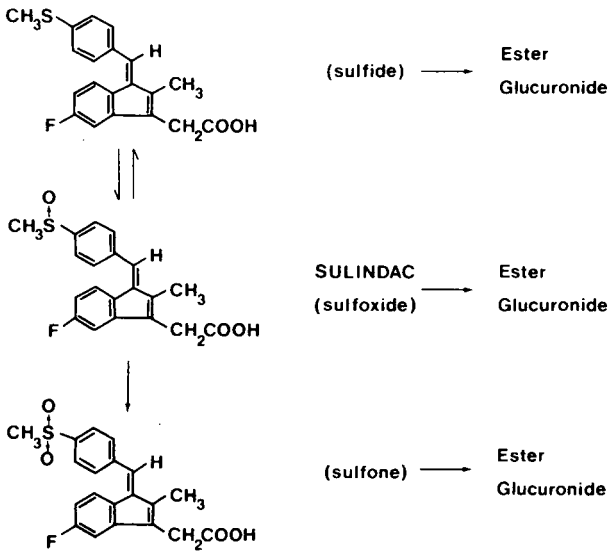

Fig. 1. Metabolism of sulindac in man.

their respective glucuronides [11]. Studies on sulindac pharmacokinetics should properly include measurement of all three oxidative forms of sulindac. Previously reported assay methods have often been too tedious and/or impractical for routine analysis of clinical samples. The mass spectrometric method of Walker et al. [13] offers insufficient accuracy at lower concentrations (< 0.5 μg/ml). The radioimmunoassay procedure of Hare et al. [14] requires lengthy extraction and paper chromatography steps in order to achieve assay specificity. Gas chromatography with electron-capture detection has been employed for measuring sulindac and its metabolites, but this method requires pre-column derivatization and does not adequately resolve sulindac from its sulfone metabolite [15]. Dusci and Hackett [16] have utilized high-performance liquid chromatography (HPLC) with UV detection (254 nm) for simultaneous quantitation of sulindac and its metabolites in serum. However, their method includes a time-consuming evaporation step and is not applicable to assay of urine samples.

Our objective has been to develop a sensitive HPLC assay for quantitation of sulindac and its metabolites in both plasma and urine.

MATERIALS AND METHODS

Chemicals

Sulindac, sulindac sulfone, sulindac sulfide and the *cis*-5-chloro analogue of sulindac sulfide were all obtained from Merck Sharp & Dohme Research Labs. (West Point, PA, U.S.A.). These compounds were found to be > 99% pure by HPLC with UV detection at 254 nm except for a minor impurity (about 3%) in the 5-chloro sulfide analogue. Standard solutions were prepared in methanol and were found to be stable at 4°C. Methanol and 1-chloro-

butane were both HPLC grade solvents (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Drug administration

Four male volunteers (ages 30—45 years) each received a 200-mg Clinoril® tablet (MSD) from the same lot after overnight fasting. Blood samples were periodically withdrawn from a forearm vein through an indwelling catheter and immediately placed in a heparinized centrifuge tube (Venoject, Kimble-Terumo, Elkton, MD, U.S.A.). Samples were centrifuged and plasma was stored at −20°C. Urine was also collected at specified intervals, beginning with a pre-drug sample. The volume of each urine sample was measured promptly after collection and then stored at −20° C.

Extraction of plasma

One ml plasma, 100 μl methanol (containing standards when preparing standard curve) and 50 μl internal standard solution (0.03 mg/ml, 5-chloro sulfide analogue in methanol) were each placed in a 40-ml glass extraction tube and thoroughly mixed. 1-Chlorobutane (25 ml) and 1.0 *N* hydrochloric acid (150 μl) were then added, and the tube shaken for 10 min on a mechanical shaker. After phase separation by centrifugation at 1500 *g* for 10 min, 20 ml of the 1-chlorobutane layer were transferred to a second 40-ml centrifuge tube containing 250 μl 0.4 *N* sodium hydroxide. This tube was shaken mechanically for 10 min and then centrifuged at 1500 *g* for 10 min. The final 1-chlorobutane layer was discarded by aspiration and 50 μl of the aqueous phase was injected into the chromatograph.

Extraction and hydrolysis of urine

For measurement of unconjugated compounds, 200 μl urine, 100 μl methanol (containing standards when preparing standard curve) and 50 μl internal standard solution were thoroughly mixed in a 40-ml glass extraction tube. 1-Chlorobutane (25 ml) and 0.5 *N* sodium citrate buffer (pH 3.0, 1.0 ml) were then added. After mechanical shaking for 10 min and centrifugation for 10 min at 1500 *g*, 20 ml of the organic phase were transferred to another 40-ml extraction tube containing 250 μl 0.4 *N* sodium hydroxide. This tube was again shaken and centrifuged, and the 1-chlorobutane layer was discarded. Fifty microliters of the aqueous phase were injected into the chromatograph.

For measurement of conjugated compounds, 4.0 ml urine were first mixed with 600 μl 1 *N* hydrochloric acid and washed twice with 10-ml portions of 1-chlorobutane. Two milliliters of solvent-washed urine were then alkalinized with 200 μl 4 *N* sodium hydroxide and kept at room temperature for 30 min. A 150-μl aliquot of 5 *N* hydrochloric acid was then added, and aglycones were extracted from 200 μl of this solution according to the method for unhydrolysed urine.

Reversed-phase chromatography

Instrumentation included a Spectra-Physics Model 8000 high-performance liquid chromatograph equipped with a UV detector (254 nm), oven (29°C), electronic peak integrator and a Spherisorb 10-μm ODS column (Applied

Science, State College, PA, U.S.A.). The mobile phase consisted of methanol—0.4 *N* sodium acetate buffer (pH 4.0) with a flow-rate of 1.2 ml/min. An initial volume ratio of 63:37 was maintained for 6 min to isocratically elute sulindac and its sulfone metabolite, and then increased to 80:20 to elute sulindac sulfide and the sulfide internal standard.

RESULTS

Evaluation of assay methods

Typical chromatograms for extracts of human plasma and urine, with and without standards, are shown in Figs. 2 and 3. Small peaks in plasma interfered somewhat with the sulindac peak. This interference was equivalent to 0.05—0.25 μg/ml (mean value, about 0.10 μg/ml) sulindac in plasma from six persons who had not received Clinoril and was found to remain constant throughout the day for any one individual. Thus, even low concentrations (0.1—0.2 μg/ml) can be reported for sulindac in plasma provided a pre-drug blank is obtained and subtracted from post-drug values. No background peaks in plasma interfered with the assay of the two metabolites; hence, the lower limit of sensitivity for these compounds was less than 0.1 μg/ml in plasma. Peaks found in normal urine did not interfere with any of the four standard peaks, and lower limits of assay sensitivity were less than 0.2 μg/ml for each compound in urine.

Extraction recovery was determined by comparing detector response to standards injected directly on the column with the response to standards extracted from plasma and urine. Mean recoveries for sulindac, sulindac sulfone and sulindac sulfide were 89, 92 and 93% from biological samples, respective-

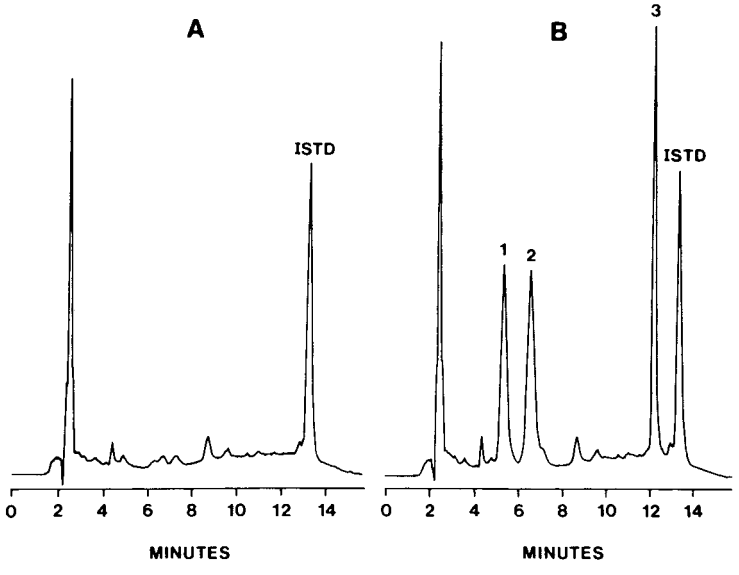

Fig. 2. Chromatograms of plasma extract for blank plasma with internal standard (ISTD) (A) and for plasma spiked to 2 μg/ml each of sulindac sulfone (1), sulindac (2) and sulindac sulfide (3) plus 1.5 μg/ml internal standard (B).

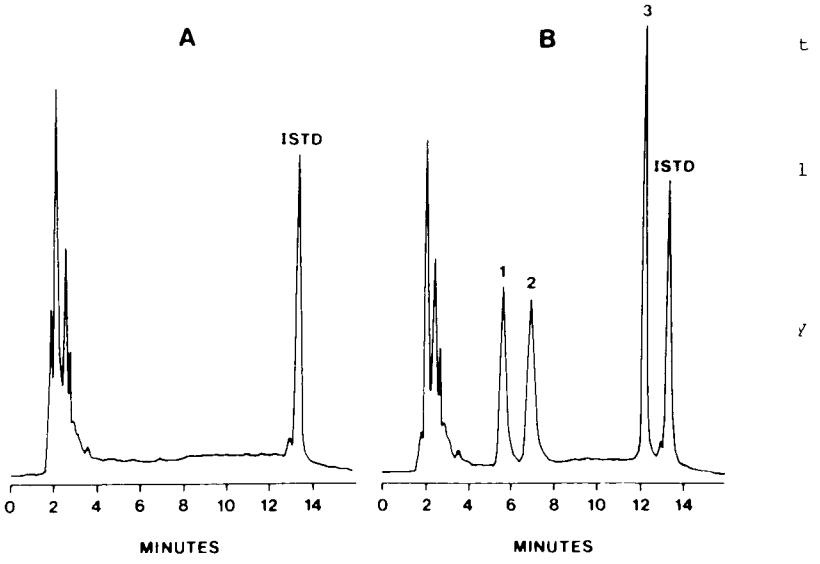

Fig. 3. Chromatograms of urine extract for blank urine with internal standard (ISTD) (A) and for urine spiked to 10 μg/ml each of sulindac sulfone (1), sulindac (2) and sulindac sulfide (3) plus 7.5 μg/ml internal standard (B).

ly. Recoveries for sulindac and sulindac sulfone were constant over the entire concentration range of investigation; therefore, peak areas without internal standardization were used as the basis of quantitation. Recovery of the sulfide metabolite was more variable; however, the sulfide internal standard compensated well for this variation. Standard curves for the various compounds were linear up to at least 40 μg/ml in plasma and 100 μg/ml in urine. Correlation coefficients (least squares method) for plasma standard curves (0.1, 0.4, 1.0, 2.0, 5.0 and 10.0 μg/ml) were typically 0.9998, 0.9997 and 1.000 for sulindac, sulindac sulfide and sulindac sulfone, respectively. Correlation coefficients were equally good for urine standard curves (0.2, 1.0, 2.0, 5.0, 10.0, 25.0 and 50.0 μg/ml). Random variability for the assay methods was

TABLE I

VARIATION IN REPLICATE STANDARDS EXTRACTED FROM HUMAN PLASMA AND URINE

Type of sample	Concentration (μg/ml)	Coefficient of variation (%)		
		Sulindac	Sulindac sulfone	Sulindac sulfide
Plasma (N = 4)	0.4	2.5	6.3	11
	2.0	2.4	1.0	6.9
	10	3.8	3.4	0.7
Urine (N = 6)	5.0	3.7	4.1	
	50	2.7	2.7	

assessed by preparing in replicate high and low standards in both urine and plasma (Table I).

Aspirin and indomethacin (at concentrations up to 50 μg/ml) did not interfere with the assay for sulindac and its metabolites.

Application of assay to clinical samples

The concentrations of sulindac and its two major metabolites were measured in plasma after a single 200-mg dose of Clinoril (Fig. 4). The magnitude

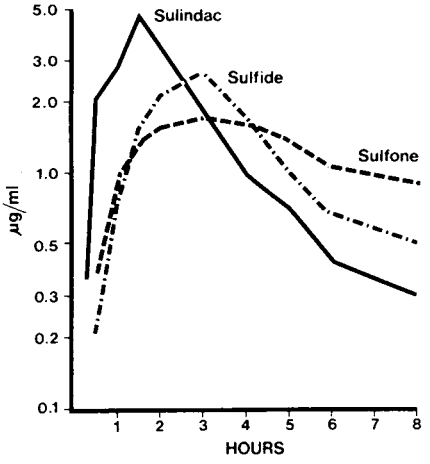

Fig. 4. Concentrations of sulindac, sulindac sulfone and sulindac sulfide in plasma after oral administration of 200 mg Clinoril. Mean values for four subjects are presented.

and shape of the concentration—time curves are similar to that of previously published work [11]. Table II indicates the urinary concentration and total excretion of sulindac and its various metabolites for 8 h after a 200-mg dose of Clinoril. A comparison between this table and historical results is not possible, since earlier work on the urinary excretion of sulindac after a 200-mg dose did not distinguish between glucuronide metabolites and unconjugated sulindac and sulindac sulfone at early time intervals.

DISCUSSION

We have described a sensitive HPLC method which can be readily applied to studies on the bioavailability, metabolism and clearance of sulindac in man. The advantages of this method over a previously published HPLC technique [16] can be attributed to an improved extraction procedure. Thus, the previous method is not applicable to urine samples, includes a time-consuming evaporation step and, due to excessive UV-absorbing contaminants in the solvent front, elutes sulindac and sulindac sulfone prior to achieving a level detector baseline. In addition, the previous assay did not recommend daily extraction of standards from serum, despite intersubject variation in pre-drug serum blanks. For the present work, we have utilized the most universally available UV detector wavelength (254 nm). However, we have recent-

TABLE II

EXCRETION OF SULINDAC AND ITS METABOLITES AFTER 200 mg CLINORIL, ORALLY

Values represent means for 4 subjects. Only trace amounts of sulindac sulfide and its glucuronide were found in urine.

	0—1 h	1—2 h	2—3 h	3—4 h	4—6 h	6—8 h	Total 0—8 h
Sulindac (μg/ml)	42.5	148	100	43.5	13.6	6.44	
(mg)	2.11	6.42	3.72	1.40	1.29	0.66	15.6
Sulindac glucuronide* (μg/ml)	56.1	177	126	67.7	21.3	8.45	
(mg)	1.74	5.63	3.56	1.75	1.54	0.52	14.7
Sulfone (μg/ml)	1.49	7.02	8.18	9.79	3.89	2.55	
(mg)	0.07	0.23	0.25	0.30	0.37	0.25	1.47
Sulfone glucuronide* (μg/ml)	13.4	59.5	62.3	63.3	36.3	24.4	
(mg)	0.55	1.78	1.90	1.78	2.88	2.15	11.0

*Conjugate values are reported as mg or μg/ml of aglycone measured after hydrolysis.

ly found that detection at 280 nm can provide more accurate measurement for low concentrations of sulindac, since, at this wavelength, background peaks from plasma do not interfere with any of the drug assay peaks. While detector sensitivity for sulindac sulfide is diminished at 280 nm relative to 254 nm, the assay is still fully reliable down to 0.1 μg/ml for each compound in plasma.

Automation of the assay with an autosampler (Waters Intelligent Sample Processor Model 710B) allows one worker to process over 60 samples (including standards) each day. We now routinely use sodium metabisulfite solution (0.5% in 0.4 *N* sodium hydroxide, prepared daily) instead of 0.4 *N* sodium hydroxide for the back-extraction procedure, as this completely abolishes the slow oxidation of sulindac and sulindac sulfide that can occur during overnight exposure of these compounds to dilute alkali.

In conclusion, we have found that by introducing an improved extraction procedure, HPLC with UV detection can be utilized for measuring sulindac and its metabolites in both plasma and urine. In fact, no other method presently offers the same high degree of assay sensitivity, ease of execution and adaptability.

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CHROMBIO. 927

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CLOMIPHENE USING POST-COLUMN ON-LINE PHOTOLYSIS AND FLUORESCENCE DETECTION

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SUMMARY

A method has been developed for the extraction and quantitation of the ovulatory stimulant drug clomiphene from blood plasma. The *cis*- and *trans*-isomers were separated by normal-phase chromatography using chloroform—methanol as the mobile phase. After eluting from the column, the clomiphene was passed through a PTFE photolysis coil irradiated by a powerful UV lamp, resulting in conversion of the isomers to highly fluorescent species. The derivatised material was then detected using a fluorescence spectrometer. Use of this method enables a substantial improvement in sensitivity over UV detection and has permitted the measurement of plasma clomiphene levels in patients receiving clomiphene therapy.

INTRODUCTION

Clomiphene (Clomid, Merrell, Fig. 1) is a non steroidal triphenylethylene compound which is currently used as an ovulatory stimulant. It is the drug of choice in the normoprolactinaemic anovulatory woman who has gonadotrophin and oestrogen production. In properly selected patients 70% can be expected to ovulate, however the risk of multiple pregnancy in those who conceive is about 20% [1].

The mode of action of the drug is not clearly understood but it appears that clomiphene stimulates the release of gonadotrophin by a direct action

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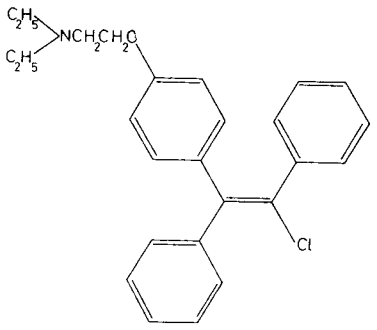

Fig. 1. Structure of clomiphene.

on the hypothalamic/pituitary axis and by reducing the inhibitory influence of endogenous oestrogens.

Studies with ^{14}C-labelled clomiphene [2, 3] indicate that it is readily absorbed after oral administration and is excreted mainly via the faeces. The half-life for the administered radioactivity in an oral tracer dose is five days. However, the drug is still present in the faeces up to six weeks following administration, and thus enterohepatic recycling is suspected [4]. No information is available on the plasma levels present after a single dose but studies of a closely related drug, tamoxifen, have indicated concentrations of 80 pmol/ml after a single dose of 10 mg/m^2 body surface area [5]. After administration of a single dose of 100 mg of clomiphene citrate, peak concentrations of 25—250 pmol/ml of plasma could therefore be expected. Thus an analytical method capable of detecting clomiphene plasma levels below 1 ng/ml is required.

Clomiphene as normally administered consists of a mixture of the *cis*- and *trans*-isomers. In order to derive meaningful pharmacokinetic data from such samples, the method utilised to determine the drug should be able to distinguish between the two isomers, as studies have shown that only the *cis*-isomer is active [6, 7]. Clomiphene however has a relatively low extinction coefficient for UV absorption (ϵ = 10,450 l/mol cm at 298 nm) which would not permit quantitation of nanogram amounts of the drugs using UV detection.

Recent work on tamoxifen [5, 8—10] has made use of the conversion of stilbene-based molecules by UV irradiation to highly fluorescent phenanthrene derivatives. This conversion has also been used in the assay of diethylstilbestrol [11].

In this paper we describe a method to analyse clomiphene levels in plasma. The isomers are extracted from the plasma and separated by high-performance liquid chromatography (HPLC) on a silica column. The eluted isomers are passed through a photolysis coil irradiated by a powerful UV lamp, resulting in conversion of the isomers to highly fluorescent species. These derivatised products are then detected using a fluorescence spectrophotometer. The use of photochemical reactions for post column derivatization has recently been reviewed [12].

EXPERIMENTAL

Reagents

Analytical-grade chloroform, methanol and diethyl ether were redistilled before use. It was necessary to ensure that the chloroform and methanol used in the HPLC mobile phase were very carefully dried to maintain constant retention times. Thus methanol was redistilled from magnesium and iodine and stored over molecular sieve while chloroform was redistilled from anhydrous calcium chloride and stored over molecular sieve. Diethyl ether was redistilled and stored over sodium. *trans*-Clomiphene citrate and clomiphene citrate (a mixture of the *cis*- and *trans*-isomers) were generously donated by Wm.S. Merrell Company (Sydney, Australia).

Equipment

The HPLC system (Fig. 2) incorporated a Perkin-Elmer Series 3B pump fitted with a septum injector, and either a Perkin-Elmer Model 3000 or a Model 650-10S fluorescence spectrometer. For some experiments a Perkin-Elmer Model LC75 UV detector was used. Separation was achieved on a DuPont Zorbax (6 μm) Sil silica column (25 cm $\times$ 4.6 mm I.D.). The mobile phase was chloroform—methanol (80:20). The photochemical reactor consisted of a 3-m length of 0.3 mm internal diameter PTFE-tubing wound around the silica window section of the cooling waterjacket on an Hanovia medium-pressure photochemical mercury lamp. The exposed sections of the lamp were wrapped in aluminium foil to reduce stray radiation.

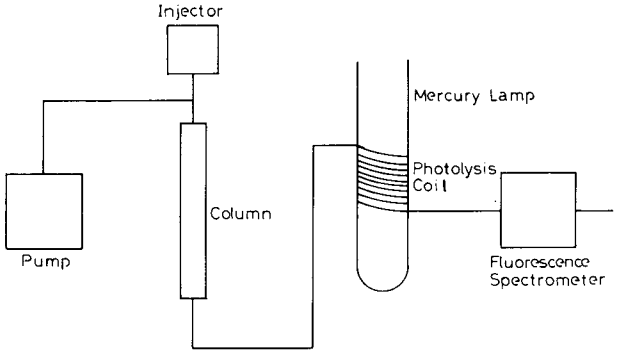

Fig. 2. HPLC system for on-line photolysis.

Procedure

Samples of whole blood, taken by venipuncture at varying times after administration of the dose, were obtained from patients undergoing clomiphene therapy. The blood was collected in tubes containing lithium heparin, which were then centrifuged. The plasma was transferred to small sample vials and stored at -20°C until analysed.

Samples of plasma (3 ml) were transferred into 20-ml centrifuge tubes containing 1 ml of borate buffer, pH 9. The tube was vortexed and 9 ml of redistilled AR diethyl ether were added. The tube was vortexed for a further

2 min to ensure thorough mixing and then centrifuged at 1600 *g* for 15 min. The ether layer was transferred to a 10-ml centrifuge tube and dried by the addition of 1 g of anhydrous sodium sulphate. The tube was then centrifuged at 1600 *g* for 10 min and the ether layer transferred to another 10-ml centrifuge tube. The ether was evaporated to dryness with a stream of air and the extracted material was redissolved in 75 μl of chloroform—methanol (80:20). The tube was vortexed for 2 min to dissolve any material adhering to the walls of the centrifuge tube, and 10 μl of the resultant solution were injected into the chromatograph. The extraction efficiency was 70% at 30 ng/ml.

RESULTS AND DISCUSSION

Chromatography

A representative chromatogram obtained from a standard solution of clomiphene (1 μg/ml) in methanol is shown in Fig. 3. Separation of the *cis*- and *trans*-isomers can be clearly seen and the areas of the peaks are in proportion to the relative concentrations of the respective isomers in the solution (*cis* 55%, *trans* 45%). By comparison with a chromatogram obtained using a UV detector set at 298 nm inserted before the photolysis coil, there is a significant improvement in signal-to-noise ratio with little degradation of the chromatographic resolution.

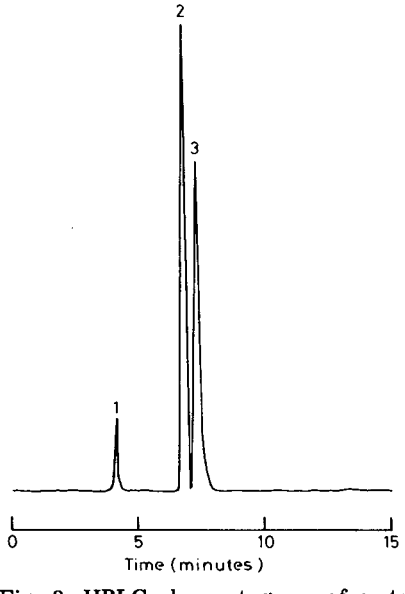

Fig. 3. HPLC chromatogram of a standard solution of clomiphene in methanol (1 μg/ml). Peaks: (1) impurity peak from methanol, (2) *cis*-clomiphene and (3) *trans*-clomiphene.

If the mercury lamp is switched off before the elution of the clomiphene isomers from the column, no signal is obtained from the fluorescence spectrometer.

Emission and excitation wavelengths of the photolysed derivatives were obtained by dissolving clomiphene in the mobile phase and exposing 3-ml

quartz cuvettes containing the solution to a UV lamp for 30 min. The emission and excitation spectra were scanned, and the solutions, which were previously not fluorescent, emitted strongly at 367 nm when excited at 257 nm.

Due to the on-line nature of the derivatisation, lengthy residence times of the eluted isomers within the photolysis coil would result in photochemical destruction of the derivatised isomers while short transit times would result in incomplete conversion of the clomiphene isomers. The variation in photolysis yield with flow-rate was determined by injecting a standard solution of clomiphene at varying flow-rates. The flow-rate response of the detector itself was measured by substituting naphthalene injections for the clomiphene and the photolysis flow-rate response was then corrected to yield the optimum flow-rate of 0.8 ml/min for the 3-m coil.

Linear calibration graphs passing through the origin were obtained for both isomers over a concentration range of 0—45 ng/ml after extraction from spiked plasma samples and were constructed from a measurement of the peak heights of the derivatised isomer peaks. Correlation coefficients of 0.989 and 0.984 were obtained for the *cis*- and *trans*-isomers respectively. An internal standard was not used in the assay since, despite an extensive evaluation, a suitable compound has not yet been found which satisfies the numerous criteria for an adequate internal standard.

Minimum detectable levels (signal-to-noise ratio $>$ 2) for the two isomers were determined to be 145 fmol (60 pg) using a Perkin-Elmer 650-10S fluorescence spectrometer. This represents a minimum detectable level of 350 pg/ml in plasma. On the Perkin-Elmer Model 3000 fluorescence spectrometer the minimum detectable level for each isomer was 615 fmol (250 pg), whilst with UV absorption detection of the underivatised isomers the minimum detectable level was 12.5 pmol (5 ng).

Fluorescence spectroscopy

Stop-flow fluorescence spectra were obtained from plasma extracts by halting the flow of the mobile phase during elution of the peak, and scanning the emission and excitation spectra. The results for the *cis*-isomer are shown in Fig. 4 and can be seen to correlate very well with the stop-flow spectra standards. The spectra obtained from the *trans*-isomer were similar to those shown in Fig. 4. The spectra have been corrected for the slight fluorescence observed from the chloroform—methanol solvent when excited at 257 nm.

The structures of the photolysis products have not yet been confirmed but by analogy with the work on tamoxifen [5, 10] it is expected that the reaction will involve ring closure to form the phenanthrene derivatives. This conclusion is also in agreement with the conversion of stilbene and triphenylethylene to phenanthrene derivatives by UV irradiation [13, 14].

Both the *cis*- and *trans*-isomers will form phenanthrenes of very similar structure and would therefore be expected to show similar emission and excitation spectra, as is observed.

Blood levels

The analytical procedure described above has been used in a preliminary study of the plasma clomiphene levels of a group of patients undergoing clomiphene therapy.

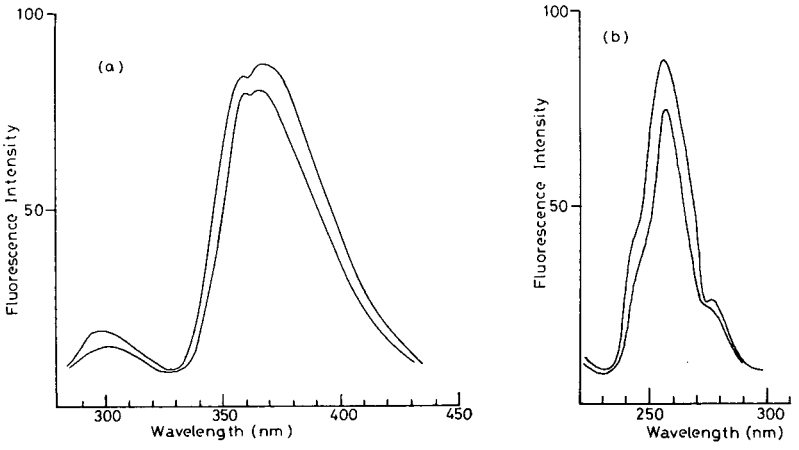

Fig. 4. Fluorescence spectra obtained by halting the flow of mobile phase during elution of *cis*-clomiphene. (a) Emission spectra with excitation at 257 nm; (b) excitation spectra with emission monitored at 367 nm. The upper traces are from spiked plasma samples while the lower are from a patient receiving clomiphene therapy.

A sample chromatogram is shown in Fig. 5 and the levels tabulated in Table I. The results show several interesting features. First, although the dosage form as administered contains 55% of the *cis*-isomer, the concentrations of the two isomers extracted from the plasma are not in the same ratio. The concentration of the *cis*-isomer has apparently fallen to approximately 35% of the total isomer concentration. This may be due to a number of factors relating to the distribution and metabolism of the drug.

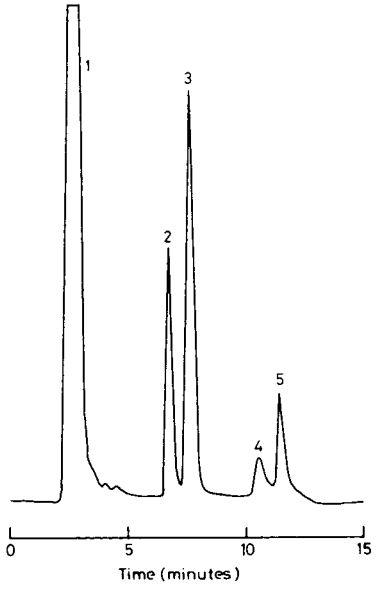

Fig. 5. HPLC chromatogram of a plasma sample from patient J. Peaks: (1) solvent peak, (2) *cis*-clomiphene, (3) *trans*-clomiphene, (4) and (5) clomiphene metabolites.

TABLE I

CLOMIPHENE LEVELS IN PATIENT PLASMA SAMPLES DETERMINED USING ON-LINE POST-COLUMN PHOTOLYSIS AND FLUORESCENCE DETECTION

Sample identification	Time after dose (h)	Dosage (mg)	*Cis*-isomer (ng/ml)	*Trans*-isomer (ng/ml)
M	2.0	100	6.9	23.1
McG	3.0	100	14.6	30.4
A	3.25	100	9.4	23.1
S	2.5	150	24.1	42.7
J	3.25	150	42.3	80.9
B	3.25	150	39.1	>60

Secondly, from Fig. 5 the appearance of several other peaks can be observed. These must have a similar structure to clomiphene in order to be detected using the photolysis—fluorescence system, and it is postulated that these represent metabolites of the clomiphene. Another pair of peaks, not shown in Fig. 5, but of very much lower concentration is also observed.

Table I shows that there is good correlation between the dose administered and the levels observed in the plasma samples. The levels show a significant increase as the dosage level is raised and are around the expected values based upon the work on tamoxifen [5]. In all cases the *cis*:*trans* ratio is significantly below the ratio present in the dosage form.

Work is proceeding to confirm the identity of the derivatives, to elucidate the structure of the metabolites, and to investigate aspects of the pharmacokinetics of clomiphene. The results of these investigations will be presented in a subsequent publication.

CONCLUSION

A method has been developed for the extraction and quantitation of the *cis*- and *trans*-isomers of clomiphene in plasma. The use of an on-line derivatisation by UV irradiation, coupled with fluorescence detection has enabled determination of clomiphene levels below 1 ng/ml of plasma. The sensitivity and selectivity of the technique will permit investigation of the pharmacokinetics of clomiphene.

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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS FOR CHLOROQUINE IN BODY FLUIDS APPLICATION TO STUDIES OF DRUG RESISTANCE IN *PLASMODIUM FALCIPARUM*

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SUMMARY

A high-performance liquid chromatographic method has been developed for the sensitive determination of chloroquine in body fluids. The method has been applied to quality-control assay of World Health Organization (WHO) In-Vitro, Macro-Test Kits for the assessment of susceptibility of *Plasmodium falciparum* to chloroquine. Experiments utilizing [^{14}C]chloroquine demonstrated that water was not capable of efficiently desorbing chloroquine from the inside surfaces of kit vials. The addition of blood to the vials effectively desorbs chloroquine. Subsequent addition of the blood to aqueous base followed by hexane extraction permits quantitation by reversed-phase, ion-pair high-performance liquid chromatography utilizing ultraviolet detection at 344 nm. The method is capable of determining as little as 20 ng of chloroquine per vial. This method, utilizing the methyl ether of 9-anthracenemethanol as internal standard, can quantify chloroquine in 1 ml of blood or urine with a minimum detection limit of 20 ppb* (ng/ml). Measurement of blood levels of chloroquine in persons contracting falciparum malaria while following a prophylactic regimen complements in-vitro drug susceptibility measurements in characterizing resistant strains of the parasite.

INTRODUCTION

Resistance of certain strains of *Plasmodium falciparum* to chloroquine has been reported from a number of malarious areas in the world [1]. In order to effectively monitor the occurrence and spread of resistant parasites, the World Health Organization (WHO) has developed standardized test kits for assessment of the response of *P. falciparum* to chloroquine. The WHO In-Vitro, Macro-Test

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

0378-4347/81/0000—0000/$02.50

Kits, based on the work of Rieckmann and Lopez-Antunano [2], each contain fifteen series of vials, each series containing seven vials with 5 mg of glucose and 0.25—3.0 nmol of chloroquine. In addition there are 30 control vials and 9 vials containing 1.25 nmol for use in special investigations. In use, 1 ml of defibrinated blood from an infected patient is added to each of the vials and incubated at 37°C for 24 h, followed by assessment of the effect of increasing levels of chloroquine on the maturation of the parasites. Growth at the 1.5-nmol level of chloroquine and above indicates parasite resistance.

The use of the WHO test kits can standardize assessments of *P. falciparum* resistance to chloroquine around the world, but to do so the kits must be carefully quality-controlled. Although a number of other approaches have been used in the past to quantify chloroquine in blood, including ultraviolet (UV) spectroscopy [3], fluorescence spectroscopy [4] and gas chromatography [5], the use of high-performance liquid chromatography (HPLC) with UV detection at 344 nm appeared to promise the best combination of convenience, sensitivity and specificity for the desired assay.

Another aspect of the evaluation of chloroquine resistance in *P. falciparum* is the measurement of blood levels of chloroquine in persons contracting falciparum malaria while following a prophylactic regimen. The demonstration of normally effective blood levels of chloroquine coincident with the presence of parasitemia suggests the presence of a resistant strain of the parasite. Further, a method used for measuring blood levels should be capable of measuring as little as 10 ppb chloroquine since this level represents the lower end of the effective range [6, 7] in plasma. Corresponding whole-blood values are substantially higher [8, 9].

The present study details the development of methodology which provides accurate quality-control assay of the small quantities of chloroquine used in the test kits. This methodology can be used also to determine blood levels of chloroquine in cases of suspected chloroquine-resistant malaria, to establish whether therapeutic levels have indeed been attained. The urine levels corresponding to therapeutic blood levels are in the ppm range and thus easily measured by the method.

The potential for adsorptive losses of chloroquine during analysis has been studied, and the method includes precautions designed to make such losses negligible.

EXPERIMENTAL*

Standards

Standard chloroquine base, desethylchloroquine base and chloroquine diphosphate were supplied by Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.).

The methyl ether of 9-anthracenemethanol, used as internal standard, was synthesized by methylation of the alcohol using sodium hydride and methyl iodide in dimethyl sulfoxide. A 1-g quantity of 9-anthracenemethanol was

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

dissolved in 4 ml of dimethyl sulfoxide in a screw-cap test tube, and about 50 mg of hexane-washed sodium hydride and 0.3 ml of methyl iodide were added. The mixture was shaken for 30 min. A 10-ml quantity of water was added followed by extraction using four 4-ml quantities of benzene. Evaporation of the combined benzene extracts followed by vacuum drying yielded slightly over a gram of the product. The melting point range of the crude product was 82—87°C. The structure of the product was verified using proton nuclear magnetic resonance spectroscopy and gas chromatography—mass spectrometry. HPLC analysis using the reversed-phase, ion-pair conditions of the chloroquine analysis showed the presence of a small impurity peak eluting immediately after the main peak. Recrystallization from hexane raised the melting point to a range of 86—88°C but did not completely remove the impurity. The latter has no effect on the analysis.

[^{14}C] Chloroquine used in the adsorption studies was kindly provided by Dr. Coy D. Fitch, St. Louis University Medical Center. It had been purchased from New England Nuclear (Boston, MA, U.S.A.) and had a specific activity of 1.66 mCi/mmol.

Reagents

Non-radioactive chloroquine diphosphate used in the [^{14}C] chloroquine work was from Sigma (St. Louis, MO, U.S.A.). Sodium hydride (50% dispersion in mineral oil), methyl iodide and 9-anthracenemethanol were obtained from Aldrich (Milwaukee, WI, U.S.A.). Hexane and methanol were glass-distilled solvents, available from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was deionized and passed through a SEP-PAK C_{18} cartridge, available from Waters Assoc. (Milford, MA, U.S.A.), prior to use. Sodium heptanesulfonate was from Eastman Organic Chemicals (Rochester, NY, U.S.A.). The blood used for the standards and test-vial incubation was freshly collected and preserved using acid citrate dextrose (ACD).

Equipment

The liquid scintillation counting was performed using Beckman Models LS-230 and LS-7500 liquid scintillation spectrometers.

Fluorescence measurements were performed using a Perkin-Elmer Model MPF-2A spectrophotofluorometer. The excitation and emission wavelengths were set at 332 nm and 385 nm, respectively, while a 10-nm slit-width was set for both entrance and exit slits.

The HPLC apparatus consisted of two Waters Model M6000A solvent delivery systems and a Waters Model 660 solvent programmer, coupled to a Waters Model U6K loop injector, a Varian Vari-chrom variable-wavelength absorbance detector and a Whatman Partisil-10 ODS-3 (25 cm × 4.6 mm) reversed-phase column.

Analysis of test-kit vials using water extraction and fluorescence quantification

Each test-kit batch contained fifteen series of seven vials per series, with vials nominally containing 0.25, 0.50, 0.75, 1.00, 1.50, 2.00 and 3.00 nmol of chloroquine. Each was also to contain 5 mg of glucose. Five series were analyzed for each test-kit batch. A spectrofluorometric method from the literature [4]

was adapted for assay of chloroquine in the WHO test-kit vials. One milliliter of demineralized water was added to each vial, followed by vortexing and the subsequent addition of 1.0 ml of pH 9.5 borate buffer. Each vial was vortexed and the contents transferred to a 0.5-ml micro-cuvette for measurement of fluorescence intensity. The quantity originally present in each vial was determined by comparison with a standard curve which was established by determining the fluorescence intensity of solutions made by the dilution of 3.0-ml aliquots of each standard with 3.0 ml of buffer. Standard curves were linear over the range of interest.

Adsorption studies using [^{14}C] chloroquine

Standard dilutions, prepared in water, contained ^{14}C-labeled chloroquine and non-radioactive chloroquine (NRC) in appropriate concentrations for vial fortification. Control vials from a WHO test-kit batch, containing glucose only, were charged with 0.25—3.0 nmol of [^{14}C] chloroquine, using 50 μl of the appropriate standard solution per vial in each case. Counting of 50-μl aliquots of each standard was performed using a xylene—dioxane—2-ethoxyethanol (1 : 3 : 3) counting solution [10] to verify the activity of the standards.

In analysis of the fortified vials, a gentle flow of dry nitrogen was used to remove water from each vial. A 1.0-ml quantity of either defibrinated blood or demineralized water was added to vials at each level, followed by 37°C incubation for 2 h.

Silanized vials were fortified and incubated similarly to the control vials. These vials had been acid-washed, water-rinsed, acetone-rinsed and dried, followed by silanization using 5% dimethyldichlorosilane in toluene. Rinsing with methanol and subsequent drying completed the treatment. For these tests 50 μl of 100 mg/ml glucose was added prior to fortification with the 50 μl of [^{14}C] chloroquine solution.

Analysis for [^{14}C] chloroquine in the vials was essentially by the method of Fitch [11]. For each incubated vial the blood or water was quantitatively transferred to a screw-cap test-tube containing 0.5 ml of 5 *N* sodium hydroxide and 0.1 ml of 4.09 mg/ml aqueous NRC. A 1.0-ml quantity of water was used as a rinse. The pipet was pre-rinsed using about 0.5 ml of 0.32 mg/ml NRC. A 5-ml quantity of 1.5% isoamyl alcohol in heptane was added, followed by capping (PTFE insert) the test tube, 20 min reciprocal shaking and centrifugation for 5 min at 270 *g*. A 4.0-ml aliquot of the heptane layer from each was then transferred to a corresponding scintillation vial, 15 ml of toluene-based cocktail added and the samples counted.

To each of the original vials was added 0.10 ml of 4.09 mg/ml NRC and 1.0 ml of water. Each solution was then quantitatively transferred to a corresponding test tube containing 0.5 ml of 5 *N* sodium hydroxide and 0.1 ml of 0.32 mg/ml NRC and the sample extracted and counted as outlined above.

Analysis of test-kit vials and clinical samples using blood incubation followed by HPLC—UV quantification

In the analysis of vials, standards were prepared by fortifying clean vials with known quantities of chloroquine diphosphate and 5 mg glucose each and carrying these through the procedure to yield a standard curve. A 1.0-ml quantity of

whole blood with ACD as anticoagulant/preservative was added to each vial, standard and sample alike, and the vials incubated for 20 min at 37°C. Each sample was then quantitatively transferred by silanized Pasteur pipet to a silanized screw-cap test tube containing 0.5 ml of 5 *N* sodium hydroxide. A 2.5-ml quantity of demineralized water was used as a rinse in the transfer. A 3.0-ml quantity of hexane was added to each sample tube. Each tube was capped and shaken for 30 min using a reciprocal shaker. A 50-μl quantity of octanol was added, followed by centrifugation, to aid in the separation of layers. Internal standard solution was then added (200 μl of a 5.6 μg/ml solution of the methyl ether of 9-anthracenemethanol in ethyl acetate) to a silanized 3-ml Reacti-vial (Pierce, Rockford, IL, U.S.A.) after which a 2.0-ml aliquot of the hexane layer was also placed in the Reacti-vial. The sample in each vial was evaporated using a heating block held at 45°C to leave only residual octanol. The hexane refluxed during evaporation, continually washing the sides of the Reacti-vial. A 20-μl quantity of mobile phase concentrate [methanol—water—acetic acid (72.5:25.0:2.5) with 0.0125 *M* sodium heptanesulfonate] was added to the cooled vial. Injections of 15-μl quantities of standards and samples on the chromatograph using UV detection permitted quantitation.

The reversed-phase column was operated in the ion-pair mode using a mobile phase of methanol—water—acetic acid (80:19:1) with 0.005 *M* sodium heptanesulfonate. The flow-rate was 1.0 ml/min (55 bar). UV absorbance of eluting compounds was measured at 344 nm with a detector sensitivity of 0.05 a.u.f.s. Peak heights from the corresponding recorder trace were used for quantitation. The ratio of the chloroquine peak height to the internal standard peak height was plotted against quantity of chloroquine for each standard to establish a curve. Comparison of that calculated ratio for each sample to the standard curve was then made.

WHO In-Vitro, Macro-Test Kits were assayed for chloroquine upon receipt from the facility in which they were manufactured in Manila, The Philippines.

Clinical samples containing chloroquine in whole blood, plasma and urine were analyzed as above with the sample added to the 5 *N* sodium hydroxide in a screw-cap test tube and then carried through the remainder of the procedure. A 2.5-ml quantity of demineralized water was added to give the desired concentration of sodium hydroxide before extraction. For urine samples, final dilution was to 1.0 ml, and quantitation was by external standard comparison with fortified urine standards similarly treated.

RESULTS AND DISCUSSION

Desorption of chloroquine from glass by water and by blood

Earlier studies have demonstrated the adsorption of organic bases on untreated glass [12—15], even in dilute aqueous acid [14]. The indications of chloroquine adsorption on glass in the present study, seen in the fluorescence assay of test-kit vials, were confirmed by the experiments utilizing ^{14}C-labeled chloroquine. Blood is clearly much better able to desorb chloroquine from untreated vials than is water (Table I). These results suggest the necessity of adding blood to test-kit vials prior to analysis for chloroquine.

Silanized vials also have an appreciable tendency to adsorb chloroquine in

TABLE I

BLOOD/VIAL AND WATER/VIAL DISTRIBUTION OF CHLOROQUINE FOUND USING [^{14}C]CHLOROQUINE FORTIFIED (A) WHO IN-VITRO MACRO-TEST KIT VIALS (BATCH 10) AND (B) PRETREATED (SILANIZED) VIALS

Quantity of chloroquine (nmol)	(A) Macro-test kit vials				(B) Silanized vials			
	Percent of added counts found	Distribution of found counts (%)			Percent of added counts found	Distribution of found counts (%)		
		Blood	Water	Vial		Blood	Water	Vial
0.25	96.4	98.2		1.8	98.1	98.9		1.1
0.25	95.6	97.2		2.8	97.9	98.8		1.2
1.25	91.1	97.4		2.6	95.1	99.4		0.6
1.25	90.7	97.6		2.4	95.2	99.7		0.3
3.00	90.5	97.1		2.9	95.1	99.4		0.6
3.00	88.9	97.4		2.6	94.7	99.3		0.7
0.25	89.6		12.4	87.6	91.9		91.7	8.3
1.25	86.4		33.2	66.8	100.0		98.3	1.7
3.00	88.5		52.0	48.0	101.0		99.1	0.9

competition with water (Table I). The approximately 0.02 nmol adsorbed as indicated by the data in Table I represents 6.5 ng of chloroquine. This demonstrates the care necessary to avoid or compensate for losses of chloroquine when analyzing at the ppb (ng/ml) level.

Characterization of the HPLC method for chloroquine in blood

Standard curve data for the method are summarized in Table II. Fig. 1 shows representative HPLC—UV traces. The data demonstrate that the linearity of the system is quite good as is the precision at the 0.82 nmol per vial (263 ppb in blood) level. The range represented encompasses that corresponding to the test-kit vials and to concentrations normally found in the whole blood and plasma of treated individuals. Taking a 3.0-ml sample and a larger fraction of the extract for injection permits accurate detection of as little as 5 ppb (ng/ml) chloroquine.

Originally, 1.5% isoamyl alcohol in heptane was used as the extracting solvent for the HPLC method. The use of hexane, followed by the addition of 50 μl of octanol prior to centrifugation, was substituted for several reasons. Hexane is more volatile and thus more easily evaporated from the sample than heptane. Octanol serves as well as isoamyl alcohol to break emulsions during extraction and to minimize adsorption of chloroquine on glass surfaces. Further, octanol serves as a "keeper" for the internal standard during evaporation. With isoamyl alcohol the last of the alcohol must be removed to avoid an interference in the chromatogram, although heating of the sample appreciably beyond dryness causes sublimation of the internal standard and correspondingly high results for the method. The presence of the residual octanol yields a negative dip in the chromatogram, but at a point (retention time = 4.6 min)

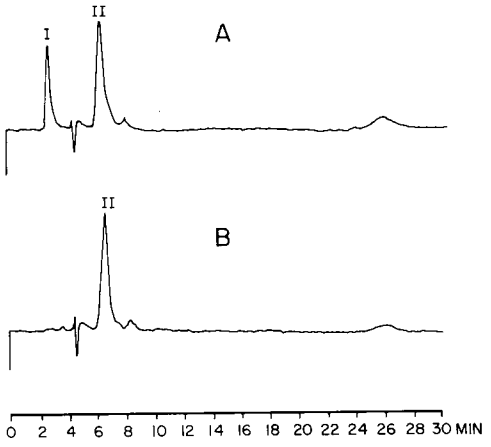

Fig. 1. Representative traces for the analysis of chloroquine by reversed-phase, ion-pair HPLC using the methyl ether of anthracenemethanol as internal standard. (A) Standard containing 262.9 ng/ml chloroquine base in blood; (B) blood blank. Peaks: I, chloroquine; II, internal standard.

which does not interfere with the analysis. Also, the HPLC peaks are sharper than is the case when isoamyl alcohol is used.

The solvent used to reconstitute the samples prior to injection is designed to provide adequate solubility for both chloroquine and the internal standard along with mobile phase compatibility, to ensure good peak shapes.

It was demonstrated that, after reconstitution, the samples could be stored for no longer than one day prior to injection to avoid some loss of precision. The samples were injected on the same day that they were reconstituted; in this way 14—16 samples can be processed at a time.

Cleaning and silanizing the glassware between each glassware use was found to be important. Inadequately deactivated surfaces adsorb appreciable amounts of chloroquine, and a beading test using water showed that silanized surfaces lost much of their hydrophobic character after one use. Further, rinsing of glassware with demineralized water and acetone sometimes failed to remove all of the chloroquine from the previous analysis. Treating the glassware between each use guarantees removal of the last traces of chloroquine by the 6 *N* hydrochloric acid together with the formation of a fresh, inert, hydrophobic surface by silanization for the subsequent analysis.

A typical chromatogram, illustrated in Fig. 1A, exhibits several interesting features. The chromatogram is free from interfering peaks even though the amount of extract injected represents the quantity of chloroquine in 0.20 ml of blood. Indeed, the hexane extraction provides sufficiently specific extraction that a 3.0-ml blood sample may be taken and an injection of extract equivalent to 1.2 ml of blood made when the concentration in the sample requires and quantity of sample permits. This reduces the minimum detection limit for the method to 5 ng/ml. The retention times of chloroquine and internal standard are 2.6 and 6.4 min, respectively. Other characteristics of the chromatogram include the negative dip due to octanol at 4.6 min, the small quantity of impurity in the internal standard appearing at 8.2 min and the broad peak at 26.0

TABLE II

STANDARD-CURVE DATA FOR CHLOROQUINE IN WHOLE BLOOD BY HPLC—UV

Relative standard deviation (n = 4) at 0.822-nmol level = 2.32%.

x = Chloroquine added		y = Chloroquine/internal standard peak height ratio	x' = Chloroquine calculated* (ng)
nmol	ng		
0.00	0.00	0.00	0.72
0.205	65.73	0.206, 0.231	62.2
0.822	262.9	0.931, 0.955, 0.938, 0.903	262.7
1.541	493.0	1.794, 1.753	499.4
3.082	986.0	3.487, 3.500	983.1

*Calculated from the least squares straight line $y = mx + b$ for which m = 0.00356, b = −0.00255; r^2 = 0.9997.

min, due apparently to blood lipids. In practice an injection is made and then a second sample introduced after 10 min. No further injection is made until the lipid peak from the second injection has eluted, requiring a total of 40 min from the time of the first injection. Another peak due to a blood component appears in some samples and is seen at a retention time of 5.0 min in Fig. 1.

The determination of chloroquine in WHO test-kit vials

In comparison of analyses of vials from a WHO test-kit batch by the water extraction—fluorescence and blood incubation—HPLC methods, results by the former method are quite low for the lower nominal value vials where the effect of chloroquine adsorption is amplified (Table III). HPLC quantification of blood-incubated, test-kit vials was chosen over fluorescence because in our hands the former provides better precision and greater selectivity and is proce-

TABLE III

COMPARISON OF RESULTS OF ANALYSES OF CHLOROQUINE TEST-KIT VIALS BETWEEN METHOD A USING WATER EXTRACTION WITH FLUORESCENCE QUANTIFICATION AND METHOD B USING BLOOD INCUBATION WITH HPLC—UV QUANTIFICATION

Average of 5 values except where noted.

Batch 3, nominal (nmol)	Quantity of chloroquine (nmol)	
	Method A	Method B
0.25	0.056 ± 0.009	0.234 ± 0.093
0.50	0.098 ± 0.120	0.457 ± 0.102
0.75	0.49 ± 0.09	0.735 ± 0.289
1.00	0.66 ± 0.08	1.02* (1.09, 0.94)
1.50	1.20 ± 0.13	1.41**
2.00	1.83 ± 0.26	1.84* (1.65, 2.03)
3.00	2.81 ± 0.41	2.85* (2.96, 2.73)

*Two values.

**Single value.

durally simpler than a fluorescence method designed to provide comparable sensitivity.

HPLC analysis of chloroquine in clinical samples

The method used in clinical analysis is the same as that for the kits except for the elimination of blood incubation and subsequent transfer to the extraction test tube. The application of the method to clinical samples, however, requires some conjecture as to the interpretation of the analytical results. McChesney et al. [16] state that, of identifiable chloroquine-related species in the urine of treated human subjects, an average of 70% was unchanged drug and 23% was desethylchloroquine. An independently developed HPLC method [17], published subsequent to the completion of the present study, includes plasma analysis data for a patient in which the chloroquine concentration was found to be approximately ten times that of desethylchloroquine.

In experiments employing the present method, authentic desethylchloroquine proved to have a retention time and sensitivity comparable to that for chloroquine. Efficiency of extraction is 70% for the metabolite compared to about 85% for chloroquine. Since the compensating standards contain chloroquine alone, the value determined by the method will represent slightly less than the sum of the two compounds, expressed as chloroquine, if desethylchloroquine is present.

Table IV includes the results of the analyses for chloroquine in the body fluids of two patients who developed falciparum malaria after returning to the United States from separate travels in East Africa. Both had utilized a chloroquine diphosphate prophylactic regimen (300 mg as base per week) before, during and after their respective trips. A limited sample of blood from Patient 1 was available and some of this was used for in-vitro susceptibility testing of the

TABLE IV

ANALYSIS FOR CHLOROQUINE IN BODY FLUIDS OF PATIENTS DEVELOPING PLASMODIUM FALCIPARUM PARASITEMIA WHILE UNDERGOING CHEMOPROPHYLAXIS

Sample matrix	Concentration of chloroquine base, ppb (ng/ml)			
	Patient 1		Patient 2	
	Individual results	Mean	Individual results	Mean
Whole blood	108	—	198.2 205.4	201.8
Plasma	40.0	—	39.8	—
Serum	—	—	29.0 33.6	31.3
Urine	19,580 19,870 19,920 20,170	19,885 ± 242	17,510 17,360 17,600 17,360	17,460 ± 11

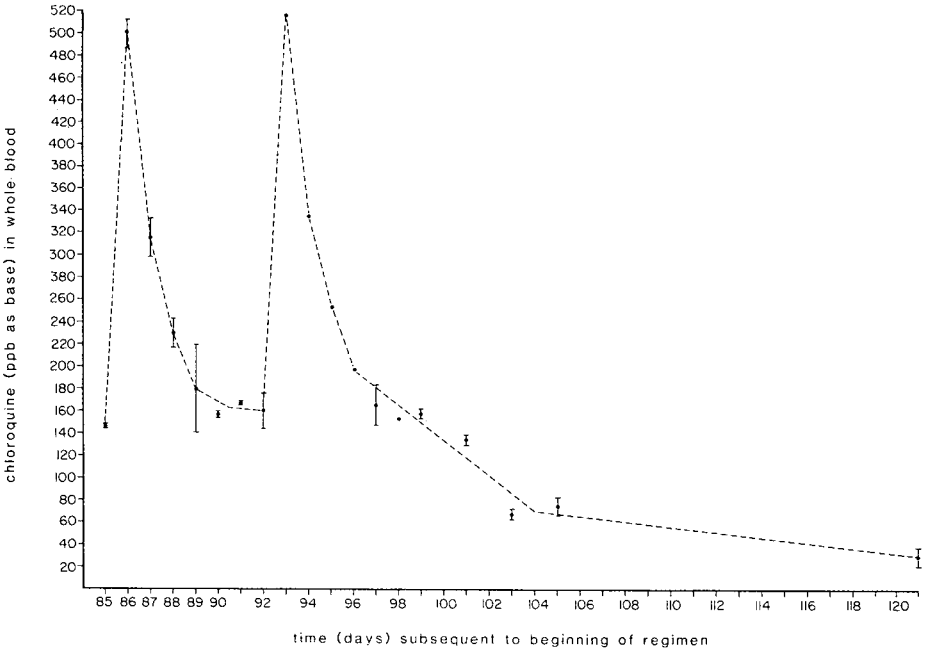

Fig. 2. Whole-blood concentrations of chloroquine in a volunteer during and subsequent to the thirteenth week of a prophylactic regimen utilizing chloroquine diphosphate (300 mg as base). Chloroquine was ingested immediately subsequent to sampling on days 85 and 92. No chloroquine was ingested subsequent to day 92. The ends of the bars extending above and below a data point represent the individual results of duplicate determinations.

parasites. The values in Table IV for Patient 1 were determined from 0.7 ml of whole blood, 1.0 ml of plasma and 1.0-ml aliquots from a urine sample.

The whole-blood drug levels in the two patients may be compared with those found (Fig. 2) in a volunteer whose blood was analyzed during and subsequent to the thirteenth week of a regimen of 300 mg as the base per week. The whole-blood chloroquine concentrations found in the patients were less than those found in the volunteer on the corresponding day subsequent to tablet ingestion, but the levels present are within a normally effective range [6] and should effectively prevent the development of chloroquine-susceptible *P. falciparum*. Detailed case studies including parasitological investigations are to be published elsewhere [18].

There are at least two advantages in the use of whole blood, rather than plasma, for chloroquine determination in clinical studies involving chloroquine resistance. Subjects treated with chloroquine exhibit higher whole-blood than plasma levels, so that determination of the former yields more precise and accurate values at low blood concentrations. Analysis of whole blood yields a fundamental value which may be confidently compared from sample to sample, while analysis of a blood fraction, e.g. plasma, allows the potential for inaccurate results due to in-vitro drug redistribution by hemolysis or other processes [19, 20]. A leukocyte count is normally available for clinical whole-blood samples, so that this factor may be taken into account [7]. The use of the present method permits the gathering of extensive whole-blood chloro-

quine baseline data which will be useful in the characterization of future falciparum malaria cases in which resistance of the parasite to chloroquine is suspected.

ACKNOWLEDGEMENTS

We are indebted to Dr. Coy D. Fitch, Director, Division of Endocrinology, St. Louis University School of Medicine, for the [^{14}C] chloroquine, the use of his laboratory facilities and for his valuable counsel in the radiolabeled experiments. We would also like to thank Mr. Hugh Flax for careful technical assistance.

This work was supported in part by the World Health Organization (WHO) through a WHO Contract Technical Agreement and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ENDRALAZINE AND TWO OF ITS METABOLITES IN HUMAN PLASMA

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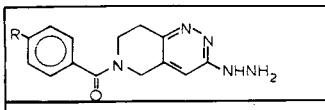
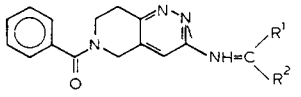
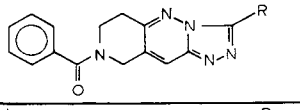
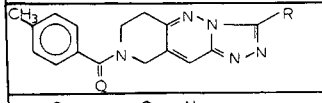
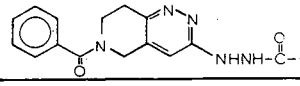
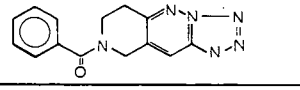
SUMMARY

Endralazine (I) is a new antihypertensive which is chemically and pharmacologically related to hydralazine and dihydralazine. A sensitive high-performance liquid chromatographic—fluorescence assay for the drug and two of its metabolites [methyltriazoloendralazine (VII) and hydroxymethyltriazoloendralazine (VIII)] in human plasma was developed. After conversion of I and its internal standard to triazolopyridopyridazine derivatives the latter and metabolites were separated by high-performance liquid chromatography and detected using their fluorescence. The limits of detection of the assay were 1 nmol/l for I and VII and 0.1 nmol/l for VIII. Intra-assay coefficients of variation were 2.5—5.1% for I (range 1000—10 nmol/l), 4.2—4.5% for VII (range 100—5 nmol/l) and 3.4—5.7% for VIII (range 100—1 nmol/l). Following oral administration of 5 and 10 mg of I to two normal volunteers (slow acetylators) peak plasma levels of I occurred between 0.75 and 1 h after the dose, and declined in a biexponential fashion. The terminal half-life ranged from 2.8—3.7 h. These results contrast with those obtained for hydralazine in plasma where in vitro and in vivo half-lives were < 30 min.

INTRODUCTION

Endralazine (6-benzoyl-3-hydrazino-5,6,7,8-tetrahydropyrido[4,3-*c*]pyridazine mesylate, I) is a new drug with antihypertensive properties [1—5] and is related to hydralazine and dihydralazine both chemically and pharmacologically. When given to man, a single oral dose of 10 mg of endralazine appears to elicit a response similar in magnitude to that obtained for approximately 50—100 mg of hydralazine [5].

Endralazine and hydralazine appear to undergo similar routes of metabolism, that is, acetylation, hydrazone formation and hydroxylation [5]. However,

	I. R=H II. R=CH_3	Endralazine Endralazine internal standard
	III. R^1=CH_3; R^2=COOH IV. R^1=CH_2CH_2COOH; R^2=COOH V. R^1=CH_3; R^2=CH_3	Endralazine pyruvic acid hydrazone Endralazine α-ketoglutaric acid hydrazone Endralazine acetonide
	VI. R=H VII. R=CH_3 VIII. R=CH_2OH IX. R=CH_2CH_3	Endralazine derivative with formic acid Endralazine acetylation metabolite Endralazine acetylation metabolite Internal standard for VII
	X. R=H XI. R=CH_2OH	Endralazine internal standard derivative Internal standard for VIII
	XII.	Endralazine acetylation metabolite
	XIII.	Endralazine derivative with nitrous acid

these studies suggest that I may not exhibit the same acetylator phenotype determined differences in its metabolism which have been described for hydralazine [6]. Similarly, preliminary work [7] has suggested that I may not undergo the very rapid reaction with endogenous α-keto acids which has been described for hydralazine [6, 8].

It appears that to date there are no pharmacokinetic studies of I and its metabolites in humans reported. To this end, sensitive high-performance liquid chromatographic (HPLC) assays for I and the acetylation metabolites, VII and VIII, in human plasma were developed. The reaction rate of I with endogenous pyruvic acid in human plasma at 37°C in vitro and its pharmacokinetics following oral administration to two normal volunteers were studied using the method developed.

MATERIALS AND METHODS

Reagents and materials

All reagents were analytical grade and aqueous solutions were prepared using glass-distilled water. Chloroform was Nanograde from Mallinckrodt (St. Louis, MO, U.S.A.). Specially purified acetonitrile (210 nm cut-off, Unichrom from Ajax Chemicals, Melbourne, Australia) was used for HPLC. Endralazine mesylate (I), methylendralazine (II) the hydrazone metabolites (III and IV), and the acetylation metabolites (VII, VIII and XII) were provided by Sandoz (Basle, Switzerland). Although metabolite V has been identified [5], the pure substance was not available for these studies.

Standards

Stock solutions of I and II were prepared in methanol (200 μmol/l each) and stored in glass at 4°C. These solutions were prepared fresh weekly. Stock solu-

tions of the acetylation metabolites, VII and VIII (20 μmol/l of each) and their internal standards, IX (300 μmol/l) and XI (100 μmol/l) were also prepared in methanol and were stable for at least one month at 4°C. A solution containing I (10 μmol/l), VII and VIII (1 μmol/l of each) in water, was used to prepare the appropriate plasma standards at the time of each assay run or study. The internal standard mixture was also prepared fresh, prior to each assay run, and contained II (5 μmol/l), IX (7.5 μmol/l) and XI (2.5 μmol/l) in water.

Peak area and peak height ratios of the drug and metabolites to their internal standards, were determined for plasma standards and unknowns and quantification performed by reading unknown values from a plotted standard curve.

Syntheses

Synthesis of VI. 100 mg of I and 500 μl of 90% formic acid were heated at 90°C for 1 h in a loosely stoppered glass tube. The mixture was cooled, water added (1 ml) and the mixture neutralized with solid sodium bicarbonate. The precipitated solid was filtered, washed with distilled water and dried under vacuum at 20°C for 24 h. The solid had a melting point of 153—155°C and gave a single peak with retention time 4.2 min using the HPLC conditions described below. The electron impact mass spectrum determined at 20 eV, probe temperature 150°C using a Model AEI MS-30 mass spectrometer showed the molecular ion (*m/e* 279) and a fragmentation pattern consistent with structure VI.

Synthesis of internal standard XI. Methylendralazine (II) (50 mg) and 250 μl of 90% glycolic acid were heated at 90°C for 1 h in a stoppered glass tube. The mixture was cooled and neutralized with solid sodium bicarbonate. The precipitated solid was filtered, washed with water and dried under vacuum at 21°C. The solid had a melting point of 216—217°C and gave a single peak with retention time 26.3 min using the HPLC conditions described below. The field desorption mass spectrum was consistent with the structure of the glycolic acid ester of XI and showed a molecular ion with mass 381. This ester (850 μg) was readily and completely hydrolyzed to XI by treatment with 1 ml of 1 *N* aqueous sodium hydroxide at 21°C for 2 h. The solution was then neutralized with hydrochloric acid and diluted to 100 μmol/l with water and used as the stock solution (stable for at least one month at 4°C). It gave a single peak with retention time 5.8 min by HPLC.

Synthesis of internal standard IX. Fifty milligrams of I and 250 μl of 90% propionic acid were heated at 90°C for 1 h in a stoppered glass tube. The mixture was cooled, diluted with water (0.5 ml) basified (pH 12) with 1 *N* aqueous sodium hydroxide and extracted with chloroform (5 ml). The chloroform was evaporated under nitrogen at 45°C leaving an oil which slowly solidified. Using the HPLC conditions described below the solid gave a single peak with retention time 14.0 min. The electron impact mass spectrum determined at 20 eV with a probe temperature of 170°C showed the molecular ion (*m/e* 307) and a fragmentation pattern consistent with structure IX.

Reaction of I with formic acid

To optimize the conversion of I to the fluorescent derivative VI the conditions of the reaction with formic acid were closely studied. A methanolic solu-

tion of I was prepared (10 μmol/l) and 100 μl (containing 1 nmol of I) aliquoted into glass culture tubes (diSPo tubes from Scientific Products, McGaw Park, IL, U.S.A.) (75 × 12 mm) and the methanol removed under a stream of pure nitrogen at 45°C. A 50-μl aliquot of 90% aqueous formic acid was added to the tubes and they were sealed with firmly fitting plastic caps. Pairs of tubes were incubated at room temperature (21°C) and at 80 and 90°C in a heating block for 0.5 h. Other tubes were incubated at 90°C and pairs removed 10, 15, 30, 45 and 60 min after commencement of incubation. A 450-μl aliquot of mobile phase, 1.5 m*M* phosphoric acid—acetonitrile (82:18) was added to each tube and 100 μl then injected into the chromatograph. The peak area of VI was compared with that of a known amount of synthesized material injected into the chromatograph.

Blood collection and assay procedure

Venous blood samples (10 ml) were drawn into plastic syringes and transferred to ice cold polypropylene tubes containing 125 I.U. of lithium heparin. The blood was then aliquoted into conical polypropylene tubes (TC-10 centrifuge tube from Medical Plastics, Melbourne, Australia) and centrifuged at 8000 *g* in an Eppendorf Model 5412 centrifuge for 30 sec. The plasma was immediately transferred to an ice cold polypropylene tube. Plasma (1 ml) was transferred to a 15-ml glass stoppered tube; 100 μl of the internal standard mixture containing II (5 μmol/l), IX (7.5 μmol/l) and XI (2.5 μmol/l) were added followed by 5 ml of chloroform. Extraction was carried out immediately by vortexing for 30 sec and the phases were separated by centrifugation (5 min at 1100 *g*). The aqueous layer was removed by vacuum aspiration and discarded, and the organic layer was poured into autosampler tubes (75 mm × 12 mm, diSPo tubes from Scientific Products), and evaporated at 45°C under a stream of pure nitrogen. 90% formic acid (50 μl) was added and the mixture was vortexed gently. The tubes were then tightly capped and incubated at 90°C in a heating block for 30 min. The formic acid was evaporated at 90°C under a stream of pure nitrogen, 1 drop of ammonia added to hydrolyse the formic acid esters of the hydroxymethyltriazolo compounds and then this was also evaporated. The residue was reconstituted in mobile phase, 1.5 m*M* phosphoric acid—acetonitrile (82:18) and 100 μl injected into the chromatograph.

High-performance liquid chromatography

The chromatograph used (Spectra-Physics Model SP 8000) was equipped with a ternary solvent system, helium degas and automatic data reduction facilities. A 10-μm RP-8 reversed-phase column (Spectra-Physics, Santa Clara, CA, U.S.A.) was used at a column temperature of 55°C. The instrument was operated in the constant flow mode and the mobile phase consisted of 1.5 m*M* aqueous phosphoric acid—acetonitrile (82:18) with a flow-rate of 2 ml/min.

All solvent lines from the column to the detector were carefully thermally insulated. The column effluent was monitored using a fluorescence detector (Schoeffel, Model 970) at an excitation wavelength of 230 nm with an emission cut-off filter allowing 90% transmission at 389 nm. The fluorimeter sensitivity was 3.5, range 0.1 μA full scale and time constant 6.0 sec. Samples were injected automatically using a 100-μl injector loop and an autosampler (Spec-

tra-Physics Model 8010). All files for instrument operation and integration were stored on disc (Spectra-Physics Model SP 4010 disc module).

Reproducibility and recovery

Intra-assay reproducibility for the endralazine and metabolite assay was determined by assaying five replicate plasma samples containing added amounts of drug and metabolites at concentrations ranging from 0.1—1000 nmol/l (Table I). Day-to-day reproducibility was not determined directly because of the in vitro reaction of the parent drug with α-keto acids in plasma which could occur during freezing and thawing of samples. This precluded storing a bath of frozen samples and assaying one within each assay run. An estimate of day-to-day precision was obtained by examining the variability in the slope of the standard curves for ten consecutive runs on different days.

Recoveries of I, VII and VIII from plasma in the assay method were determined by injecting known amounts of the endralazine derivative (VI) and the metabolites (VII and VIII) into the chromatograph and comparing the peak areas with those obtained for plasma standard of known concentration.

TABLE I

INTRA-ASSAY REPRODUCIBILITY FOR THE ASSAY OF ENDRALAZINE AND METABOLITES

Five replicate determinations at each concentration.

Drug/metabolite	Coefficient of variation (%) at concentrations (nmol/l)						
	1000	100	50	10	5.0	1.0	0.1
I	2.5	1.2	2.7	5.1	—	17.3	—
VII	—	4.2	—	4.0	4.5	18.6	—
VIII	—	3.4	—	—	4.8	5.7	19.8

Specificity of the HPLC assays

The endralazine hydrazones (III and IV) and the uncyclized acetylation metabolite (XII) were added to plasma to give a final concentration of 10 μmol/l and carried through the assay procedure described above. If conversion to either the endralazine derivative (VI) or to the metabolites VII and VIII occurred, the percentage converted was determined in each case. In addition, a number of fluorescent drugs and their metabolites (Table II) were added to plasma and subjected to the conditions of the assay procedure. If a peak was obtained the retention time was recorded.

Stability of extracted samples

To determine whether any change occurred in samples after chloroform extraction, standards containing I, VII and VIII in a concentration of 100, 10 and 10 nmol/l respectively were extracted with chloroform as described above. One pair of samples (duplicates) was immediately processed as described above and injected into the chromatograph. Additional pairs were allowed to stand for 2 and 4 h after vortexing without centrifugation and then processed as

TABLE II

RETENTION TIMES OF A NUMBER OF FLUORESCENT DRUGS AND THEIR METABOLITES

Drug	Retention time (min)
Endralazine metabolite VIII	3.2
Endralazine derivative VI	4.2
Internal standard XI	5.8
Endralazine metabolite VII	7.0
Internal standard derivative X	8.7
Internal standard IX	14.0
3-Hydroxymethyl-*s*-triazolo[3,4-*a*]phthalazine*	2.3
s-Triazolo[3,4-*a*]phthalazine*	3.3
3-Methyl-*s*-triazolo[3,4-*a*]phthalazine*	5.3
4-Hydroxypropranolol**	25.5
N-Desisopropylpropranolol**	46.7
Propranolol	112
Atenolol	NP***
Metoprolol	NP
Timolol	NP
Frusemide	6.0
Methyldopa	NP
Prazosin	NP

*Hydralazine metabolites.
**Propranolol metabolites.
***No peak observed with a retention time less than 2 h.

before. These samples were then injected into the chromatograph and peak area ratios to the internal standards obtained for each sample.

Reaction of I in plasma in vitro at 37°C

Fresh venous blood from a non-medicated normal volunteer was heparinized and centrifuged immediately. The plasma pyruvic acid level was estimated using an enzyme assay kit (Boehringer diagnostic kit). To half of the plasma was added endralazine to provide a final concentration of 1.0 μmol/l and the mixture maintained at 37°C in a water bath. Samples (1 ml) were taken at times 0, 5, 10, 15, 30, 45 min and 1.0, 1.5, 2.0, 2.5 and 3 h after the addition of I and were assayed for I, VII and VIII by the method described. To compare the rates of reaction of I and hydralazine at 37°C, a duplicate experiment was carried out simultaneously with 1.0 μmol/l of hydralazine using the same plasma. Levels of hydralazine were determined by HPLC using the method described previously [9].

Pharmacokinetic study

Two slow acetylators of sulphadimidine [10] were each given 5-mg and 10-mg oral doses of endralazine with 500 ml of water, on separate occasions more than one week apart. Blood samples were drawn 0, 10, 20, 30, 40, 50 min and 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 h after the dose and the plasma assayed for I, VII and VIII using the method described.

RESULTS

HPLC assay for endralazine and metabolites

Chromatograms obtained for the assay of I, VII and VIII are shown in Fig. 1. Intra-assay coefficients of variation are shown in Table I. Recovery of I was 84% over the concentration range 1.0—1000 nmol/l, 92% for VII over the range 1.0—100 nmol/l and 85% for VIII over the range 0.1—100 nmol/l. The variation in the slope of the standard curves was 3.5%, 5.6% and 9.9% for I, VII and VIII, respectively. Limits of detection (determined at peak height twice noise) were 1 nmol/l (0.3 ng/ml) for I and VII and 0.1 nmol/l (0.03 ng/ml) for VIII following injection of half the extracted sample on column. No change in peak areas or heights was observed in extracted samples which were allowed to stand for 2 and 4 h before centrifugation and removal of the chloroform layer. Similarly no change occurred over 24 h in derivatized samples reconstituted in mobile phase and allowed to stand at 21°C.

Reaction of I and the internal standard II with aqueous formic acid at 90°C for 30 min resulted in complete conversion to the respective fluorescent derivatives VI and X. Shorter derivatization times or lower temperatures resulted in reduced recovery of VI. Other approaches to the derivatization of I using trifluoroacetic anhydride, propionic anhydride, dansyl chloride, *o*-phthalaldehyde and formaldehyde were unsuccessful.

Specificity

At a level of 10.0 μmol/l the endralazine hydrazones III and IV interfered in the estimation of I to the extent of 0.7% and 0.9%, respectively. The un-

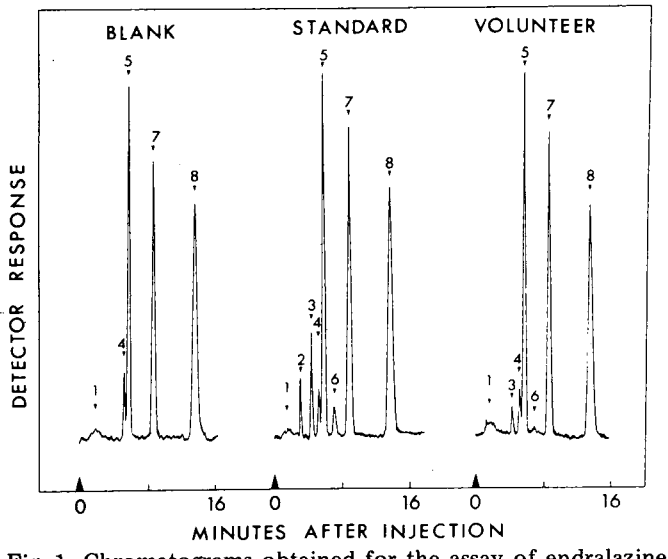


Fig. 1. Chromatograms obtained for the assay of endralazine and metabolites in blank plasma, in a plasma standard containing 100 nmol/l of endralazine and 10 nmol/l of VII and VIII and in plasma from a volunteer following an oral dose of endralazine (5 mg) and containing 26 nmol/l of I and 1.4 nmol/l of VIII. Peaks: 1 = plasma peak; 2 = VIII; 3 = VI; 4 = plasma peak; 5 = XI; 6 = VII; 7 = X; 8 = IX.

cyclized acetyl metabolite XII interfered in the assay of I to the extent of 4.5% and in the assay of VII to the extent of 5.1%. Since plasma levels of XII are apparently very low [5] this represents insignificant interference in the assay. However, longer derivatization times with formic acid at 90°C resulted in increased conversion of XII to both VI and VII. The retention times of other drugs and metabolites carried through the assay procedure are summarized in Table II.

Reaction of I in plasma in vitro at 37°C

I did not react as rapidly as hydralazine with endogenous pyruvic acid in plasma in vitro at 37°C. The first order half-life for hydralazine was 11.0 min compared with 3.8 h for I (Fig. 2).

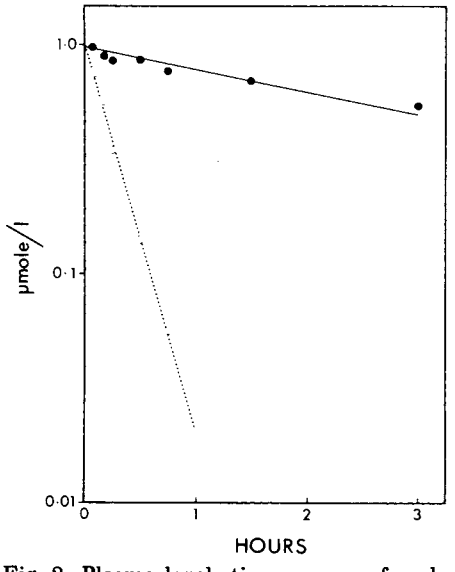


Fig. 2. Plasma level—time course of endralazine and hydralazine (1 μmol/l of each) following their addition to fresh plasma at 37°C containing endogenous pyruvic acid (88 μmol/l). ●——● = I; o • • • o = hydralazine.

DISCUSSION

Endralazine (I) and two of its metabolites, VII and VIII were extracted in good recovery from plasma at physiological pH and separated by HPLC. Separate internal standards were used for each component, and I and its internal standard (II) were selectively derivatized with formic acid to yield the triazolopyridopyridazines, VI and X. The latter and VII and VIII were detected by fluorescence allowing levels less than 1 nmol/l to be measured in plasma. No significant interferences by known metabolites of I [5] and other fluorescent drugs and their metabolites were observed in the assays. Extraction of plasma samples without subsequent derivatization demonstrated that VI was not a naturally occurring metabolite of I in the plasma of healthy volunteers. Repro-

ducibility of quantification at low plasma levels was sufficient for single-dose pharmacokinetic studies.

Attempts to extend the assay methodology developed for hydralazine [9] to the assay of I were unsuccessful. In this case the tetrazolo derivative of I (XIII) formed by treatment with weak nitrous acid was insufficiently fluorescent to allow small plasma levels to be measured. Endralazine itself, the hydrazone metabolites (III and IV) and the uncyclized acetyl metabolite (XII) had no inherent fluorescence under the chromatographic conditions presently described.

Although endralazine reacted with pyruvic acid in plasma in vitro at 37°C, the rate of this reaction was considerably slower than for hydralazine under identical conditions (Fig. 2). At 4°C the reaction of I in freshly drawn plasma was sufficiently slow (half-life = 23 h) that samples could be stored for up to 1 h without significant losses of I (< 1%). This allowed a number of samples to be drawn rapidly in pharmacokinetic studies and to be extracted as a group after storage at 4°C for up to 1 h.

Following oral administration of 5 and 10 mg of endralazine to two slow acetylators, plasma levels of I peaked between 0.75 and 1.0 h after the dose and then declined biexponentially (Fig. 3). Terminal half-lives ranged from 2.82 to 3.76 h and did not appear to be affected by dose. The ratios of the area under the plasma level—time curve from time zero to infinity (AUC_0^∞) of I for the 10- and 5-mg doses were 1.8 and 1.9 in the two subjects. Plasma levels of the acetylation metabolite VII were detected in both subjects but did not exceed 2 nmol/l. Metabolite VIII was not detected in plasma and prior hydrolysis of its conjugates may be necessary before detection is possible.

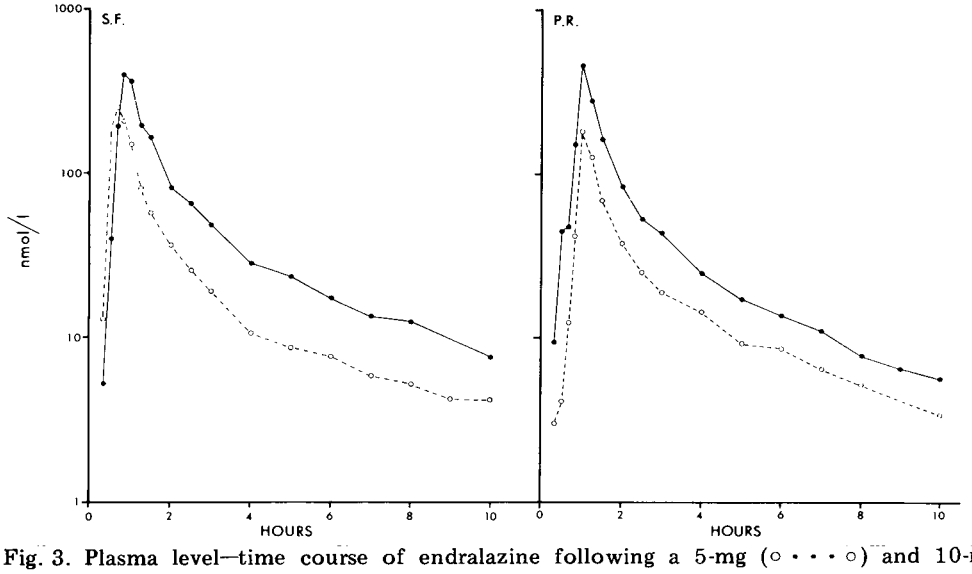


Fig. 3. Plasma level—time course of endralazine following a 5-mg (○ • • • ○) and 10-mg (●——●) oral dose of endralazine to two slow acetylators.

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CHROMBIO. 934

DETERMINATION OF RANITIDINE AND ITS METABOLITES IN HUMAN URINE BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method using ion-pair high-performance liquid chromatography is presented for determining ranitidine, ranitidine N-oxide, ranitidine S-oxide and desmethyl ranitidine in the urine from four volunteers, given on separate occasions an intravenous and oral dose of 100 mg ranitidine. This method has been used to study the metabolism and pharmacokinetics of ranitidine by man. It was found that the elimination half-life of ranitidine ranged from 110—246 min. The mean renal clearance of ranitidine in these four volunteers was 512 ml/min.

INTRODUCTION

Ranitidine hydrochloride is a new histamine H_2-receptor antagonist effective in the treatment of peptic ulcers [1]. Metabolic studies in rat and dog using [^{14}C] ranitidine showed that the ranitidine was mainly metabolised by oxidation to give metabolites I, II and III (Fig. 1). The relative amount of each metabolite formed was found to vary with the species [2]. Thin-layer chromatographic analysis of the urine from volunteers given oral and intravenous doses of ranitidine showed that ranitidine was the major component present, compound I was the major metabolite, and small quantities of compounds II and III were also present. As part of the clinical investigations on ranitidine it was necessary to develop a quantitative method for the determination of ranitidine and its metabolites in human urine. A two-step solvent extraction procedure which separates ranitidine from its metabolites has been described for the determination of ranitidine in plasma by high-performance liquid chromatography (HPLC) [3]. The reversed-phase HPLC system used [3] did not com-

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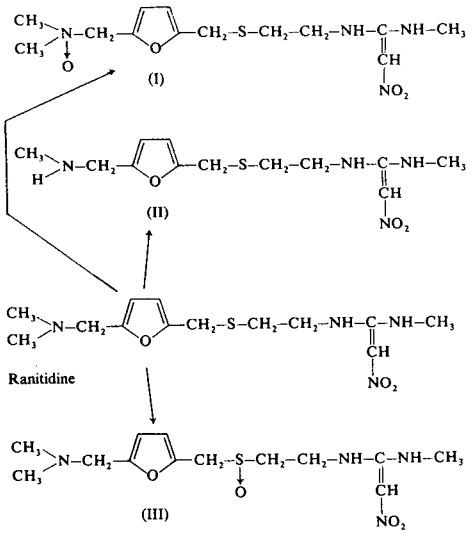

Fig. 1. Formulae of ranitidine and its three metabolites. Metabolite I (ranitidine N-oxide), Metabolite II (desmethyl ranitidine) and Metabolite III (ranitidine S-oxide).

pletely resolve ranitidine from metabolite II and could not be used for the quantitative determination of ranitidine in urine.

The possibility of using ion-pair HPLC for the determination of ranitidine and its metabolites in urine was investigated and an HPLC method using sodium lauryl sulphate as a counter-ion has been developed. This method has been used to study the pharmacokinetic renal clearance of ranitidine and the metabolism of the drug by man.

EXPERIMENTAL

Reagents and materials

Unless otherwise stated all reagents were of analytical grade. The sodium lauryl sulphate was obtained from Cambrian Chemicals (Croydon, Great Britain). Ranitidine hydrochloride, ranitidine N-oxide, (I), ranitidine S-oxide (III) and desmethyl ranitidine (II) hydrochloride were synthesised in the Chemistry Division of Glaxo Group Research Labs. (Ware, Great Britain).

Chromatography

A Spectra Physics 740B dual reciprocating pump (Spectra Physics, St. Albans, Great Britain) was used to deliver eluent to the WISP automatic sample injector (Waters Assoc., Northwich, Great Britain). Stainless steel columns (5 × 100 mm) from Shandon Southern Products (Runcorn, Great Britain) were packed with 5-μm particles of Spherisorb ODS (Phase Separations, Clwyd, Great Britain) and maintained at 45°C in a DuPont 860 air circulating oven [DuPont (U.K.), Hitchin, Great Britain].

The mobile phase was pumped at 1.0 ml/min, and a Pye LC3 variable-wavelength UV detector (Pye Unicam, Cambridge, Great Britain) was set at 320 nm and 0.01 a.u.f.s. to monitor the eluate. Chromatograms were recorded on a

Bryans 28000 pen recorder (Bryans Southern Instruments, Surrey, Great Britain), input 0—10 mV, chart speed 5 mm/min, and also on a Spectra Physics SP4050 printer plotter, ×5 attenuation, chart speed 0.25 cm/min. Peak areas were recorded and integrated using the Spectra Physics SP4000 Chromatography Data System. Data reduction was performed using a pre-programmed procedure resident in the Spectra Physics SP4100 computing integrator. A least squares fit to a quadratic equation was applied to the calibration data, and the coefficients of best fit were stored and used for the calculation of sample concentration.

Calibration and accuracy

Ranitidine hydrochloride (44.7 mg) was weighed and transferred with approximately 30 ml of control human urine into a 100-ml volumetric flask. Fresh aqueous solutions of metabolites I, II and III were prepared containing 1 mg of metabolites I and III and the equivalent of 1 mg base of metabolite II per ml and 2 ml of each of these solutions were added to the volumetric flask. The volume was made up to 100 ml with control human urine, and a series of standards covering the ranges 0—400 μg ranitidine per ml and 0—20 μg of metabolite per ml was prepared by dilution of this solution with an appropriate volume of control human urine. The range of standards was chosen to cover the concentration of ranitidine and its metabolites likely to be present in urine during 24 h after a dose of 100 mg ranitidine. The lowest standard for ranitidine corresponded to 0.8 μg/ml of urine. The accuracy and precision of the method was evaluated by analysing a sample of urine containing 80 μg ranitidine, 3.97 μg metabolite I, 4.01 μg metabolite II and 4.06 μg metabolite III per millilitre.

Human pharmacokinetic and metabolism studies

Doses of 100 mg ranitidine were administered to volunteers either intravenously or orally. Serial samples of blood and urine were collected during the 0—24 h period after giving the drug. The samples were frozen immediately after collection and stored at −20°C until analysed.

Urine analysis

Samples of urine were thawed out at room temperature and 0.25 ml of each was diluted to 2.5 ml with distilled water using a Fisons Model LFA diluter (Fisons Scientific Apparatus, Loughborough, Great Britain). A 15-μl aliquot of a sample was injected on to the chromatography column by means of a WISP automatic sample injector. The precision of the injector of the WISP was better than 1%. Therefore, the urine was analysed by direct injection and no internal standard was used. Standards were prepared containing 0—400 μg ranitidine and 0—20 μg of metabolites I, II and III per ml of urine. Volumes of 15 μl of these standards and the unknown urine samples were used for analysis by HPLC.

Serum analysis

The blood samples were allowed to clot, the serum separated and the ranitidine concentration determined by HPLC [3].

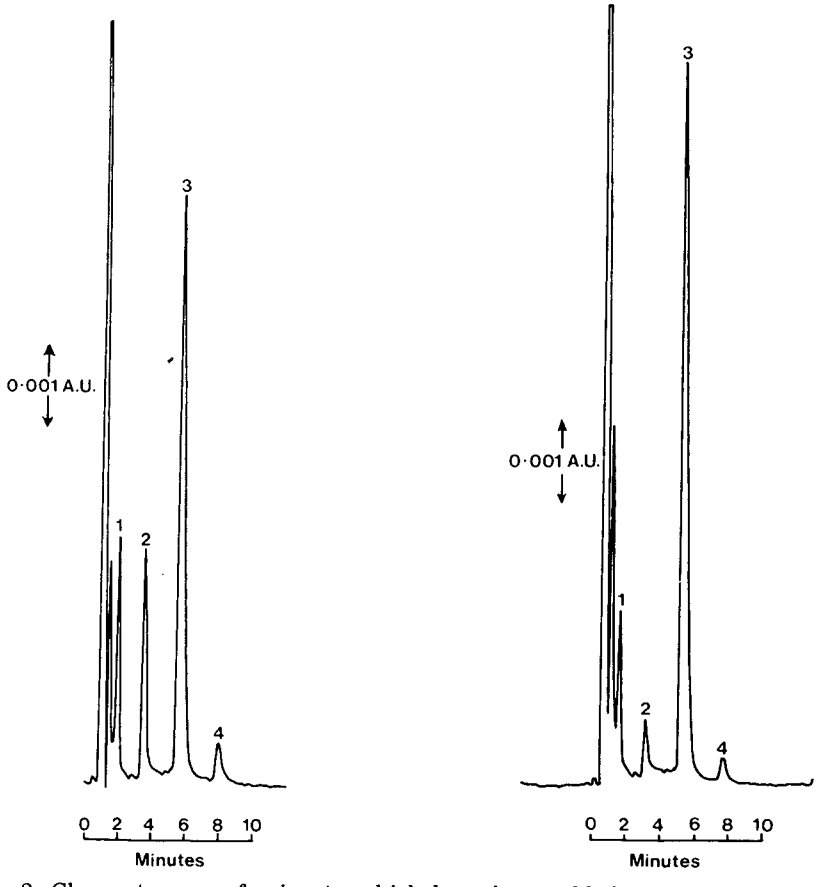

Fig. 2. Chromatogram of urine to which have been added ranitidine and Metabolites I, II and III. Peaks: 1, I 20.8 μg/ml; 2, III 20.4 μg/ml; 3, ranitidine 101 μg/ml; and 4, II 10.0 μg/ml.

Fig. 3. Chromatogram of a urine collected during 2—4 h after an intravenous injection of 100 mg ranitidine. Peaks as given in Fig. 2. The concentration of ranitidine, Metabolites I, II and III in this sample correspond respectively to 113, 11.7, 5.6 and 5.6 μg/ml.

TABLE I

ACCURACY AND PRECISION OF THE DETERMINATION OF RANITIDINE AND ITS METABOLITES BY ION-PAIR HPLC

These data are based on 8 injections of the same standard.

	Ranitidine	Metabolite I	Metabolite II	Metabolite III
Mean concentration (μg/ml)	80.89	3.95	3.96	4.12
S.D.	1.19	0.05	0.54	0.36
C.V.	1.47	1.28	13.54	8.47
Actual concentration (μg/ml)	80.00	3.97	4.01	4.06
Concentration determined expressed as % of standard	101.1	99.5	98.8	101.5

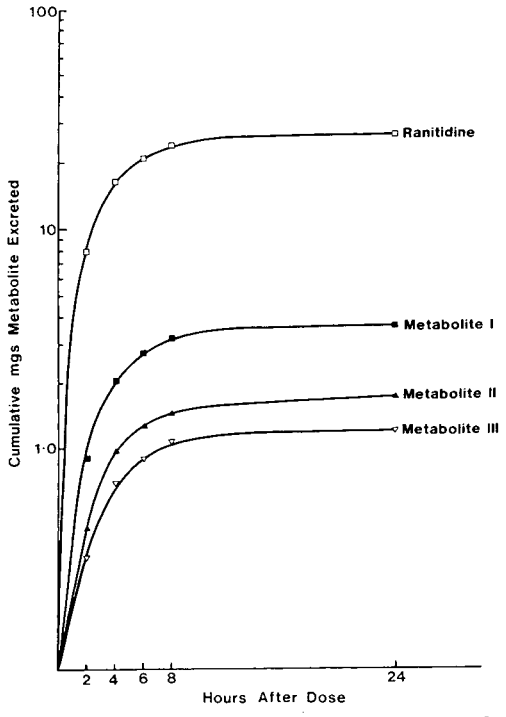

Fig. 4. Plot of mean cumulative excretion of ranitidine and its metabolites in urine from four volunteers given a single oral dose of 100 mg ranitidine.

Elimination half-life

The Sigma minus method [4] was used to calculate the elimination half-life. This is based on plotting on a log scale the amount of drug not yet excreted against the time at the end of each excretion interval in hours. The slope of the line is the elimination half-life in hours.

Renal clearance

The renal clearance values were obtained by plotting the urinary excretion rate against the plasma concentration at the mid-point time of each urine sample. The slope of the line represents the renal clearance [4].

RESULTS AND DISCUSSION

A series of methanol water systems containing 0.005 mol/l concentrations of either sodium sulphate, disodium hydrogen sulphate, sodium dihydrogen phosphate, sulphuric acid or phosphoric acid were investigated as mobile phases in the presence of 0.005 mol/l sodium lauryl sulphate. It was found that the mobile phase consisting of 600 ml methanol, 400 ml of distilled water containing 0.0005 mol/l sodium dihydrogen phosphate and 0.005 mol/l sodium lauryl gave the most efficient and the best chromatographic separation of ranitidine and its three metabolites. This mobile phase has been used over a long period with no evidence of column deterioration. The retention times of raniti-

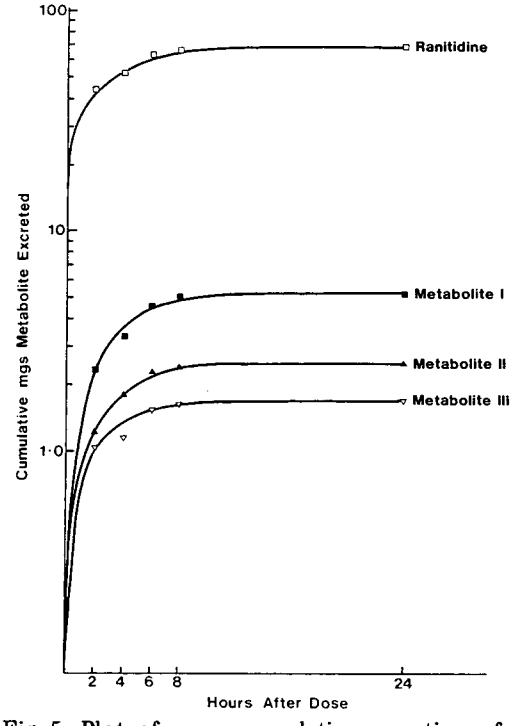

Fig. 5. Plot of mean cumulative excretion of ranitidine and its metabolites in urine from four volunteers given a single intravenous dose of 100 mg ranitidine.

dine and its metabolites in the system chosen were for (I), 1.9 min; (III), 3.9 min; ranitidine, 5.8 min and (II), 7.9 min.

A chromatogram of a urine standard containing 100 μg/ml ranitidine, 20 μg/ml of metabolites I and III, and 10 μg/ml of metabolite II is shown in Fig. 2. The relationships between the concentration of ranitidine, metabolites I, II and III and peak area were found to be linear over the range 0—400 μg/ml for ranitidine and 0—20 μg/ml for each of the three metabolites. The accuracies of determination of ranitidine and the three metabolites are similar. The reproducibility of the method was determined by analysing the results of eight replicate injections of a solution containing 80.89 μg ranitidine, 3.95 μg metabolite I, 3.96 μg metabolite II and 4.12 μg metabolite III per millilitre. Metabolite I which elutes first as a sharp peak was determined with an average coefficient of variation of 1.28%. Metabolite II which has the longest retention time, was determined with a coefficient of variation of 13.54% (Table I). Metabolite III which elutes with a retention time between that of metabolite I and ranitidine was determined with an average coefficient of variation of 8.47%.

Fig. 3. is a chromatogram of a urine sample collected during 2—4 h after an intravenous injection of 100 mg ranitidine. Ranitidine is the major component in the urine, I is the major metabolite and small amounts of II and III are present.

In Figs. 4 and 5 are shown the cumulative urinary excretion of ranitidine and

the three metabolites during 24 h after either an oral or intravenous dose of 100 mg ranitidine. The mean ± standard deviation of the cumulative 0—24 h urinary excretion of ranitidine after intravenous injection was 68.2 ± 7.9% compared with 26.6 ± 6.2% after an oral dose.

The percentage bioavailability, calculated as shown in eqn. 1, was 39% (range 30—51%).

$$\% \text{ bioavailability} = \frac{\begin{array}{c}\text{Amount excreted in 0—24 h urine} \\ \text{after 100-mg oral dose}\end{array}}{\begin{array}{c}\text{Amount excreted in 0—24 h urine} \\ \text{after 100-mg intravenous dose}\end{array}} \times 100 \quad (1)$$

The low bioavailability value found after an oral dose of ranitidine could be due to poor absorption or metabolism of the drug during its passage through the gastrointestinal tract and liver. Ranitidine is a highly water soluble drug with a log P value of 0.2 and pK_a values of 8.2 and 2.7 and it should be well absorbed from the gastrointestinal tract. The percentages (± S.D.) of both the intravenous and the oral dose excreted as metabolites during the first 24 h after administration of ranitidine were respectively for I, 5.1 ± 1.43%, 3.7 ± 0.65; II, 2.4 ± 0.5%, 1.7 ± 0.44%; and III, 1.7 ± 0.69%, 1.2 ± 0.4% of the dose. The similar values for the percentage of the dose of ranitidine excreted in the urine as metabolites after either an oral or intravenous dose of ranitidine would suggest that (a) the oral dose of ranitidine had not been extensively metabolised or (b) that metabolism may have occurred and the metabolites had been preferentially excreted via the bile rather than the urine. The biliary and urinary excretion of [^{14}C]ranitidine and its metabolites have been studied in dogs. Radio thin-layer chromatography of the bile and urine showed that ranitidine N-oxide, the major metabolite, was preferentially excreted via the bile [5]. Man has been shown to metabolise ranitidine in a similar manner to the dog [2] and it could be that in man an oral dose of ranitidine is more extensively metabolised than an intravenous dose, and that there is preferential excretion of the metabolites in the bile.

The elimination half-life of ranitidine after intravenous injection calculated from the urinary excretion data ranged from 110—248 min.

The development of the HPLC assay for urinary ranitidine has been used in conjunction with the plasma ranitidine assay [3] to determine the renal clearance of ranitidine after intravenous administration of the 100-mg dose of ranitidine. The mean renal clearance value obtained was 512 ml/min. This high renal clearance indicated that apart from glomerular filtration there is also extensive tubular excretion of ranitidine.

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CHROMBIO. 939

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF A NEW β-LACTAM ANTIBIOTIC, 6059-S (MOXALACTAM)

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SUMMARY

Reversed-phase high-performance liquid chromatography was applied to the quantitative determination of a new β-lactam antibiotic, 6059-S, and its *R*- and *S*-epimers were resolved. The procedure was also applied to pharmaceuticals and human urine samples. Chromatographic separation was effected on a bonded hydrophobic stationary phase with two mobile phases: methanol—phosphate buffer for the resolution of the epimers and methanol—tetra-*n*-butylammonium phosphate for the quantitation of 6059-S. For the determination of 6059-S in human urine, the latter mobile phase was used successfully without interference by the other urine components. An in vivo experiment was conducted by administering intravenously 1 g of 6059-S to seven volunteers and analysing their urine by chromatographic and microbiological assays, and a comparison of the results gave a correlation coefficient of 0.9954. One-compartment model analysis of the time-course data revealed that 6059-S was excreted in urine intact with a rate constant of 0.433 h^{-1}.

INTRODUCTION

A new semi-synthetic β-lactam antibiotic, 7β-[2-carboxy-2-(4-hydroxyphenyl)acetamido]-7α-methoxy-3-{[(1-methyl-1H-tetrazol-5-yl)-thio]-methyl}-1-oxa-1-dethia-3-cephem-4-carboxylic acid disodium salt. 6059-S (Moxalactam) (I) [1], was discovered to be highly active against a broad range of gram-negative microorganisms, including those resistant to other cephalosporins, and to have a wide antibacterial spectrum [2].

Determinations of antibiotic drugs are conventionally performed by microbiological assay. Such methods require a relatively long time and sometimes result in difficulties when more than one antibiotic drug is administered or an antibiotic is accompanied by one or more active decomposition products. Sometimes 6059-S is accompanied by a small amount of a microbiologically

active decomposition product, decarboxy-6059-S (II), and a non-active one, 1-methyl-1H-tetrazole-5-thiol (III).

Diastereoisomers of many penicillins or cephalosporins occur. As shown in Fig. 1, 6059-S, which closely resembles cephalosporins or cephamycins in structure, also consists of *R*- and *S*-epimers owing to an asymmetric carbon atom in the side-chain. In recent years, much work has been published on the high-performance liquid chromatographic (HPLC) separation of β-lactam antibiotics. However, no HPLC separation of their diastereoisomers has been reported except for ampicillin [3], cephalexin [3], 7-ureidoacetamidocephalosporins [4] and carbenicillin [5].

This paper describes the determination of 6059-S and the separation of its *R*- and *S*-epimers in the quality control of pure substances and in human urine by reversed-phase HPLC. The results obtained by HPLC and microbiological assays are compared.

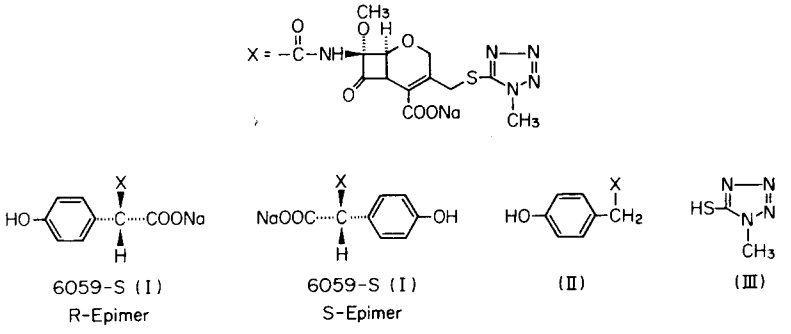

Fig. 1. Structures of 6059-S and related compounds.

EXPERIMENTAL

Materials and reagents

The compounds 6059-S (I), decarboxy-6059-S (II) and 1-methyl-1H-tetrazole-5-thiol (III) were synthesized at Shionogi Research Laboratory (Osaka, Japan). Guaranteed-reagent grade monobasic potassium phosphate (Kanto, Tokyo, Japan), monobasic sodium phosphate (Wako, Osaka, Japan) and dibasic sodium phosphate (Wako) and reagent-grade *o*- and *p*-acetanisidide (Eastman-Kodak, Rochester, NY, U.S.A.) were used. A 10% solution of reagent-grade tetra-*n*-butylammonium hydroxide (Wako) was used as a constituent of the mobile phase in paired-ion chromatography. Water was purified by using an ion-exchange column, reverse osmosis and finally a single distillation. HPLC-quality methanol (Nakarai, Kyoto, Japan) was used.

Apparatus

The liquid chromatograph consisted of a Waters Model 6000A pump, a Type U6K injector (Waters Assoc., Milford, MA, U.S.A.) or a Rheodyne Model 7120 injector, and a Model 440 UV detector (Waters Assoc.) or a Japan Spectrooptics UVIDEC-100 variable-wavelength UV detector. A pre-

packed column of Nucleosil $10C_{18}$, particle size 10 μm (30 cm × 4 mm I.D.), was obtained from Macherey, Nagel & Co. (Düren, G.F.R.). For urine analysis, a guard column packed with Nucleosil $10C_{18}$ in a 5 cm × 4 mm I.D. stainless-steel tube was connected to the analytical column. Quantitation was based on integration of peak areas using a Chromatopac EIA (Shimadzu, Kyoto, Japan).

Chromatographic procedure

Two mobile phases were applied: methanol—0.05 *M* monobasic potassium phosphate solution (5:95) adjusted to pH 6.5 (eluent A) and methanol—0.005 *M* tetra-*n*-butylammonium phosphate solution (25:75) which was prepared by combining a 10% solution of tetra-*n*-butylammonium hydroxide with a mixed solution of dibasic sodium phosphate and monobasic sodium phosphate to make the pH 6.0 (eluent B).

The mobile phase was passed through a membrane filter (0.4 μm) to remove any particulate matter and dissolved gases prior to use. All experiments were run at room temperature. The flow-rates were 2.0 ml/min for eluent A and 1.0 ml/min for eluent B, and the inlet pressures were about 14 and about 10 MPa, respectively. The detector was operated at 254 nm.

Determination of R- and S-epimer ratios and quantitation of 6059-S in pharmaceutical raw materials

Approximately 0.5 mg of sample was dissolved in 1 ml of distilled water at room temperature. Immediately, 5 μl of the sample solution were introduced on to the chromatographic column under the above operating parameters using eluent A. Ratios of the *R*- to the *S*-epimer were determined from the ratios of their peak areas.

For quantitative determination, a 60 mg/l internal standard solution of *p*- or *o*-acetanisidide was prepared. Reference 6059-S, with a known water content, was accurately weighed into a volumetric flask, then dissolved and diluted as required with the internal standard solution to give a range of concentrations from 0.5 to 0.1 mg/ml. A 5-μl volume of a standard solution of 6059-S was injected into the column under the above operating parameters using eluent B. Calibration graphs for 6059-S were prepared by plotting the standard sample concentration corrected for the water content against the ratio of the sum of the peak areas of the *R*- and *S*-epimers to the internal standard peak area. Sample solutions of pharmaceutical raw materials were prepared in a similar manner to those of the standards and subjected to HPLC under the same conditions as the standards.

In vivo experiments and determination in human urine

The in vivo experiments were conducted with two groups of three (T) and four (F) healthy male volunteers. In each experiment, 1 g of 6059-S was given intravenously to each volunteer. The ratios of the *R*-epimer to the *S*-epimer of the dosed drugs were 1.11 and 1.05 for the T and F groups, respectively. In the T group, urine samples were taken just before and at 1, 2, 4, 6, 8 and 12 h after administration, frozen, stored at −20°C and measured within 1 week. On another day, urine samples of the F group were

taken just before and at 1, 2, 4, 6 and 8 h after drug administration, and analysed in the same way as for the T group.

After thawing, 1—2-ml urine samples were passed through Sep-Pak cartridges (Waters Assoc.) for preliminary sample clean-up, and the eluates were injected directly into a liquid chromatograph under the above operating parameters using eluent B.

For the calibration procedure, 1 g from the same batch as the dosage drug was dissolved in distilled water and diluted with water to give a concentration range of 10—0.1 mg/ml. Calibration graphs for 6059-S were prepared for the ranges 10—2, 3—0.3 and 1—0.1 mg/ml by plotting the standard sample concentration against the sum of the peak areas of *R*- and *S*-epimers for the concentration range desired from the predicted concentration in urine samples. Ratios of the *R*- to the *S*-epimer were determined from the ratios of their peak areas together with the quantitation of 6059-S.

Microbiological assay of 6059-S

Concentrations of 6059-S were determined by the band culture assay method, using as the test organism *E. coli* 7437, which has been described previously [2]. Standard solutions of 6059-S were prepared at concentrations of 8, 4, 2, 1, 0.5 and 0.25 μg/ml by dissolving 1 g from the same batch as the dosage drug in a 0.1 *M* potassium phosphate buffer solution (pH 7.0). The urine samples were diluted to a concentration below 8 μg/ml prior to assay.

RESULTS AND DISCUSSION

Separation of R- and S-epimers

There are many diastereoisomers of penicillin, cephalosporin and cephamycin antibiotics owing to the existence of an asymmetric carbon in the side-chain of the β-lactam ring. Determination of the diastereoisomeric purity of antibiotics is important in order to evaluate the antimicrobial activity of the isomers. Recently, Salto [3] separated completely the diastereoisomers of ampicillin and cephalexin by reversed-phase chromatography, and Young [4] also separated some diastereoisomeric 7-ureidoacetamido cephalosporins in a similar manner.

We were able to separate the diastereoisomers of 6059-S completely by reversed-phase chromatography using 0.05 *M* phosphate buffer (pH 6.5) containing 5% methanol, as shown in Fig. 2. Under these conditions, an impurity, 1-methyl-1H-tetrazole-5-thiol (III), was eluted early but another impurity, decarboxy-6059-S (II), was not eluted, probably owing to the difference in the degree of hydration based on monobasic and dibasic acids. With gradient chromatography, increasing the methanol content in the same mobile phase, the peak of decarboxy-6059-S (II) is, of course, observed.

The former of the two peaks of 6059-S on the chromatogram shown in Fig. 2 was identified as that of the *R*-epimer of 6059-S, based on comparison with the retention time of the crystallized diammonium salt* of 6059-S free

*This compound was first crystallized by Dr. K.S. Yang of Lilly Res. Lab., Eli Lilly & Co., Indianapolis, IN, U.S.A.

acid, the structure of which was determined to be that of the *R*-epimer by X-ray diffraction [6].

The pure *R*- or *S*-epimer obtained by preparative chromatography was re-chromatographed immediately under the same conditions and gave only one peak. This result indicates that the ratios of the *R*- to the *S*-epimer do not change and no decomposition product is produced during this chromatographic procedure. On the UV spectra, no difference in absorbance between the *R*- and *S*-epimers was observed at any wavelength. Accordingly, the ratios of the peak areas of the *R*- to the *S*-epimer represent the ratios of their contents.

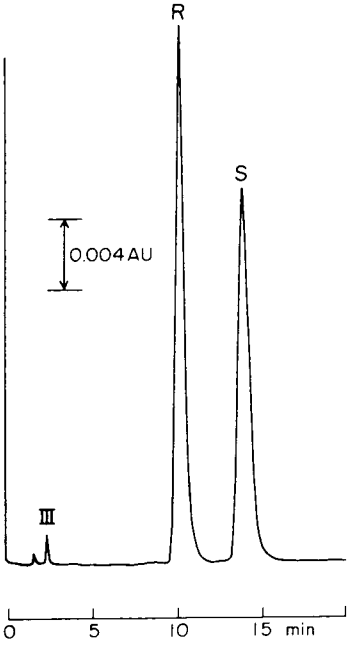

Fig. 2. Determination of the ratio of the *R*- to the *S*-epimer in a pharmaceutical material. Conditions as in text (eluent A); R, *R*-epimer; S, *S*-epimer; III, 1-methyl-1H-tetrazole-5-thiol.

Quantitative determination of 6059-S

Decarboxy-6059-S (II), one of the degradation products, was not eluted under isocratic conditions using eluent A as described under Experimental. Information about this compound, however, is important for the determination of the purity of pharmaceutical raw materials and commercial products and the metabolites in biological materials, because it has some antibacterial activity [7].

The compound 6059-S was eluted at a convenient time under the chromatographic conditions using eluent B, although the separation of the epimers was incomplete. The chromatography allows adequate elution of decarboxy-6059-S (II) without overlapping by the internal standard, *o*- or *p*-acetanisidide.

An example of the chromatogram is shown in Fig. 3. The eluates corresponding to the two 6059-S peaks were fractionated and each fraction was re-chromatographed immediately under the same conditions. No extra peaks were observed on the chromatogram. From this result, decomposition of 6059-S during the chromatographic procedure can be neglected. Two peaks of compounds II and III observed in Fig. 3 arose from impurities in the sample material itself.

The determination of 6059-S was based on the ratio of the combined peak area of the epimers to the peak area of the internal standard measured by an integrator. The calibration graphs obtained by this method showed good linearity in the range 0.5—0.1 mg/ml. We confirmed that mannitol or glucose added to the pharmaceutical preparation did not interfere with the determination of 6059-S.

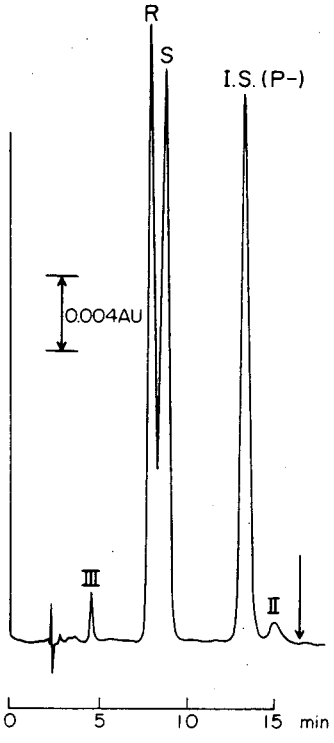

Fig. 3. Determination of 6059-S in a pharmaceutical material. Conditions as in text (eluent B; 25% methanol); volume injected; 5 μl; 0.4 mg/ml; R, *R*-epimer; S, *S*-epimer; II, decarboxy-6059-S; III, 1-methyl-1H-tetrazole-5-thiol; I.S.(P-), *p*-acetanisidide; arrow, *o*-acetanisidide (I.S.) to be eluted.

Determination of 6059-S in human urine

Chromatography using eluents A and B was tested for determining 6059-S in human urine. Interfering absorptions at the retention times of the 6059-S epimers were found in the chromatogram of control urine using eluent A or methanol-free eluent A. Fig. 4 shows a typical chromatogram obtained using

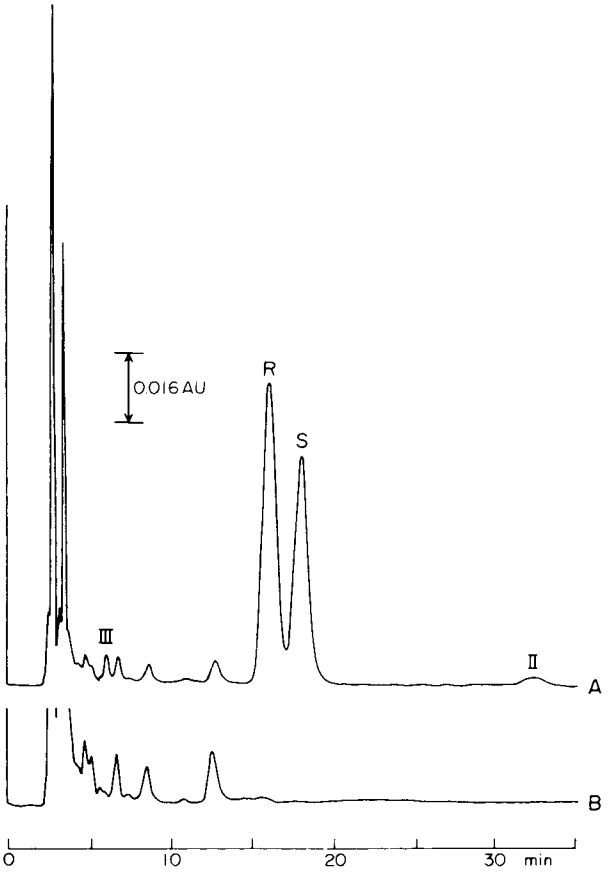

Fig. 4. (A) Chromatogram of human urine excreted following administration of 6059-S. Conditions as in text (eluent B; 20% methanol); volume injected; 3 μl; R, *R*-epimer; S, *S*-epimer; II, decarboxy-6059-S; III, 1-methyl-1H-tetrazole-5-thiol; *R* + *S*, 3.2 mg/ml. (B) Chromatogram of control human urine.

eluent B with a methanol content of 20% for a sample of urine containing 3 mg/ml of 6059-S from a volunteer. The front and rear peaks of 6059-S corresponding to compounds III and II, respectively, originate from the dosed material. During the time-course assay experiment, the amounts of both compounds in urine remained almost constant. In the experiment employing spiked urine samples, the recovery in the clean-up procedure by use of the Sep-Pak C_{18} cartridge proved to be quantitative, and the detection limit was about 0.01 mg/ml.

Table I gives the results for the amounts of 6059-S excreted in the urine of seven volunteers, determined by HPLC, together with the bioassay data and for the ratios of the *R*- to the *S*-epimer obtained by HPLC. The average accumulated excretion recoveries up to 12 h for the T group and up to 8 h for the F group were 795.7 and 782.3 mg, respectively. The actual average amounts of 6059-S injected were determined to be 975 mg from analysis of the residue in an injector and a bottle. Consequently, the corrected average

TABLE I

EXCRETED AMOUNTS AND RATIO OF *R*- AND *S*-EPIMERS OF 6059-S IN URINE FOLLOWING A SINGLE INTRAVENOUS ADMINISTRATION OF 1 g TO VOLUNTEERS

Dosage *R/S* ratio: T, 1.11; F, 1.05.

Group	Subject	Result	Time after administration (h)						
			0—1	1—2	2—4	4—6	6—8	8—12	Total*
T	M.O.	HPLC (mg)	338	152	152	71	38	29	780
		Bioassay (mg)	365	153	146	68	36	29	797
		R/S ratio	1.35	1.08	1.07	0.94	0.90	0.90	1.15
	S.O.	HPLC	311	168	152	74	44	35	784
		Bioassay	364	164	151	71	40	32	822
		R/S ratio	1.35	1.13	1.07	1.01	0.97	0.95	1.17
	M.H.	HPLC	363	175	163	68	31	23	823
		Bioassay	364	172	164	63	30	24	817
		R/S ratio	1.26	1.11	1.02	0.94	0.89	0.90	1.12
F	H.T.	HPLC	311	167	185	93	44		800
		Bioassay	337	194	186	81	35		833
		R/S ratio	1.27	1.16	1.03	0.97	0.96		1.14
	H.I.	HPLC	323	172	182	88	43		808
		Bioassay	354	173	178	84	40		829
		R/S ratio	1.18	1.06	1.08	0.95	0.98		1.09
	M.K.	HPLC	296	160	178	97	45		776
		Bioassay	314	167	173	83	42		779
		R/S ratio	1.12	1.08	1.01	1.01	0.98		1.06
	S.M.	HPLC	302	146	169	76	52		745
		Bioassay	305	154	184	85	56		784
		R/S ratio	1.16	1.10	1.06	0.98	1.05		1.10

*T, 0—12 h; F, 0—8 h.

recoveries were 81.6% for the T group and 80.2% for the F group. The excretion rate—time curves are given in Fig. 5. One-compartment model analysis of the time-course data revealed that 6059-S dosed intravenously was excreted in urine in the intact form with a rate constant of 0.433 ± 0.047 h^{-1} (n = 7).

In Table I, it is noteworthy that the ratios of the *R*- to the *S*-epimer changed in the time-course of excretion. The early ratios were higher than that in the dosed 6059-S drug itself, but decreased with time to become lower than that of the dosed drug. The spiked human urine samples (pH 7.0), with initial ratios of the *R*- to the *S*-epimer of 1.27 and 0.71, were allowed to stand for 2 h at 37°C in order to examine whether interconversion between the two epimers occurs in the bladder. As both ratios remained almost constant with time, we can regard the ratios determined on collected urine samples as true values for excretion. The fact that the ratios of the *R*- to the *S*-epimer in total urine approached the level in the dosed drugs suggests that no interconversion between the two epimers occurs in the human body. Recently, Yamada et al. [8] reported that the faster excretion of the *R*-

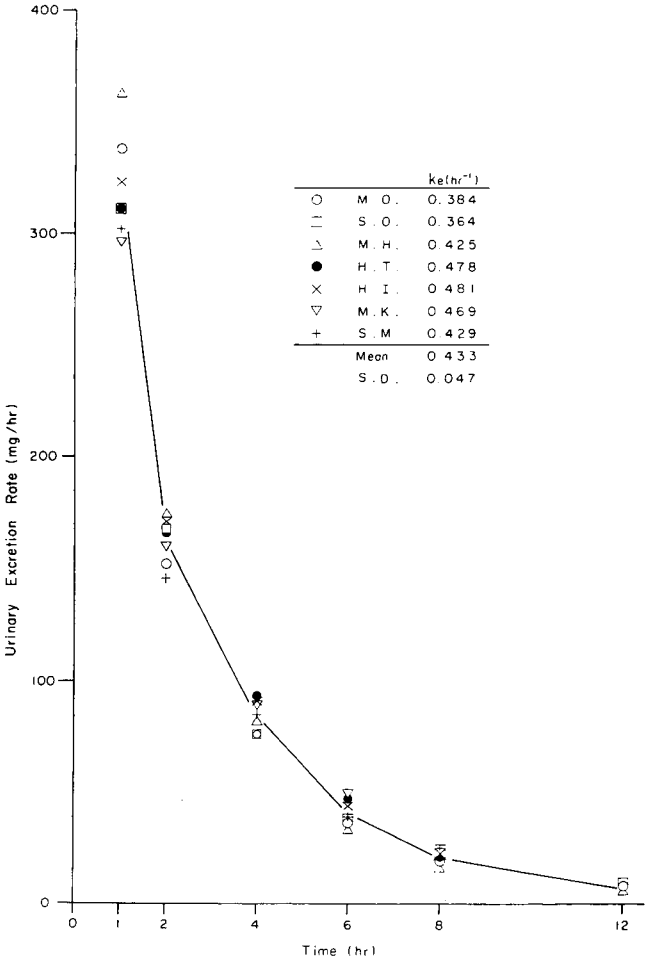

Fig. 5. Urinary excretion rate of 6059-S after intravenous administration of 1 g of 6059-S.

epimer compared with that of the *S*-epimer can be explained by the larger unbound fraction of the former in human plasma.

Correlation with microbiological assay

Fig. 6 shows the correlation of HPLC and microbiological data in Table I for the amounts of 6059-S excreted. Linear regression analysis led to the equation $y = 1.085x - 8.57$, with a correlation coefficient of 0.9954 and a coefficient of variation of 7.1%. The slope of greater than unity and the negative intercept mean that the microbiological results are higher than the HPLC results in the early stages and vice versa in the later stages. As the ratio of the *R*- to the *S*-epimer in the standard solution of 6059-S is equivalent to the dosage level and the *R*-epimer is approximately three times more active than the *S*-epimer against the test organism [9], the estimated concentrations of 6059-S in the samples tend to be higher by microbiological assay than by

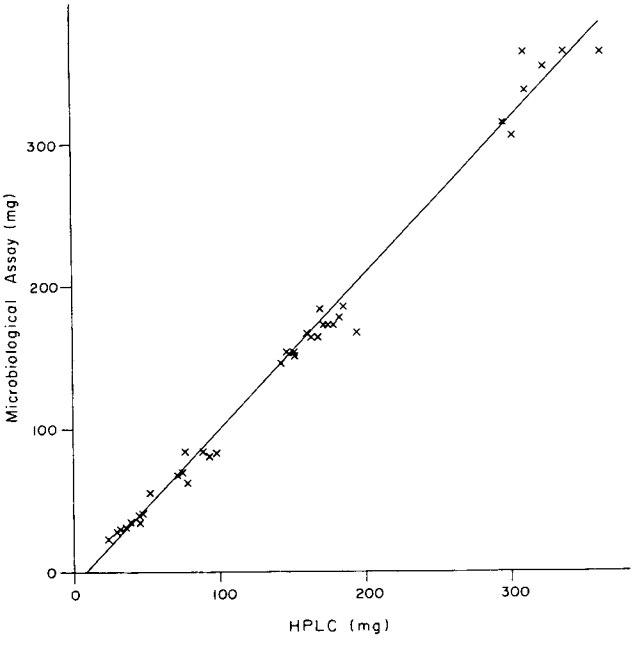

Fig. 6. Linear regression analysis of amounts of 6059-S in human urine samples determined by HPLC and microbiological assay.

HPLC when the ratio of the *R*- to the *S*-epimer in the sample is higher than the dosage level, namely in the early stages. This may explain the discrepancy between the values obtained by the two assay methods and the characteristics of the regression analysis.

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Note

Determination of a wide range of urinary amine metabolites using a simple high-performance liquid chromatographic technique

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The determination of catecholamine and indoleamine metabolites in body fluids plays an important role in both psychopharmacological research and clinical diagnosis, particularly in diseases such as phaeochromocytoma or carcinoid syndrome [1]. Qualitative screening can be performed by thin-layer chromatography; alternatively (or in addition) the quantitative determination of single compounds or small groups of compounds can be performed by gas chromatography [2], fluorimetry [3] or high-performance liquid chromatography (HPLC) [4, 5]. We report here a procedure for urinary metabolites which, although a simple screening method, has the adequate sensitivity and accuracy otherwise exhibited by the more complex HPLC separation and detection systems. It involves a simple solvent extraction, HPLC separation and UV spectroscopic detection of *p*-hydroxyphenylpyruvic acid (*p*-HPPA), 3-methoxy-4-hydroxyphenyl glycol (MHPG), 2-hydroxyphenylacetic acid (2-HPAC), 3,4-dihydroxyphenylacetic acid (DOPAC), hippuric acid (HA), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), homovanillic acid (HVA), vanillic acid (VA) and indoleacetic acid (IAA).

EXPERIMENTAL

Urine extraction

An aliquot from a 24-h urine sample collected in 25 ml of concentrated hydrochloric acid (pH 2—3) containing 15 mg of creatinine was diluted to 30 ml with water and extracted with two 30-ml volumes of diethyl ether. The pooled organic phase was dried over anhydrous sodium sulphate for 30 min and then evaporated to dryness under vacuum at 30°C. The dry deposit was dis-

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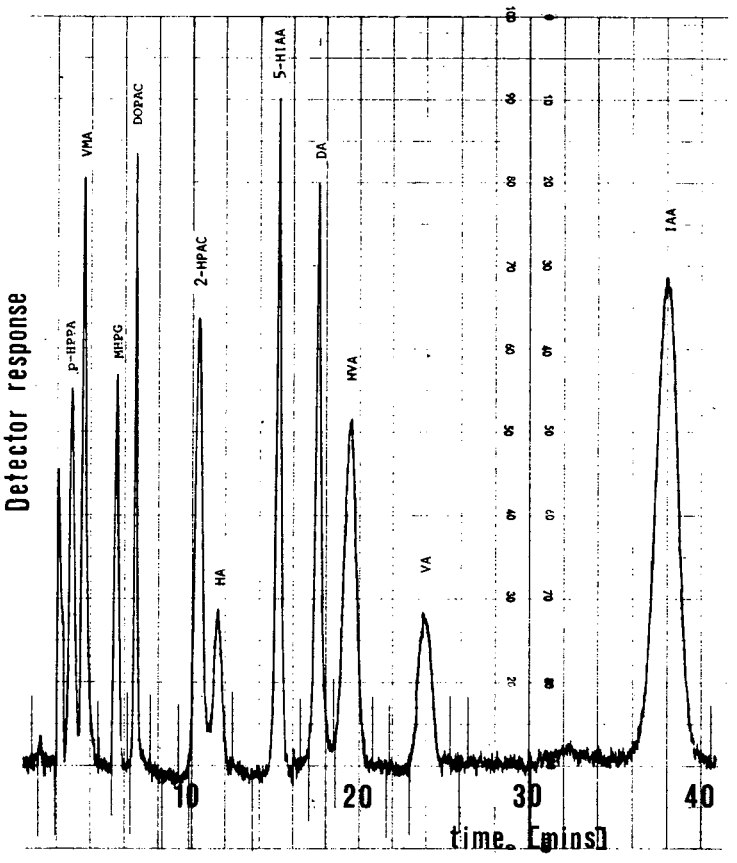

Fig. 1. Qualitative chromatographic separation of a mixture of standard compounds.

TABLE I

HPLC OF URINARY METABOLITES

Compound	Retention time (min)	Sensitivity of the method (μg per 30 ml)
p-HPPA	4.04	15
VMA	4.73	1.0
MHPG	6.53	3.5
DOPAC	8.12	0.2
2-HPAC	12.45	0.1
HA	13.08	2.0
5-HIAA	17.15	0.1
DA	19.3	0.2
HVA	22.4	0.1
VA	25.4	0.1
IAA	41.38	0.1

solved in 2 ml of 0.2 *M* acetic acid and 20 μl of the solution were injected into the HPLC system.

Chromatography and detection

HPLC was performed using a reversed-phase column (25 × 0.4 cm, 5-μm Spherisorb ODS; LDC, Riviera Beach, FL, U.S.A.) with 0.2 *M* acetic acid as eluent delivered by a reciprocating double-piston pump at 1 ml/min. Samples were introduced by a 20-μl loop injector. Detection was effected using a UV spectrometer (Spectromonitor II; LDC) set at 280 nm. An electronic integrator (minigrator; Spectra Physics, Santa Clara, CA, U.S.A.) was used to simplify peak identification and quantitation. The compounds present were determined by an external standard method; together with each batch of 5—10 samples a standard mixture was extracted and the peak areas were compared. The validity of this method was checked from time to time by addition of standards to a duplicate urine sample.

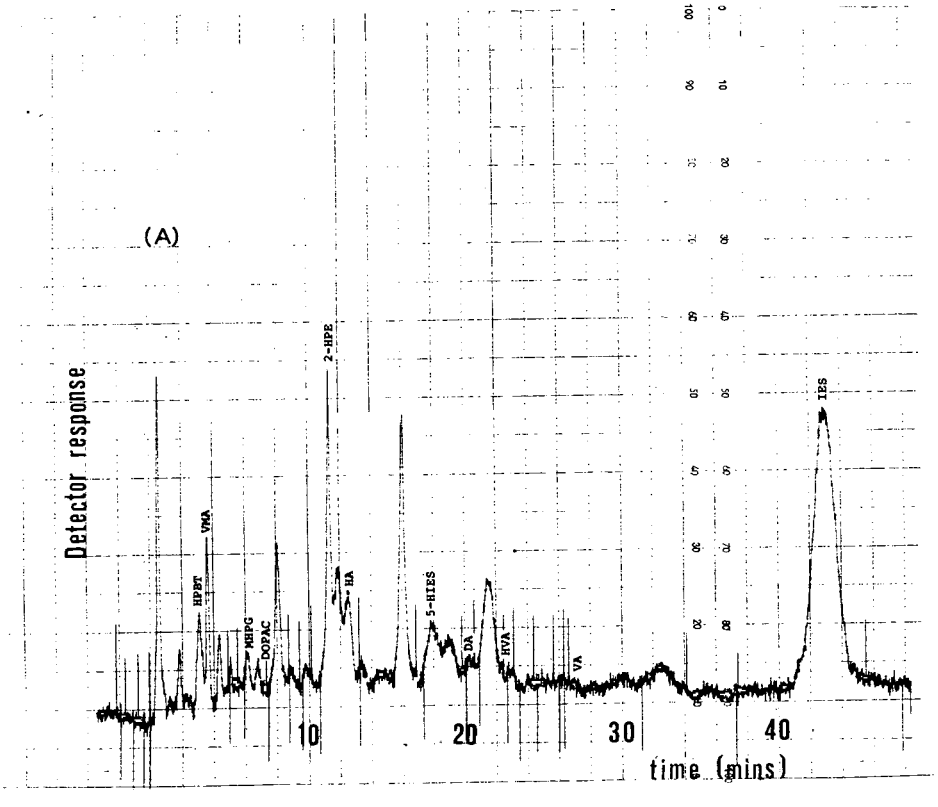

Fig. 2A.

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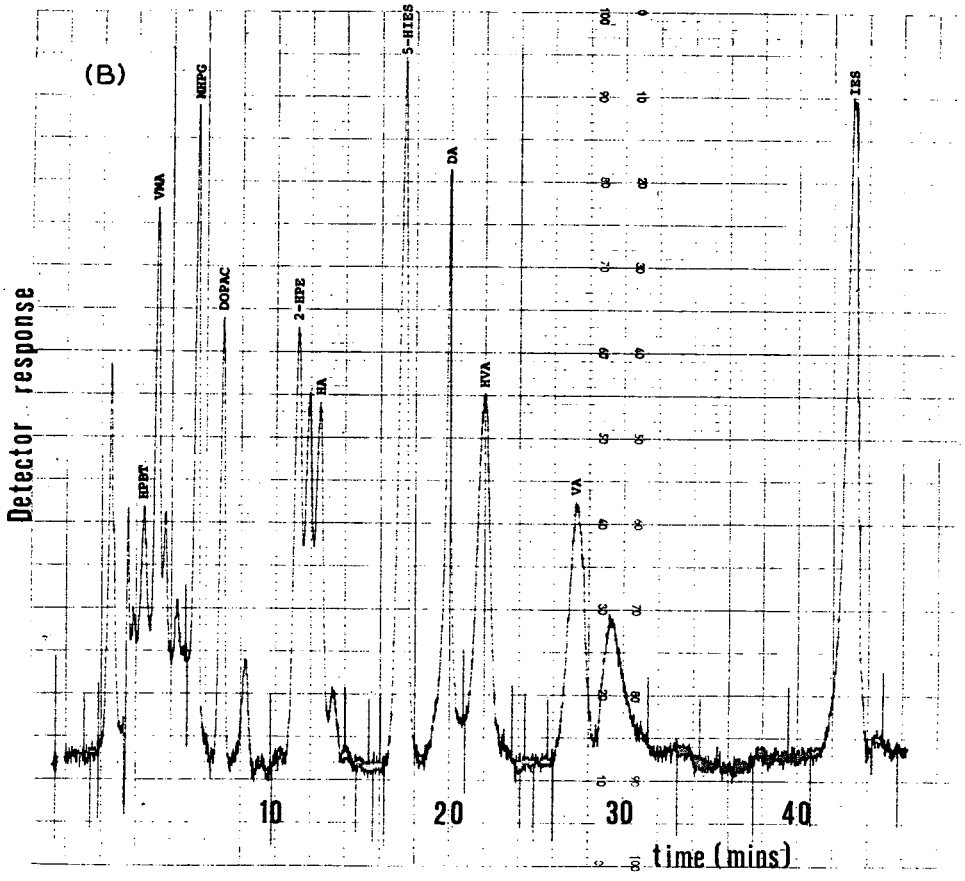

Fig. 2. Chromatograms of a normal diluted urine sample extracted as described in the text, (A) without or (B) with added standards.

RESULTS AND DISCUSSION

The sensitivity of the method with respect to each urinary metabolite is shown in Table I. The high variation between different samples reflects the variation in both the percentage extracted and the molar absorptivity at 280 nm. The sensitivity is, however, adequate for all amine neurotransmitter metabolites measured here (Fig. 1). The reproducibility of the method was established by multiple extraction from a single urine sample; this provided a mean coefficient of variation of 6% for the range of compounds studied, which compares favourably with other techniques [2—5].

It takes approximately 1 h to run a chromatogram; this time could perhaps be reduced when not all of the metabolites listed here need to be determined.

We have applied the method to the routine analysis of urine samples (Fig. 2) in the diagnosis of carcinoid syndrome, phaeochromocytoma and similar

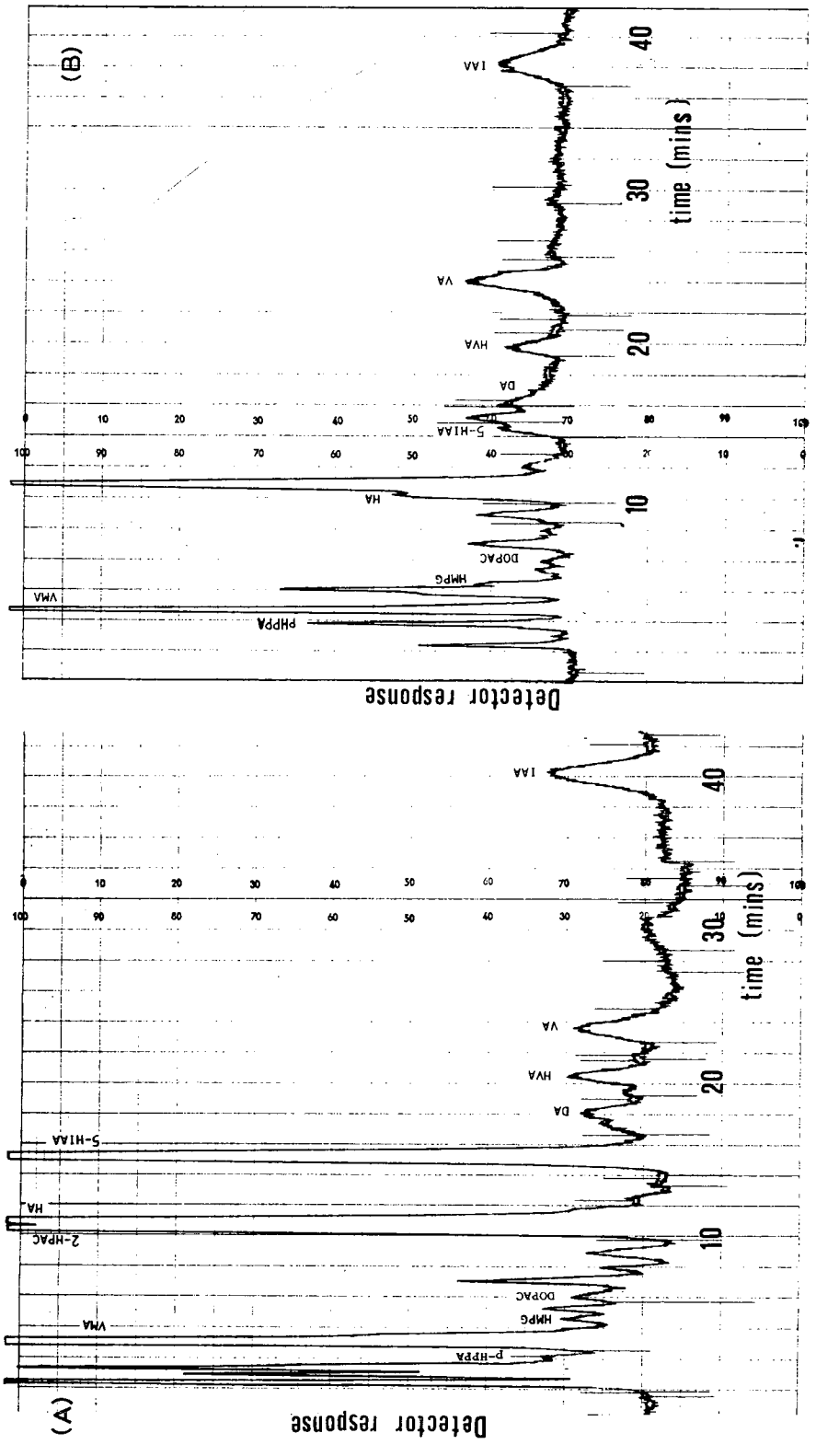

Fig. 3. Typical chromatogram of (A) a carcinoid urine sample and (B) another showing the urinary pattern of amine metabolites from a patient with anorexia nervosa.

diseases [1]. In addition, an investigation into amine metabolism in anorexia nervosa has been undertaken using this system [6]. Examples of pathological analyses are shown in Fig. 3.

In conclusion we have found this method to be a simple and accurate procedure for the determination of a wide range of urinary metabolites.

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Note

Separation of human C-apolipoproteins by high-performance liquid chromatography*

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(First received January 15th, 1981; revised manuscript received March 23rd, 1981)

The C-apolipoproteins (apo C) are part of the protein moiety of very low-density lipoproteins (VLDL) which are involved in the metabolism of lipoproteins [1]. They are a heterogeneous class of proteins and can be separated into apo C-I, C-II, C-III by column chromatography [2]. Since isoelectric focusing results in additional C-apolipoprotein bands [3, 4] the separation of apo C by column chromatography seems to be incomplete. The increasing interest in specific antisera for the immunochemical quantification of apolipoproteins needs highly purified antigens. Therefore we tried to separate the C-apolipoproteins by high-performance liquid chromatography (HPLC).

MATERIAL AND METHODS

From pooled sera of patients with primary type IV hyperlipoproteinemia very low-density lipoproteins (VLDL) were prepared by preparative ultracentrifugation (d = 1.006 g/ml, 125,000 g, 48 h, 4°C). VLDL were separated by tube slicing, delipidated with an equal volume of tetramethylurea (TMU), dialyzed against 0.05 M NH_4HCO_3 buffer (pH 8.6) for 24 h, and lyophilized. For column chromatography (Sephadex G-200 fine, 100 × 2.5 cm, 4°C) 50 mg of the TMU-soluble apolipoproteins were resolubilized in 0.05 M NH_4HCO_3 buffer (pH 8.6) containing 6 M urea [5]. The apo C-containing fractions were pooled, dialyzed and lyophilized. They were, furthermore, analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) (10%) and 8 M urea (7.5%, pH 8.6). Fractionation of the C-apolipoproteins was performed by HPLC.

*Dedicated to Prof. Dr. H. Schwiegk on his 75th birthday.

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The HPLC experiments were performed using an Altex programmer Model 420 and two Altex pumps (Model 110A) with a variable-wavelength UV detector (Kontron Corp., Munich, G.F.R.).

Solvent methanol (HPLC grade) and reagent grade sodium phosphate were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was glass-distilled from the deionized water supply of the laboratory. The HPLC column was purchased from Kontron; a pre-column was prepared in this laboratory. The column dimensions were 250 × 4.2 mm I.D., the pre-column dimensions 40 × 4.2 mm I.D. The packing was reversed-phase C-18 material (particle size 10 μm).

Chromatographic conditions were: sample injection as 15 mg/ml solutions in phosphate buffer; detection in a 10 mm path length cell (volume 7 μl) at 210 nm. Buffer A was methanol, buffer B 0.01 *M* phosphate buffer (pH 6.0). A linear gradient was supplied from 40 to 28% buffer B in 120 min. The flow-rate was 1 ml/min. Methanol was removed by drying under vacuum. The identification of the separated peaks was done by amino acid analysis and determination of the N-terminal amino acid.

RESULTS

The gel permeation chromatogram of the TMU-soluble VLDL proteins is shown in Fig. 1. The pooled C-apolipoproteins typically [6] could be separated by analytical polyacrylamide gel electrophoresis in urea (Fig. 2a) and gave a broad band in SDS (Fig. 2b). The preparative separation by HPLC resulted in 19 peaks with a yield of 80%; 17 peaks (Fig. 3) turned out to be C-apolipoproteins on the basis of amino acid analyses [7—9]. The remaining two peaks had amino acid compositions which do not correspond to any known apolipoprotein. While apo C-I represented one peak (typical amino acid composition, for example, the lack of half-cystine, tyrosine and histidine, one N-terminal amino acid residue threonine and the second residue proline from the N-terminus), apo C-II could be separated into 4 peaks (five tyrosine and one isoleucine residue, threonine as the single N-terminal amino acid and glutamic acid as the second N-terminal residue), and apo C-III into 12 peaks (two tyrosine residues, the lack of half-cystine and isoleucine, serine as the single N-terminal amino acid and glutamic acid as the second N-terminal residue). All the apo C-II or apo C-III peaks resulted in identical amino acid analyses and no atypical amino acids such as homocitrulline have been found.

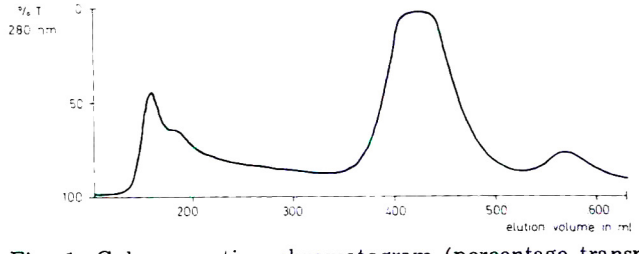

Fig. 1. Gel permeation chromatogram (percentage transmission at 280 nm) of 50 mg of TMU-soluble VLDL on Sephadex G-200 fine. The peak, containing the C-apolipoproteins, was eluted between 380 and 480 ml.

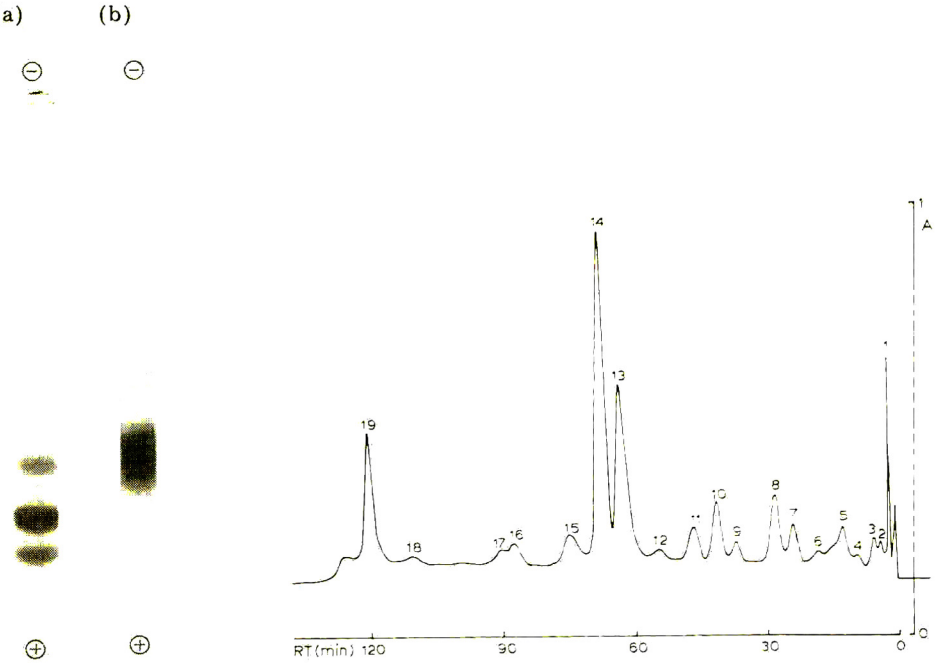

Fig. 2. Polyacrylamide gel electrophoresis (50 μg) in urea (a) and SDS (b) of the C-apolipoproteins. Bands from the cathode to the anode (in a): apo C-I, C-II, C-III$_0$, C-III$_1$, C-III$_2$.

Fig. 3. Chromatogram of C-apolipoproteins separated by HPLC under the following conditions: linear gradient from 40% to 28% buffer B (0.01 *M* sodium phosphate, pH 6.0) eluent methanol; 120 min, flow-rate 1.0 ml/min. RT = retention time. Peak 1 = apo C-I; peaks 2 and 4 = unidentified; peaks 3 and 5—15 = apo C-III; peaks 16—19 = apo C-II.

Comparable chromatograms were obtained under more acidic conditions (down to pH 2.1).

DISCUSSION

These data demonstrate that HPLC is a suitable method for the preparative separation of the heterogeneous C-apolipoproteins. This method is superior to preparative isoelectric focusing of the C-apolipoproteins (close isoelectric points, low yield and difficulty in removing the ampholyte [4]).

Apo C-III has been reported to consist of three polymorphic forms [10], which is due to different sialic acid content [11]. Whether the six polymorphic forms in isoelectric focusing [4] or the 12 forms we found in HPLC are due to differences in the sialic acid portion or due to aggregation remains to be established. Carbamylation can be excluded, since chromatography was performed at 4°C [2], the ratio of the lysine residues was constant and no atypical amino acid residues have been found.

Apo C-II is considered to be homogeneous [8] though in isoelectric focusing two forms have been reported [4]. The demonstration of four apo C-II peaks in HPLC which are identical in their amino acid composition might be

explained by differences in their carbohydrate content [12]. Whether our two unidentified peaks correspond to apo C-IV and apo C-V [3] can not be decided.

The preparation of highly purified C-apolipoprotein might be of importance not only for raising specific antibodies but also of clinical importance [13—15].

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Note

Evaluation of C_{18} Sep-Pak cartridges for biological sample clean-up for tricyclic antidepressant assays

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The most commonly used method for the clean-up of biological samples for quantitation of tricyclic antidepressants is the extraction method. The various solvents used for extraction, extraction and derivatization procedures for gas chromatographic (GC) and GC—mass spectrometric (MS) methods have recently been reviewed [1]. In our studies we have used *n*-hexane—isopropanol (9:1) for extraction and a three-step extraction procedure for clean-up of biological samples, such as plasma and urine for tricyclic antidepressant assays [2]. More recently Waters Assoc. (Milford, MA, U.S.A.) have introduced a variety of small packed (Sep-Pak) cartridges for sample clean-up for analysis by liquid chromatography or GC. A few reports have recently appeared in the literature on the use of C_{18} Sep-Pak cartridges for the clean-up of serum or plasma samples for nucleoside and warfarin analysis [3, 4]. In view of our interest in plasma tricyclic antidepressant assays we have now investigated the use of C_{18} Sep-Pak cartridges for separating tricyclic antidepressants and their desmethyl metabolites from plasma and urine samples and evaluated the advantages, specifically sample cleanliness, specificity, efficiency, reproducibility, over the conventional extraction method.

EXPERIMENTAL

Materials

All solvents are pesticide grade (Mallinckrodt, St. Louis, MO, U.S.A.). Tricyclic antidepressant drugs were all obtained from pharmaceutical research laboratories as mentioned in a previous report [2]. d_4-Imipramine and d_4-desipramine were used as internal standards for all tricyclic drugs [2]. Stock solutions of hydrochlorides of internal standards were prepared separately

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in glass distilled water to contain 1 mg/ml of d_4-imipramine and d_4-desipramine as base. A mixture of 10 μl of each standard was then diluted to 1 ml with water to give a working standard containing 10 ng/nl of each of the internal standards. In addition dideuteromethyl amitriptyline and dideuteromethyl doxepin were prepared by reduction of N-formyl derivatives of nortriptyline and desmethyl doxepin using lithium aluminum deuteride [5, 6]. C_{18} Sep-Pak cartridges were purchased from Waters Assoc.

Working standards

Solutions containing 100—500 ng/ml of different tricyclic drugs in water, and drug-free plasma samples were prepared by adding standard solutions of the drug. The drug pairs, imipramine—desipramine, amitriptyline—nortriptyline, doxepin—desmethyldoxepin and protriptyline were added to separate tubes containing 2 ml of water or control plasma samples. Fifty microliters of internal standard solution (500 ng each of d_4-imipramine and d_4-desipramine) were added to each one of the tubes. A mixture of all the seven drugs (200 ng each) and two internal standards (500 ng) was added to a control plasma sample. In another set d_2-amitriptyline and d_2-doxepin were used as internal standards for amitriptyline and doxepin, respectively.

Carbonate—bicarbonate buffer

A mixture of 5 g each of sodium carbonate and sodium bicarbonate was dissolved in 100 ml of water (pH 9.8) and stored in a refrigerator.

C_{18} Sep-Pak clean-up procedure

The cartridge is activated by passing 2 ml of methanol by pressurizing through a plastic or glass syringe followed by 2 ml of distilled water.

Sodium carbonate—bicarbonate buffer (0.5 ml) was added to the aqueous solution, or plasma solution, the mixture thoroughly mixed on a vortex mixer, and passed through the cartridge via the syringe at a flow-rate not greater than 5 ml/min followed by 1 ml of washings from the sample tube. The cartridge was then washed by passing 2 × 2 ml of distilled water through it. The effluent of the sample and washings were collected, and saved for determination of unabsorbed drugs.

Ten milliliters of solvent mixture, hexane—isopropanol (9:1) were passed through the cartridge and the eluate collected in a 15-ml glass stoppered centrifuge tube. The eluate consisted of 0.4 ml of aqueous layer from the void volume of the cartridge, and was drawn off and discarded. The organic layer was evaporated under a current of nitrogen, the residue derivatized to the trifluoroacetyl (TFA) derivative using N-methyltrifluoroacetamide according to the method described in an earlier report [2].

Gas chromatography—mass spectrometry

The GC and GC—MS—selected ion monitoring (SIM) conditions for quantitation of all tricyclic antidepressant drugs have been previously described in detail [2]. The same conditions were used in this study. All quantitations were carried out using the electron impact mode with ionization potential 70 eV, source temperature 260°C, separator temperature 250°C. Therefore

for amitriptyline and doxepin, the corresponding N-dideuteromethyl derivatives were used as internal standards and ions *m/z* 58 and 60 were monitored for SIM. In all cases in these initial evaluation studies, the complete mass spectra of the drug peaks were obtained from plasma and urine samples in order to ascertain the sample cleanliness.

RESULTS

Column efficiency

The spent efficient including water wash was extracted at pH 10.0 into hexane—isopropanol (9:1) and worked up according to the procedure described earlier [2]. Analysis by GC—MS—SIM did not show any trace of the drugs indicating complete absorption of the compounds by the C_{18} Sep-Pak column. Similarly in preliminary experiments we collected the first 10 ml of solvent eluent and separately 5 ml of second eluent. The second fraction was analyzed separately for the drug quantitation. In most cases (80% of the samples), the drug content was undetectable and in 20% of the samples it was less than 5%. It can be concluded that the extraction efficiency is over 95%. Further, recovery studies with known concentrations (100—200 ng/ml) of added standards to drug free plasma showed a mean recovery of 93.8 ± 3.5% (n = 4).

The calibration curves were linear and similar to those reported for the extraction method [2].

Final sample cleanliness

In one experiment a mixture of seven drugs and internal standards was processed through the C_{18} Sep-Pak cartridge and the drugs were simultaneous-

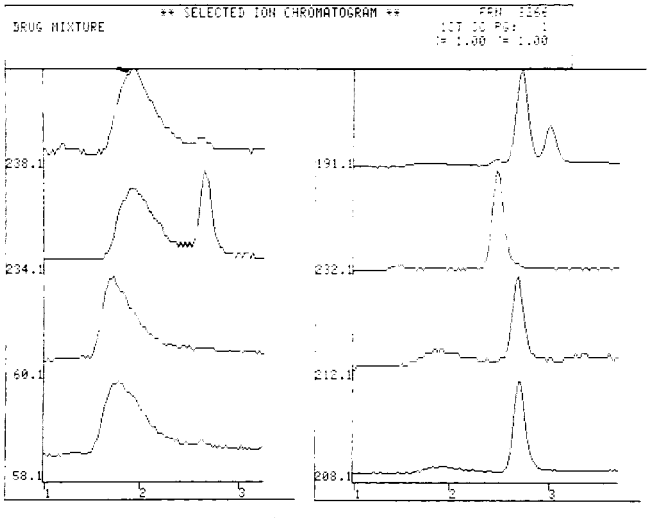

Fig. 1. SIM recordings of drug mixture and internal standards. Peaks: *m/z* 58, imipramine, doxepin and amitriptyline; *m/z* 60, d_2-doxepin and d_2-amitriptyline; *m/z* 191, protriptyline TFA; *m/z* 208, desipramine TFA; *m/z* 212, d_4-desipramine TFA; *m/z* 232, nortriptyline TFA; *m/z* 234, imipramine, desmethyl doxepin TFA; *m/z* 238, d_4-imipramine.

ly analyzed by GC—MS—SIM after derivatization. The selected ion recordings are shown in Fig. 1.

As illustrative examples, a few of the mass spectra obtained from patient plasma and urine samples processed through the Sep-Pak cartridges are given in Figs. 2—5. The mass spectra were identical with those of reference spectra obtained using five compounds under the same GC—MS conditions. In over 20 samples that we have so far processed by this method under the GC—MS conditions we have used, the spectra indicated that the sample peaks were clean and contained only the drug or the drug and internal standard. The samples were at least as clean as those obtained by the conventional extraction

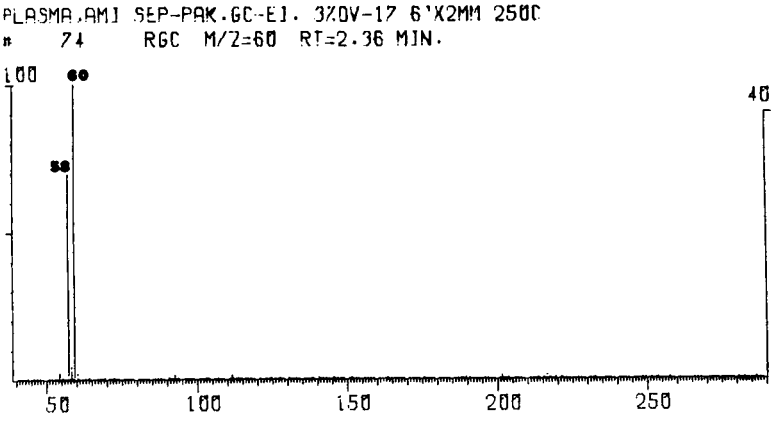

Fig. 2. Mass spectrum of amitriptyline peak from patient plasma sample processed by C_{18} Sep-Pak cartridge. Peaks: *m/z* 58, amitriptyline; *m/z* 60 and 281, d_2-amitriptyline.

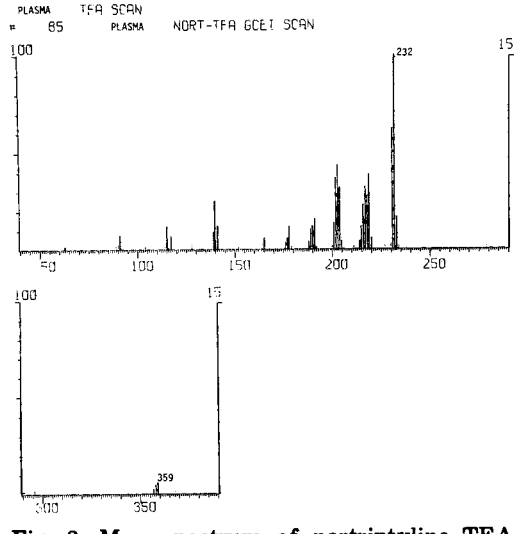

Fig. 3. Mass spectrum of nortriptyline TFA from patient plasma sample, processed by C_{18} Sep-Pak cartridge. Peaks: *m/z* 232, base peak; *m/z* 359, molecular ion.

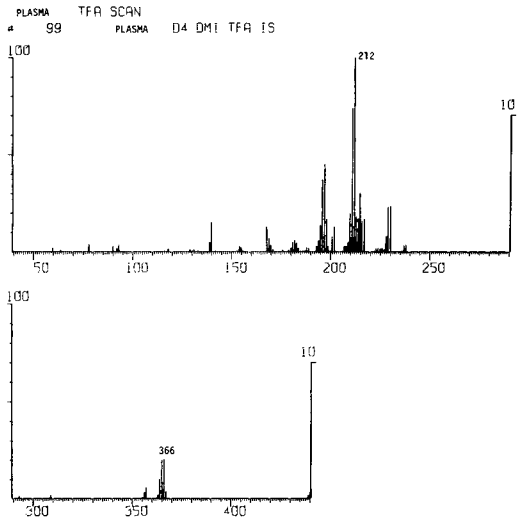

Fig. 4. Mass spectrum of d_4-desipramine TFA from the plasma sample. Peaks: *m/z* 212, base peak; *m/z* 366, molecular ion.

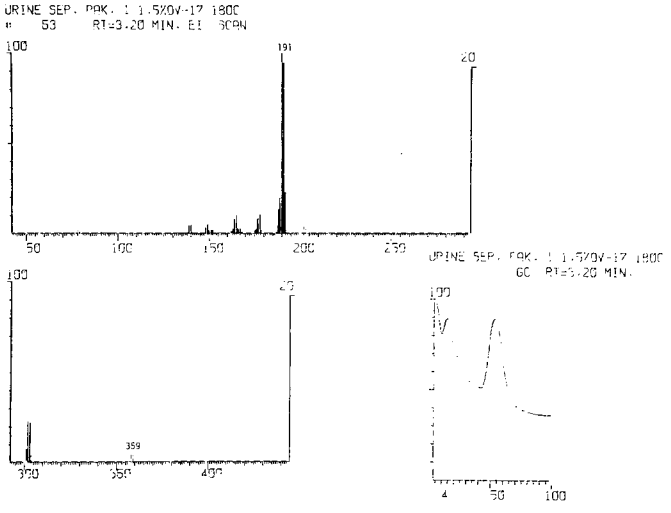

Fig. 5. Mass spectrum of protriptyline TFA from a patient urine sample.

method we have been using in our laboratories. The SIM recording for protriptyline assay is shown in Fig. 6.

Quantitative reproducibility and sensitivity

Parallel determinations of clinical samples (n = 11) were carried out for all tricyclic drugs by the two methods, one using the Sep-Pak and the second by extraction method. The results are presented in Table I. One interesting observation made during this study was that when equal aliquots of the final derivatized product of samples processed by Sep-Pak or extraction method

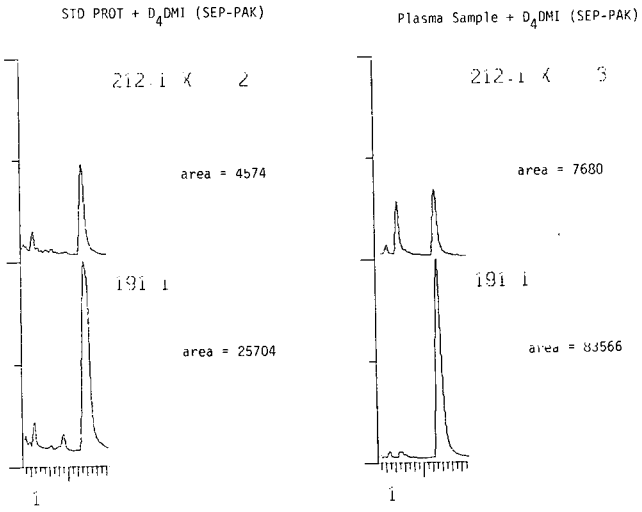

Fig. 6. SIM recording of *m/z* 191 and 212 for quantitation of protriptyline and d_4-desipramine in patient plasma, processed by Sep-Pak method.

TABLE I

PARALLEL DETERMINATIONS FOR THE TRICYCLIC DRUGS BY THE SEP-PAK AND EXTRACTION METHODS

Drug	Sample	Sep-Pak (ng/ml)	Extraction (ng/ml)
Protriptyline	Plasma	218	212
	Plasma	198	202
	Urine	2740	2700
Amitriptyline	Plasma	164	160
Nortriptyline	Plasma	172	170
Imipramine	Plasma	135	129
Desipramine	Plasma	168	170
	Plasma	404	415
	Urine	489	502
Doxepin	Plasma	100	97
Desmethyl doxepin	Plasma	65	67

TABLE II

COMPARISON OF SIM PEAK AREAS OF *m/z* 212 FOR THE INTERNAL STANDARD, d_4-DESIPRAMINE BETWEEN EXTRACTION AND SEP-PAK METHODS

Sep-Pak	*n*	Extraction (Mean ± S.D.)	*n*
82,550 ± 4200	6	78,951 ± 8601	10
81,200 ± 4096	6	59,209 ± 17,704	10

were injected into the GC—MS system, the peak areas from Sep-Pak samples were consistently higher than the peak areas from extraction samples (Table II). Further peak areas of internal standards added to biological samples varied more widely in the extraction method than in the Sep-Pak method (Table II).

DISCUSSION

In this preliminary study we have demonstrated the usefulness of C_{18} Sep-Pak cartridges for the clean-up of clinical plasma and urine samples for the analysis of tricyclic antidepressant drugs. We have also shown that the recovery of the drugs is almost quantitative (> 95%). The samples we analyzed by this method ranged in concentration from 8—410 ng/ml. In our routine clinical analysis for tricyclic antidepressants during the past two years by the extraction method, we have observed wide variations in extraction efficiencies between different plasma samples. The use of internal standards obviates this extraction problem but low recoveries can cause problems of sensitivity for quantitation of low levels of the drug. Our findings with Sep-Pak suggest more consistent and higher recoveries from plasma and urine samples. In one clinical sample where protriptyline was discontinued and desipramine was started, both drugs were monitored in the plasma sample in a single injection using a single internal standard (d_4-desipramine). The saving in time for the analysis using Sep-Pak cartridges is the most important favourable factor for this method. The three-step extraction method involves shaking, centrifuging, withdrawal of solvent or aqueous layers, in all the three steps. In parallel experiments we found that eight samples and two standards could be readied for GC—MS analysis in 40 min, while the conventional extraction methods took 150 min. Another advantage in the Sep-Pak cartridge is that the organic layer is quantitatively recoverable, while in the extraction method, only a fraction is recoverable, and sometimes losses are high due to emulsification. We are evaluating the Sep-Pak cartridges with a large number of clinical samples and if our preliminary findings are confirmed the Sep-Pak cartridges can replace the extraction method.

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Note

Gas chromatographic quantitation of two plasticizers contaminating intravenous fluids stored in plastic containers

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The plasticizer di(2-ethylhexyl) phthalate (DEHP) has been reported as a contaminant in biological fluids by several investigators. Ono et al. [1] detected measurable amounts of DEHP in peripheral blood samples of hemodialysed patients immediately after dialysis. Hillman et al. [2] measured the same plasticizer in autopsied tissue (heart and small intestine) of infants who had umbilical catheters inserted and received varying amounts of blood products. Plasticizer contamination has been reported by Roll et al. [3] in plasma and by Ishida et al. [4] during lipid analysis. The toxicity of plasticizers has also been the subject of several investigations. Calley et al. [5] have reported the LD_{50} of plasticizers in mice and also noted that DEHP shortened hexabarbital sleeping time. Aronson et al. [6] reported that DEHP significantly decreased spontaneous heart rate, coronary flow and isometric tension but elevated diastolic tension in isolated perfused heart. They also noted significant concentration changes in tissue glycogen, ATP, creatine phosphate, etc. Our toxicity studies [7] with DEHP and di-*n*-butyl phthalate (DBP) with dogs showed weight losses, incomplete excretion (especially of DBP), etc.

In addition to DEHP we have reported and quantified [by gas chromatography (GC)] and confirmed [by gas chromatography—mass spectrometry (GC—MS)] the presence of another plasticizer, DBP, in a very significant proportion (80%) of selected surgical patients [8]. Hence, in the light of our toxicity studies with plasticizers and those of other investigators, it was very important for us to determine the source of contamination. These studies were

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in turn intended to reduce plasticizer contamination in our surgical patients, especially those with impaired renal function due to incomplete excretion of the plasticizers [8]. We report here our investigations of several possible sources of plasticizer contamination.

EXPERIMENTAL

Units of whole blood and packed red cells, stored in plastic CPD solution bags and deemed unfit for human use, were secured from the hospital blood bank. Random samples of 5% glucose in water and 0.9% sodium chloride; 0.9% sodium chloride solution and lactated Ringer's solution stored in the original commercial plastic bags were obtained for study from the surgical intensive care unit's supply closet on two random days apart (4 units each). The fluids (2-ml aliquots of blood or 100 ml of crystalloid solution) were extracted with 10 ml of Dole's solution [9] [2-propanol—*n*-heptane—sulfuric acid (40 : 10 : 1)]. The homogenate was centrifuged for 10 min at 4°C, the supernatant was diluted with water (20 ml) and *n*-heptane (10 ml), the solution was agitated and the phases were allowed to separate in a separating funnel. The *n*-heptane phase was dried over anhydrous sodium sulfate, evaporated to dryness under a nitrogen stream and the residue was dissolved in 500 μl of acetone for GC analysis (4 μl). Random samples of alcohol sponges used by our phlebotomy team and the rubber stopper of the blood collecting tubes were extracted with 10 ml of Dole's solution and worked up as above.

A blank prepared using doubly distilled water was run simultaneously through the entire chemical extraction process. The same source of distilled water was used to mix the reagents. Glassware was washed in an ultrasonic washer, rinsed with prechromatographed acetone and baked in an oven overnight at 200°C.

Gas chromatographic analysis

A Hewlett-Packard 5831A gas chromatograph, equipped with a dual flame ionization detector, automatic injector, data system and a 1.8 m × 2 mm I.D. glass column with a 10% Silar-10C packing (Applied Science Labs., State College, PA, U.S.A.) was used. The carrier gas was nitrogen at a flow-rate of 30 ml/min. The column was programmed from 145 to 225°C at 2.5°C/min [8]. The injector and detector temperatures were 300°C. The plasticizers were identified on the basis of their retention times compared with those of standards (Applied Science Labs.).

RESULTS

The levels of the plasticizers detected in various solutions are presented in Table I and the following were the sources of contamination:

(a) The highest levels of DEHP were measured in the blood components stored in CPD solution and plastic bags. The levels increased with the duration of storage, ranging from 3500 to 7500 μg per 100 ml soon after the 3 weeks' expiration date (Figs. 1 and 2C). There did not appear to be any significant difference in the DEHP levels of packed red cells and whole blood. No DBP was

TABLE I

LEVELS OF PLASTICIZERS FOUND IN VARIOUS INTRAVENOUS FLUIDS AND OTHER COMMONLY USED HOSPITAL MATERIALS

System	Concentration (μg per 100 ml)	
	DBP	DEHP
Ringer's lactate	Trace	0
0.9% NaCl	1.98, 3.6	1.17, 1.30
5% glucose—0.9% NaCl	2.25, trace	0.85, 1.25
5% glucose—water	2.33, 3.5	0.95, 0.90
Blood*	—	3500—7500
Alcohol sponges	1.2, 1.30, 1.8, 1.80, 1.90, 2.85 (each sponge)	—
Rubber stopper**	—	—

*See Fig. 2 for details.
**Contained a significant amount of another plasticizer (Fig. 3).

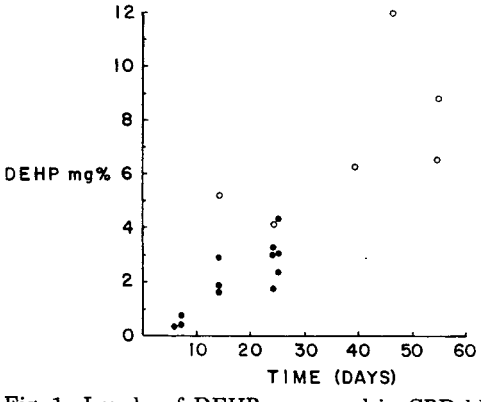

Fig. 1. Levels of DEHP measured in CPD blood stored in plastic bags. The levels of DEHP increased with the duration of storage. ○, whole blood; ●, packed cells.

detected in the above solutions.

(b) Both DEHP and DBP were measured in 0.9% sodium chloride solution, 5% glucose—0.9% sodium chloride solution and 5% glucose—water but only in ng/100 ml levels (Fig. 2B).

(c) Six alcohol sponges averaged 1.31 μg of DBP per sponge. No DEHP was detected.

(d) The red rubber stopper did not contain any DEHP or DBP. However, significant levels of another plasticizer were detected, but no attempt was made to identify it (Fig. 3).

DISCUSSION

Many plastic medical devices are possible sources of contamination with plasticizers of hospitalized patients. The devices involved in the administration of parental therapy that we have tested to date in our laboratory are significant

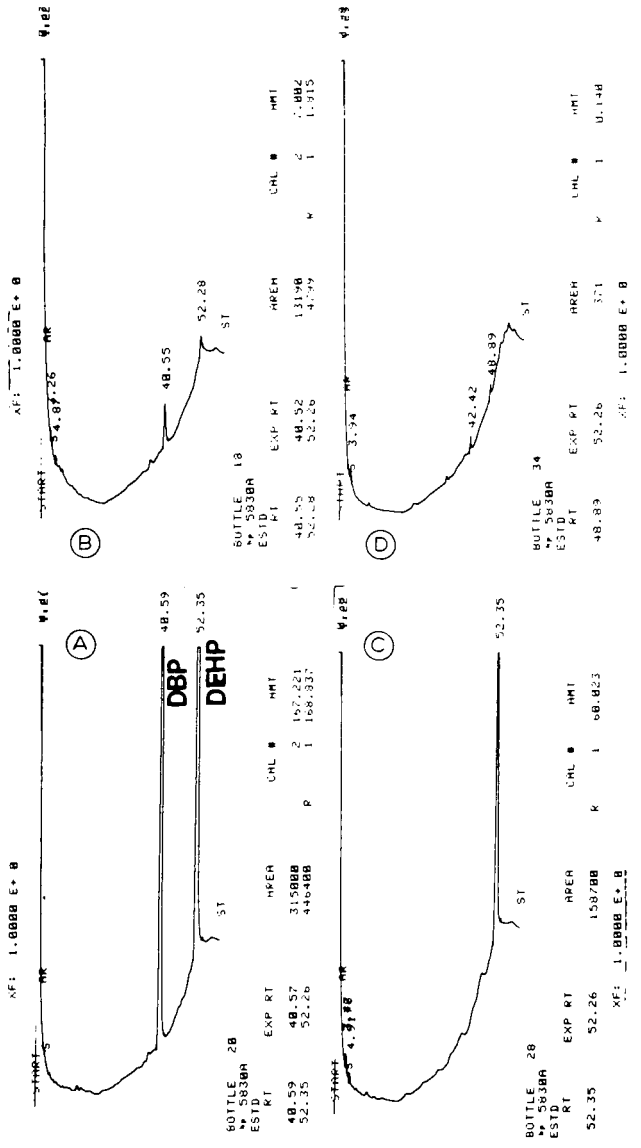

Fig. 2. Gas chromatograms of (A) DBP and DEHP standard solutions; (B) the lipid fraction of 5% G/W solution; (C) CPD stored blood; (D) distilled water blank. No DEHP or DBP were identified in the center blank (D). The highest level of DEHP was noted in the blood sample (C). Both DBP (40.55 min) and DEHP (52.28 min) were present in 5% glucose—water solution (B).

because any possible contamination will directly contaminate patients. Although other investigators [1—6] have measured the most commonly used plasticizer, DEHP, in patients and tissues, we have detected a second plasticizer, DBP, in our patients [8] and some of our crystalloids.

Our findings on DEHP contamination are in agreement with those of several other studies. Marcel and Noel [10] noted plasticizer contamination of blood

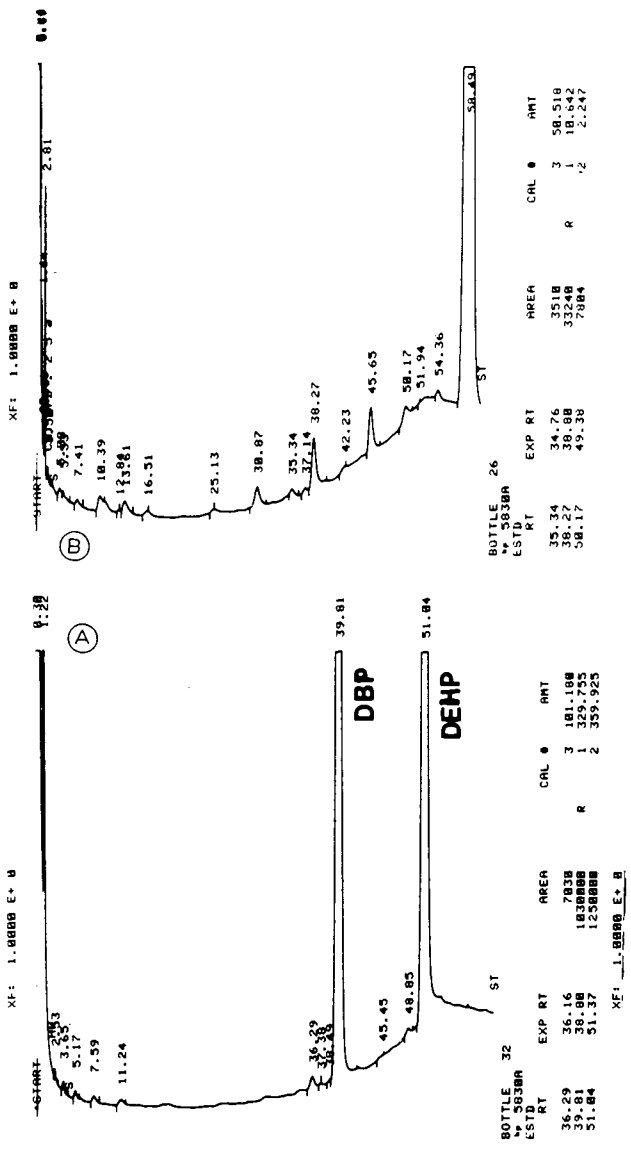

Fig. 3. Extractable lipid contents of the rubber stopper of blood collecting tubes. Composite chromatograms of (A) a standard solution of DBP and DEHP and (B) the lipid fraction extracted from a rubber stopper of a blood collecting tube. The major plasticizer, with a retention time of 58.49 min, extracted from the rubber stopper is well separated and distinguishable from DBP (39.81 min) and DEHP (51.04 min) in the standard solution. The small unidentified peak at 38.27 min can be separated by our column from DBP at 39.81 min into two separate peaks.

stored in plastic bags. Jaeger and Rubin [11, 12] detected measurable levels of DEHP in blood stored in standard plastic bags. The levels of extracted plasticizer increased with duration of storage and could be found in both the red

blood cell (10%) and plasma fractions (90%). Rossel and Bogaert [13] discussed the contamination of biological samples with tygon tubing, cork, plastic syringes, etc. Rock et al. [14] noted an increased concentration of DEHP during storage of whole blood, platelet-rich plasma and platelet concentrates.

The plasticizer DBP is recognized by several investigators as being more toxic than DEHP [5]. Our animal studies also revealed a greater toxicity from equal weight dosages of DBP and DEHP infusion [7]. Stetson and Autian [15] have extensively reviewed various plasticizer toxicity studies and believe that only a low order of acute systemic toxicity has been demonstrated in animal and human experiments. However, attention was first drawn to possible toxicity when cell cultures were destroyed when early plastic containers were used. Protein solution, e.g., blood, can extract more plasticizers from the containers. The addition of fat-soluble vitamins can also extract more of the plasticizers. We do not know the extent to which other medications added to intravenous solutions can increase the extracted plasticizer.

At present these devices have FDA approval. Except for the levels we have measured in stored blood the degree of contamination is small per device unit. However, if we take into account the total number of plastic devices, the extent of contamination could become significant. More studies on the actual contaminations and long-term toxic effects are required.

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Note

Electron-capture gas chromatographic analysis of the triazolobenzodiazepines alprazolam and triazolam

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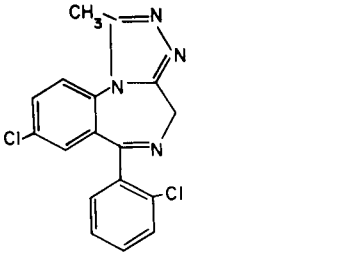
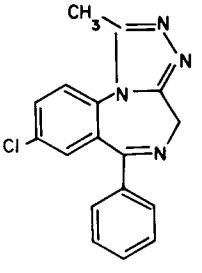
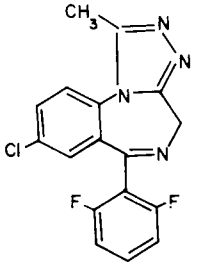
Alprazolam and triazolam (Fig. 1) are triazolobenzodiazepine derivatives used clinically as anxiolytic and hypnotic agents, respectively [1, 2]. Because



TRIAZOLAM



ALPRAZOLAM



U-31485

Fig. 1. Structural formulae of the triazolobenzodiazepines, triazolam and alprazolam. Also shown is the structure of U-31485, the analogue used as internal standard in the analysis.

of their high milligram potency, therapeutic doses of these two agents are low and concentrations in plasma are correspondingly low. Currently available pharmacokinetic data on alprazolam and triazolam have been derived from studies of the radiolabeled drugs [3—5] or with use of high-performance liquid chromatography [6]. This paper describes an electron-capture gas chromatographic (GC) assay of alprazolam and triazolam that can be utilized for quantitation of these two compounds in human plasma following single therapeutic doses.

EXPERIMENTAL

Instrumentation

The analytic instrument is a Hewlett-Packard Model 5750 gas chromatograph equipped with a 2-mCi electron-capture detector operated in the pulsed mode with a pulse interval of 150 μsec. The column is coiled glass, 1.83 m × 2 mm I.D., packed with 1% OV-17 on 80—100 Chromosorb W HP (Hewlett-Packard, Avondale, PA, U.S.A.). The carrier gas is ultrapure helium, at a flow-rate of 50 ml/min. The detector purge gas is argon—methane (95:5), at a flow-rate of 80 ml/min. Operating temperatures are: column, 290° C; detector and injection port, 310° C. The column is primed prior to each day's use by injection of 2—3 μl of a solution of purified soy phosphatides in benzene (1 mg/ml) (Asolectin, Associated Concentrates, Woodside, NY, U.S.A.).

Reagents and standards

Stock solutions are prepared by dissolving 10 mg each of triazolam, alprazolam, and of U-31485, a triazolobenzodiazepine analogue used as internal standard (Fig. 1), in a small amount of ethanol. The volume is made to 100 ml with benzene or toluene. Working standards containing 0.1 μg/ml of each compound are prepared by appropriate dilution with benzene or toluene. Stock solutions and working standards are stable for at least two months when stored in amber bottles at 4° C.

Preparation and extraction of samples

To a series of 13-ml round-bottom culture tubes equipped with PTFE-lined screw top caps is added 10 ng of the internal standard (100 μl of working standard solution). The solvent is evaporated to dryness at 40—50° C under mildly reduced pressure. A series of calibration tubes is prepared by adding 1, 2.5, 5, 7.5, 10, and 12.5 ng of alprazolam or triazolam to a series of these tubes. The solvent again is evaporated to dryness under mildly reduced pressure. A 0.5—1.0 ml aliquot of drug-free control plasma is added to each of the calibration tubes; 0.5—2.0 ml of unknown plasma is added to all of the other tubes. No other sample preparation is necessary.

To each tube are then added 3 ml of benzene (containing 1.5% isoamyl alcohol), and the tubes are agitated gently in the upright position in a Vortex-type mixer. The samples are centrifuged, and an aliquot of the organic phase is transferred to a tapered centrifuge tube. The solvent is evaporated to dryness at 40—50° C under mildly reduced pressure. The residue is redissolved in 25 μl of toluene (containing 15% isoamyl alcohol), of which 3—6 μl are injected onto the chromatograph.

Clinical pharmacokinetic study

A healthy 39-year-old volunteer participated in two clinical pharmacokinetic studies. On one occasion, he ingested a single 1.0-mg dose of alprazolam (two 0.5-mg tablets) in the fasting state. Multiple venous blood samples were drawn during the next 48 h. The samples were centrifuged, and the plasma separated and frozen until the time of assay. On another occasion, he ingested a single 0.5-mg dose of triazolam (two 0.25-mg tablets) in the fasting state. Multiple samples were drawn over the next 12 h. The plasma was separated and frozen until the time of assay. Concentrations of alprazolam and triazolam were determined using the method described above.

RESULTS

Evaluation of the method

Under the described chromatographic conditions, approximate retention times are: U-31485, 2.2 min; alprazolam, 3.3 min; triazolam, 4.1 min (Fig. 2). The relationship between plasma concentration of either drug and the peak height ratio of the drug to the internal standard is linear up to concentrations of 15 ng/ml.

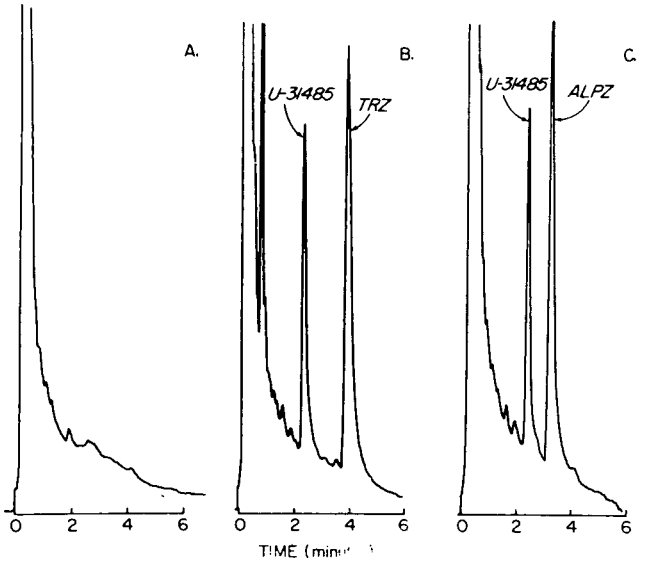


Fig. 2. (A) Chromatogram of an extract of 1 ml of drug-free control plasma sample. (B) Chromatogram of the same sample after addition of 10 ng each of triazolam (TRZ) and of the internal standard, U-31485. (C) Chromatogram after addition of 10 ng each of alprazolam (ALPZ) and of the internal standard. See text for chromatographic conditions.

The limit of sensitivity is approximately 0.25 ng of either compound per ml of plasma. Table I shows the replicability of identical samples at various concentrations. In all cases, the coefficient of variation was less than 6%. The mean deviation between 123 randomly selected replicate determinations of alprazolam was 3.9%. The between-day coefficient of variation in

TABLE I

REPLICABILITY OF IDENTICAL SAMPLES
n = 6 at each concentration.

Plasma concentration (ng/ml)	Coefficient of variation (%)	
	Alprazolam	Triazolam
1.0	4.2	5.3
2.5	4.7	3.7
5.0	5.8	3.2
10.0	3.6	2.5

the slope of consecutive standard curves was 5.2%. Residue analysis indicated that extraction of all three compounds from plasma is greater than 95% complete.

Pharmacokinetic results

After oral administration of alprazolam, a peak concentration of 22 ng/ml was measured in the sample drawn 1.0 h after dosage. Following attainment of the peak concentration, elimination proceeded thereafter with an apparent half-life of 13.2 h (Fig. 3). Administration of 0.5 mg of triazolam

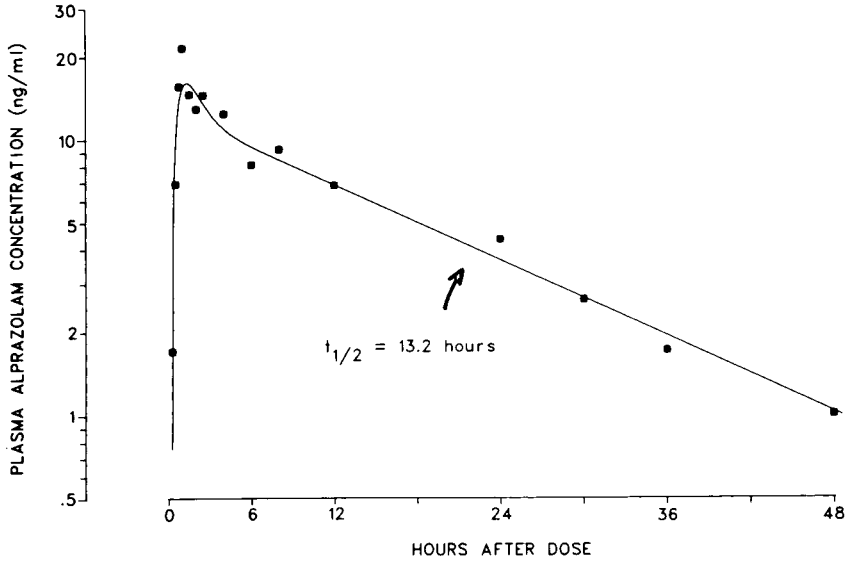


Fig. 3. Plasma alprazolam concentrations following a single 1.0-mg dose of alprazolam administered to a healthy volunteer. Solid line was determined by iterative nonlinear least-squares regression analysis using methods described previously [7].

to the same subject yielded a peak concentration of 14 ng/ml measured in the first sample drawn 0.25 h after the dose. Thereafter, the apparent elimination half-life was 3.1 h (Fig. 4).

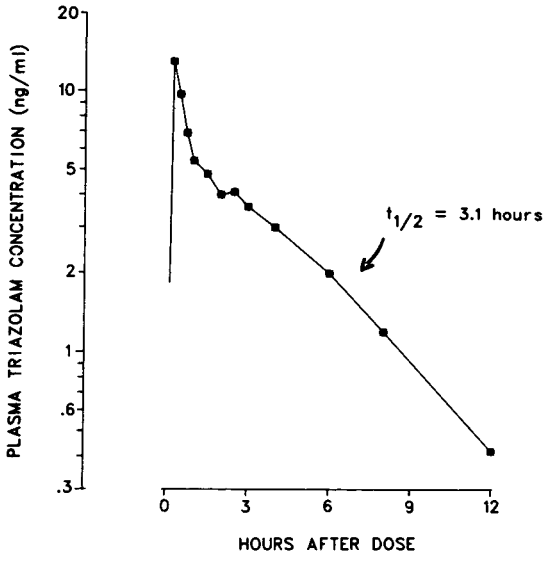


Fig. 4. Plasma triazolam concentrations following a single 0.5-mg oral dose administered to a healthy volunteer. Elimination half-life was determined by least-squares regression analysis of the terminal log—linear portion of the curve.

DISCUSSION

This paper describes a rapid sensitive method for quantitation of alprazolam and triazolam in human plasma following single therapeutic doses. The methodology is made possible by the sensitivity of the electron-capture detector. Alprazolam, triazolam, and the internal standard all are essentially quantitatively extracted from plasma into an organic solvent at physiologic pH with no sample preparation. Since chromatograms of drug-free plasma samples are consistently free of interfering contaminant peaks, cleanup of samples is not necessary. Pharmacokinetic studies in humans utilizing radioactive alprazolam and triazolam [3—5] indicated that unconjugated metabolites of these compounds appeared in only negligible amounts in human plasma and, in any case, do not yield interfering chromatographic peaks.

Kinetic properties of alprazolam and triazolam were consistent with previous reports [3—6]. Peak plasma concentrations were reached shortly after a single oral dose, indicating reasonably rapid absorption from the gastrointestinal tract. Following attainment of peak levels, the apparent elimination half-life differed between the two drugs. Alprazolam was eliminated with a half-life of approximately 13 h. This drug is intended to serve as an anxiolytic or antidepressant agent, with a recommended twice or three times daily schedule of administration [1]. The half-life of triazolam, on the other hand, is considerably shorter. This compound is used clinically as a short-acting hypnotic agent [2]. Due to its short half-life, administration on a nightly basis would lead to essentially no drug accumulation. Further studies are needed to determine individual variability in the pharmacokinetic properties of these

two compounds, as well as disease states and drug interactions that might influence their disposition.

ACKNOWLEDGEMENTS

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Note

Measurement of plasma theophylline by gas–liquid chromatography on the stationary phase SP-2510

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Theophylline (1,3-dimethylxanthine) is well established as a bronchodilator in the management of patients with asthma [1], and has proved effective in the treatment of apnea and bradycardia in premature and low birth weight infants [2, 3]. However, safe and effective use of theophylline depends on information obtained by plasma monitoring, because of pronounced intersubject differences in the biological half-life and the resultant variability in patients on the same oral dose [4, 5].

Plasma theophylline levels are routinely estimated by a variety of analytical techniques [6–24], however we were restricted to a gas–liquid chromatographic (GLC) method as this used equipment available in our laboratory. The GLC method we describe uses the support SP-2510 DA and a pre-column of SP-2250 DA. This column was developed for the estimation of the anticonvulsant drugs [25, 26]. It was desirable and convenient to extend its use for the estimation of plasma theophylline, thereby allowing routine laboratories the flexibility of using the one column for both types of analysis. A number of previously published methods had achieved this on the support 3% OV-17 [14, 21, 23], but here theophylline requires derivatisation to the butyl or pentyl derivatives, whereas the method we describe eliminates this. As the procedure utilises a nitrogen–phosphorus detector, good sensitivity is achieved on microsamples of plasma (50 μl).

EXPERIMENTAL

Materials

Theophylline and 7-(β-hydroxypropyl)theophylline were obtained from

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Sigma (St. Louis, MO, U.S.A.). Chloroform (spectrosol) and propan-2-ol (spectrosol) were obtained from Ajax Chemicals (Sydney, Australia). The column packing 2% SP-2510 DA on 100—120 mesh Supelcoport and 3% SP-2250 DA on 100—120 mesh Supelcoport were obtained from Supelco (Bellefonte, PA, U.S.A.). The internal standard 7-(β-hydroxypropyl)-theophylline (11.91 mg) was dissolved in 100 ml of propan-2-ol. A 10-ml aliquot was diluted to 500 ml with chloroform—propan-2-ol (4:1, v/v) to give a combined extraction and internal standard solution.

Pooled plasma was prepared from expired blood packs supplied by the Blood Bank at The Wollongong Hospital.

Chromatography

A Packard 427 gas chromatograph equipped with a Model 905 NP detector was used. The instrument was fitted with a coiled glass column 0.9 m × 2 mm I.D. packed with 2% SP-2510 DA on 100—120 mesh Supelcoport, with an 8-cm pre-column of 3% SP-2250 on 100—120 mesh Supelcoport as previously described [26]. The oven temperature was maintained at 205°C with detector and injector block temperatures set at 280°C. The nitrogen detector was adjusted to a background current of 40 pA with hydrogen set at the flow-rate of 4.6 ml/min, air at 100 ml/min and the carrier gas helium at 40 ml/min. The attenuator was set at range 10 × 32 with the recorder set on 1 mV full scale deflection.

Extraction of plasma samples

A 250-μl amount of the internal standard/extracting solution was added to 50 μl of plasma, followed by vortex mixing for 30 sec. After centrifugation at 1000 *g* for 2 min, the lower layer was transferred by pasteur pipette to a conical shaped tube and evaporated to dryness with a stream of nitrogen at 40°C. The residue was reconstituted with vortex mixing in 20 μl of ethyl acetate, and 1 μl was then used for injection into the chromatograph. The recovery of plasma theophylline at the 100 μmol/l level by this method was previously found to be 76% [23].

Standard solutions

A series of plasma standards with concentrations of 25, 50, 75, 100, 125 and 150 μmol/l were prepared by adding pooled plasma to 1 ml of a stock solution of theophylline in water (4.5, 9, 13.5, 18, 22.5 and 27 mg per 100 ml) in 10-ml volumetric flasks. Aliquots of these solutions were kept frozen at −20°C and thawed at 37°C prior to use.

RESULTS AND DISCUSSION

Using the above GLC conditions, a theophylline assay involving extraction and GLC of the standards and sample from one patient would take approximately 50 min. Each additional assay would only add an extra 6 min. The internal standard, 7-(β-hydroxypropyl)theophylline proved to be a suitable internal standard, having a similar GLC response to theophylline, but having

TABLE I

RETENTION TIMES OF DRUGS AND OTHER POTENTIALLY INTERFERING COMPOUNDS IN THE GLC MEASUREMENT OF THEOPHYLLINE

Compound	Retention time (min)	Compound	Retention time (min)
Caffeine	1.0	Diphylline	16.7
Methylphenobarbital	1.4	Salicylic acid	Nil
Theobromine	1.7	Acetylsalicylic acid	Nil
Paracetamol	2.0	Uric acid	Nil
7-(β-hydroxypropyl)-theophylline	3.6	Cholesterol	Nil
Phenobarbital	4.1	Plasma peak	0.8
Theophylline	4.6	Plasma peak	1.0
Diazepam	5.9	Plasma peak	1.9
Butabarbital	Nil	Plasma peak	2.6
Amobarbital	Nil	Ethosuximide	Nil
Pentabarbitone	Nil	Valproic acid	Nil
Quinalbarbitone	Nil		

good separation from it. The separation is influenced by the length of the pre-column and we found that a length of 8 cm allowed separation of theophylline, the internal standard and all compounds listed in Table I. No interference could be detected from the anticonvulsant drugs, other xanthines and a number of commonly prescribed drugs with this method (Table I).

A number of serum peaks (Table I and Fig. 1) are characteristic of most plasma samples run by this method, even for those patients not on any medication (Fig. 2). However, these peaks do not interfere with the assay of theophylline. The standard curve is plotted as the ratio of the peak height of theophylline to that of the internal standard, versus the plasma theophylline concentration in μmol/l. This ratio is reproducible between analyses. The method is linear to 150 μmol/l, the correlation coefficient when analysed by the least-squares method was 0.9991 and the limit of detection was 5 μmol/l. The regression line for the standard curve is used to calculate the theophylline concentration in patient samples.

The within-run precision of this method was 1.75% at the 110 μmol/l level and 3.3% at the 20 μmol/l level of theophylline. This was determined by analysing the plasma of a patient fifteen times within the same assay. The between-run precision was 5.2% at the 110 μmol/l level and 8.7% at the 20 μmol/l level of theophylline. This was determined by assaying the plasma of one patient on ten separate occasions.

The theophylline standards prepared as above were stable at −20°C for at least twelve weeks. A solution of the internal standard was stored at room temperature in a sealed container and was also stable for at least twelve weeks.

We have demonstrated that theophylline in plasma can be conveniently and precisely measured utilising the commonly used anticonvulsant drug column 2% SP-2510 DA with an 8-cm pre-column of 3% SP-2250 DA. The assay requires very little plasma and is simple to perform with only a single extrac-

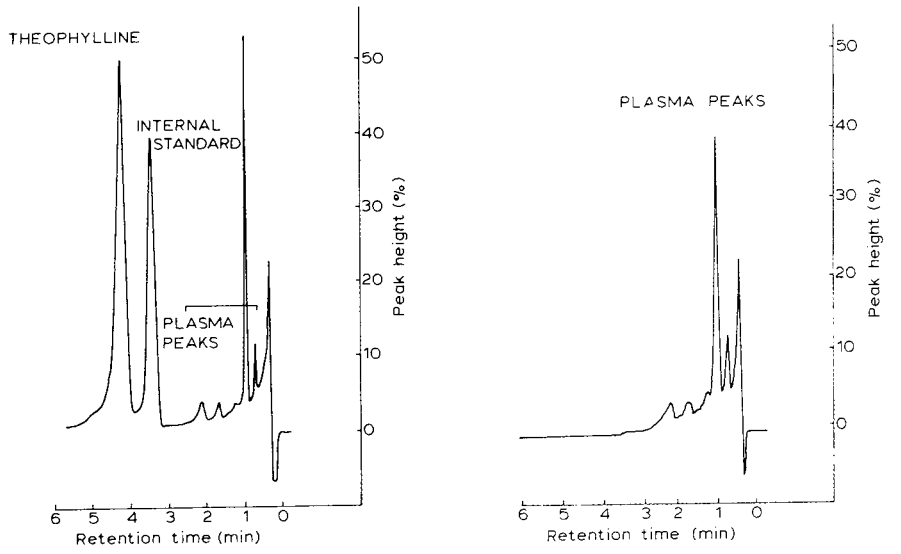

Fig. 1. GC separation of theophylline (62 μmol/l) and the internal standard, 7-(β-hydroxypropyl)-theophylline after extraction of a plasma sample obtained from an adult patient taking theophylline. Stationary phase is 2% SP-2510 DA with an 8-cm pre-column of 3% SP-2250 DA. Attenuation 320×; column temperature, 205°C; detector and injector temperature, 280°C.

Fig. 2. Chromatogram of a chloroform—propan-2-ol extract of a plasma sample from an adult patient not on theophylline medication. Stationary phase is 2% SP-2510 DA with an 8-cm pre-column of 3% SP-2250 DA. Attenuation, 320×; column temperature, 205°C; detector and injector temperature, 280°C.

tion and no derivatisation steps. Therefore this method is ideally suited for the routine estimation of theophylline in plasma. Since the development of this procedure, our laboratory has provided theophylline measurements on patients receiving theophylline medication, without encountering any difficulties.

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Note

Determination of metoprolol in plasma and urine by gas—liquid chromatography with electron-capture detection

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(First received November 18th, 1980; revised manuscript received April 3rd, 1981)

With the increasing use of β-adrenoreceptor blocking agents in the treatment of disease, there is a need for sensitive, trouble-free techniques for the measurement of plasma and urine concentrations of these drugs. Methods have been described for the determination of metoprolol but these have various problems and disadvantages. The method described by Ervik [1] required 2.0—4.0 ml of plasma, used benzene as an extraction solvent and also required 30 min per sample for chromatography, allowing only a very small number of samples to be analysed daily. It is here that the advantage of using a good extraction procedure prevails, and the method described here, in addition to allowing the analysis of 40 samples (manual injection) per day, requires only 250—500 μl of plasma and is trouble-free and sensitive with levels of metoprolol detectable down to 3.0 ng/ml, which is adequate for pharmacological studies with the drug in man. Although metoprolol is metabolized, it has been shown [2] that the four metabolites are of no pharmacodynamic significance in man and, as they do not interfere with the assay (possibly owing to the small sample size

TABLE I

MEAN PLASMA LEVELS OF METOPROLOL (ng/ml) IN TWO VOLUNTEER GROUPS RECEIVING A 50- AND A 400-mg ORAL DOSE

Dose (mg)	Time after dose (h)										
	1	1.5	2	3	4	6	8	10	24	33	48
50	56	73	55	51	28	25	20	92	4	3	0
400	538	521	479	351	291	232	152	86	10	5	0

and short derivatization time), the question of metabolites need not be considered. Table I gives some results of work carried out on metoprolol in this department using this method for the analysis of plasma samples [3].

EXPERIMENTAL AND RESULTS

Chemicals

Sodium hydroxide solution (10 *M*), hydrochloric acid (0.1 *M*), ethyl acetate (AnalaR, once distilled; Hopkin & Williams, Chadwick Heath, Great Britain), methanol (pro analysi; May & Baker, Dagenham, Great Britain) and trifluoroacetic anhydride (TFAA) (Phase Separations, Queensferry, Great Britain) were used. The extraction solvent was diethyl ether (anaesthetic grade; May & Baker)—dichloromethane (G.P.R. grade, once distilled) (2:1).

Methods

All extraction tubes should have tops that contain a paper-based liner (Searle Diagnostics, High Wycombe, Great Britain), as PTFE and rubber-based liners can produce interferences when used with electron-capture-sensitive derivatizing agents.

Derivatization was carried out in ground-glass-stoppered, conical-bottomed tubes that had beek soaked in methanol for at least 15 min to ensure cleanliness and dryness.

Plasma, urine, sodium hydroxide solution, ethyl acetate, standards and TFAA were all transferred using automatic pipettes that had been adapted to take glass Pasteur pipettes in preference to ordinary tips, which contain materials that should be avoided.

The extraction solvent and the hydrochloric acid were added using glass burettes, ensuring that the tap was made of glass and not PTFE.

Chromatography

A Perkin-Elmer F17 gas chromatograph fitted with a 10-mCi ^{63}Ni electron-capture detector and a glass column (0.5 m × 4 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q (80—100 mesh) was used.

The instrument parameters were as follows: oven temperature, 200°C; injector and detector temperatures, 300°C; pulse, 5; range, 1; attenuation, 256; and carrier gas, argon at a flow-rate of 230 ml/min.

The reasons for using such a high carrier gas flow-rate are as follows: (1) better resolution of peaks can be achieved than with a higher column temperature and a lower flow-rate; (2) improved sensitivity (same reason as above); see Figs. 2 and 3); and (3) the detector is being continuously purged and is therefore less likely to become contaminated.

Extraction

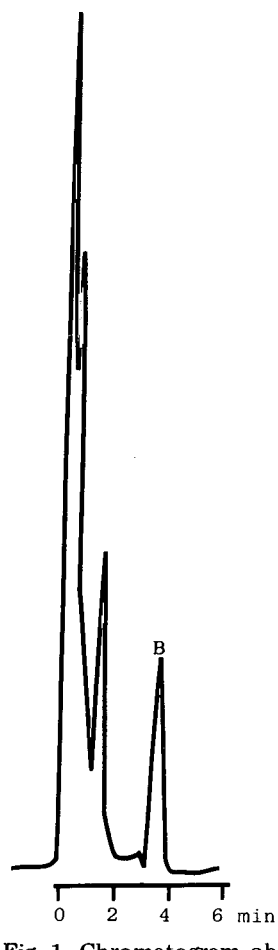
After allowing the deep-frozen plasma or urine sample to thaw at room temperature, 250—500 μl of sample are transferred into the extraction tube, then 100 μl of internal standard are added and the tube is vortexed for 3 sec (all subsequent vortexing is for 3 sec) prior to adding 100 μl of 10 *M* sodium hydroxide solution and vortexing. A 4.0-ml volume of extraction solvent is

added, the tube is vortexed, shaken for 10 min, centrifuged at 1500 *g* for 15 min, then 3.0 ml of supernatant are added to 3.0 ml of 0.1 *M* hydrochloric acid.

After vortexing, the tube is shaken for 10 min and centrifuged at 1500 *g* for 5.0 min, as phase separation is much easier than previously. After centrifugation, the supernatant is aspirated by vacuum and discarded, and the aqueous layer is made alkaline by addition of 100 µl of 10 *M* sodium hydroxide solution, followed by vortexing.

A 4.0-ml volume of extraction solvent is then added and the tube is vortexed and shaken for 10 min. Subsequently, the tube is centrifuged at 1500 *g* for 5.0 min, then 3.0 ml of supernatant are transferred into a ground-glass-stoppered conical-bottomed tube. This solvent is removed with a stream of nitrogen in a water-bath at 40°C.

After ensuring that all solvent has been removed, 100 µl of TFAA are added,



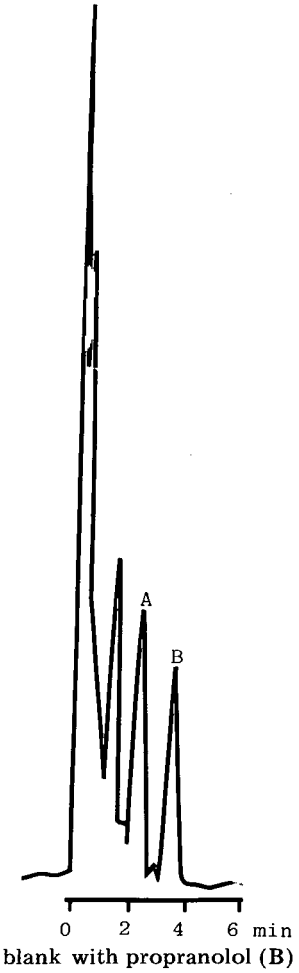


Fig. 1. Chromatogram obtained from a plasma blank with propranolol (B) added as internal standard.

Fig. 2. Chromatogram obtained from a patient plasma sample containing 295 ng/ml of metoprolol (A) and propranolol (B) added as internal standard. Conditions as in text.

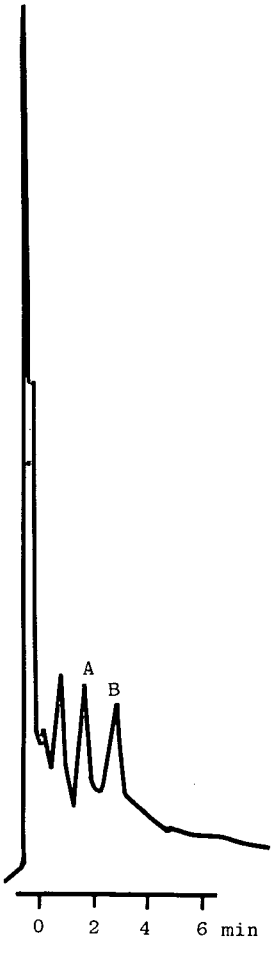


Fig. 3. Same injection as in Fig. 2, except oven temperature 230°C and carrier gas flow-rate 80 ml/min.

followed by 100 μl of ethyl acetate. The tube is then tightly stoppered, vortexed and allowed to react at 40°C for 20 min. After derivatization is complete, the tube contents are evaporated to dryness with a stream of nitrogen in a water-bath at 40°C and the residue is dissolved in 50 μl of ethyl acetate. Finally, the tube is vortexed and 1 μl of solution is injected onto the gas chromatographic column.

Typical chromatograms for plasma extracts are shown in Figs. 1 and 2 (similar chromatograms are obtained for urinary extracts).

Standards

Metoprolol. A stock solution of 1 mg/ml of metoprolol in methanol was prepared. Included in each run of patient plasma samples was a set of pooled plasma samples, to each of which was added a known amount of metoprolol (0, 50, 100, 200 and 400 ng/ml). These samples were treated in the same way as those in the test run.

Internal standard. A stock solution of 1 mg/ml of propranolol in methanol was prepared, and this was added to give a concentration of 100 ng/ml in plasma.

Calculation

To calculate the level of metoprolol in samples from patients, a calibration graph of peak-height ratio versus concentration of known standard was drawn. The graph was linear in the range 0—400 ng/ml ($r = 0.99$).

Precision

To test for fluctuation of the detector response, a single sample (230 ng/ml) was injected at five different times over a working day and the results obtained showed a mean peak-height ratio of 0.87 (standard deviation 0.03) with a coefficient of variation of 3.4%.

In order to test the reproducibility of the method, 5.0 ml of pooled plasma were spiked with 200 ng (unknown to the analyst) of metoprolol and the sample was divided into five 1.0-ml fractions and re-frozen. These fractions were analysed on different occasions over 9 days and the results obtained showed a mean level of 40.6 ng/ml (standard deviation 3.36) with a coefficient of variation of 8.2%.

DISCUSSION

Extraction

Several methods have been described for the determination of metoprolol and other β-blocking drugs and each uses a different extraction solvent. Degen and Riess [4] used diethyl ether—dichloromethane (4:1) but by increasing the polarity by doubling the proportion of dichloromethane it was found to be unnecessary to use a final clean-up stage as advocated in their method. Ervik [1] used a one-step benzene extraction, but the time saved at this stage is subsequently lost by having a very long chromatography time. Zak et al. [5] also described a method for the determination of metoprolol but it involves an exceptionally complicated and time-consuming extraction procedure.

Derivatization

Metoprolol was derivatized for 20, 40 and 60 min and it was found that there was no improvement in yield on extending the reaction time beyond 20 min.

TFAA storage

Our experience with TFAA has shown that on opening a new vial the contents are best transferred to a ground-glass-stoppered tube and stored in subdued light surrounded by a desiccating agent. These precautions have ensured consistent results and an increased shelf-life of TFAA.

Column stabilization

Before commencing a sample run, it was found that by injecting a plasma blank extract three or four times, until the peaks produced were symmetrical

(Figs. 1 and 2), the reproducibility of the method was maintained. A similar observation was also made by Kangas [6] when using an OV-17 column.

This method has been used for the analysis of over 400 samples and has proved to be straightforward and trouble-free, with good reproducibility and sensitivity, and is useful in pharmacokinetic studies on metoprolol.

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Astra (St. Albans, Great Britain) are thanked for supplying metoprolol and ICI (Macclesfield, Great Britain) for supplying propranolol.

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Note

Quantitative determination of clobazam in the plasma of epileptic patients by gas—liquid chromatography with electron-capture detection

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Clobazam (CLB), with anticonvulsant [1—4] and anxiolytic properties [5—11], was synthesized by Rossi et al. [12] in 1969, and was the first example of a 1,5-benzodiazepine. Clinical investigations have indicated that CLB is effective against various forms of epilepsy [4, 13—15], and for this reason it is now coming into use as an antiepileptic drug. There is no evidence that CLB metabolites have antiepileptic properties.

The pharmacokinetics and the metabolism of the drug have been studied by the radioreceptor technique [16] both in various animal species and in man [17, 18]. The structures of several metabolites of CLB have been clarified recently. The N-desmethylation and the 4′-hydroxylation metabolic pathways were found to be the most important ones in animals and man. The metabolism of the 1,5-benzodiazepines in the hydroxylation pathway is markedly different from that of the more diffuse 1,4-benzodiazepines in the same pathway (4′-oxidation as opposed to 3-oxidation); the metabolism in the dealkylation pathway is similar in both [18].

The concentration of N-desmethylclobazam in plasma has been found to be eight times higher than the concentration of the parent drug after long-term administration [17], whereas no information is available about the concentration of 4′-hydroxyclobazam in plasma. A method for measuring CLB and N-desmethylclobazam in guinea-pig plasma by means of a gas chromatographic (GC) technique has been published [19]; unfortunately it cannot be used to determine the concentration of CLB in the plasma of human subjects who are taking other drugs, because these cause additional interfering peaks in the chromatograms which invalidate the analysis. Another method for the determination of CLB in plasma by a fluorimetric technique [20] has been published

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but it has a low sensitivity (50 ng/ml) and it is possible that co-medications may interfere with the formation of the fluorophore of CLB.

Our purpose was to develop a simple and fast method for determining the concentration of CLB, suitable for routine clinical application and sufficiently specific with regard to the presence of other common co-medications.

EXPERIMENTAL

Reagents and standards

All chemicals were of analytical-reagent grade or better and were checked for chromatographic purity before use. Clobazam [7-chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4-(3H,5H)-dione] was kindly supplied by Hoechst (Frankfurt/M., G.F.R.). Methylclonazepam [7-nitro-1-methyl-5-(*o*-chlorophenyl)-3H-1,4-benzodiazepine-2-(1H)-one] was kindly supplied by Roche (Milan, Italy) and was employed as the internal standard. Stock solutions of CLB and methylclonazepam were prepared in methanol to give a concentration of 1 mg/ml of each compound. Plasma standards of 10, 30, 60, 150, 300 and 500 ng/ml of CLB were prepared by adding the required volumes of a standard 30 μg/ml solution of CLB to drug-free pooled plasma. Plasma standards were then divided into 1-ml samples and frozen at −20°C (calibrator samples).

Apparatus

A Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a nickel-63 electron-capture detector (ECD) was used. The glass column (0.5 m × 3 mm I.D.) was packed with GP—2% SP-2510-DA on 100—120-mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) and was conditioned as indicated by the manufacturer [21]. The GC conditions were: column temperature, 245°C; injector temperature, 300°C; detector temperature, 300°C; carrier gas (nitrogen) flow-rate, 80 ml/min. An LKB 9000 mass spectrometer equipped with a gas chromatograph was used under the following conditions: ionizing energy, 70 eV; ion source temperature, 290°C; accelerating voltage, 3.5 kV; trap current, 60 μA. The sample was introduced by either the direct inlet system (probe temperature 70°C) or the GC procedure using a similar column to that described above.

Extraction procedure

To 1 ml of plasma (calibrator samples or patient samples) were added 100 μl of the dilute internal standard (40 ng/ml in methanol), 0.5 ml of 1 *N* hydrochloric acid and 4 ml of benzene. Test-tubes were mechanically shaken for 10 min, then centrifuged at 1000 *g* for 5 min and 3.5 ml of the organic phase were evaporated to dryness in a vacuum evaporator at 45°C. The residues were dissolved in 100 μl of acetone and 1—2 μl of this solution were injected into the gas chromatograph. Calibration graphs were constructed of the peak-area ratio of CLB to methylclonazepam versus concentration of CLB. For each series of analyses a new calibration graph had to be prepared.

Recovery

Various amounts (0.3, 0.5, 1.5 and 4.0 μg) of CLB were dissolved in 10 ml

of drug-free plasma by adding a given volume of a standard solution of 30 μg/ml CLB in methanol. The plasma was divided into 1-ml samples, which were used for the recovery study. These samples were extracted as described above but without adding the internal standard. The residues were dissolved in 100 μl of acetone containing 4 ng of methylclonazepam. A second series of standards was prepared simultaneously by extracting 1 ml of drug-free plasma and then adding CLB and the marker to the dried extract at the concentrations noted above. The analytical recovery was calculated by comparing the peak-area ratios of the extracted standards to the ratios obtained from the standards to which CLB had been added after extraction. The absolute recovery was calculated by correcting the analytical recovery by a factor representing the ratio between the benzene volume added to and the benzene volume removed from the plasma and evaporated during the extraction procedure.

Linearity

The linearity was calculated by using the results obtained from the calibration graphs (from 10 to 500 ng/ml).

Reproducibility

Four plasma samples of 10 ml each (containing 100 ng, 600 ng, 1.5 μg and 4 μg of CLB) were prepared by adding given volumes of a standard 30 μg/ml solution of CLB in methanol. The plasma was divided into 1-ml samples and was kept frozen at −20°C until taken for analysis. The analyses were performed ten times, about once every twelve days over a period of 4 months using the above method.

Interference from other drugs or substances

To determine the potential usefulness of the procedure, we checked for possible interferences from other antiepileptic drugs (phenobarbital, mephobarbital, primidone, carbamazepine, phenytoin, ethosuximide and valproic acid), some of the most important benzodiazepines (diazepam, 3-hydroxydiazepam, oxazepam, N-desmethyldiazepam, clonazepam, nitrazepam, prazepam, flurazepam, flunitrazepam, lorazepam, medazepam, bromazepam and chlordiazepoxide) and some other drugs and substances [caffeine, gluthetimide, amobarbital, quinidine, theophylline, cholesterol, α-methyl-α-phenylsuccinimide, iminostilbene, 5-(*p*-methylphenyl)hydantoin and 5-(*p*-methylphenyl)-5-phenylhydantoin] by chromatographing physiological samples and pure standards at normal therapeutic or physiological concentrations.

Plasma of patients

Samples of blood from patients receiving CLB orally twice daily (at 8 a.m. and 8 p.m.) were taken in heparinized test-tubes just before the morning dose. The plasma was separated as soon as possible, immediately frozen and stored at −20°C until taken for analysis.

RESULTS AND DISCUSSION

Preliminary experiments indicated that GP—2% SP-2510-DA was the most

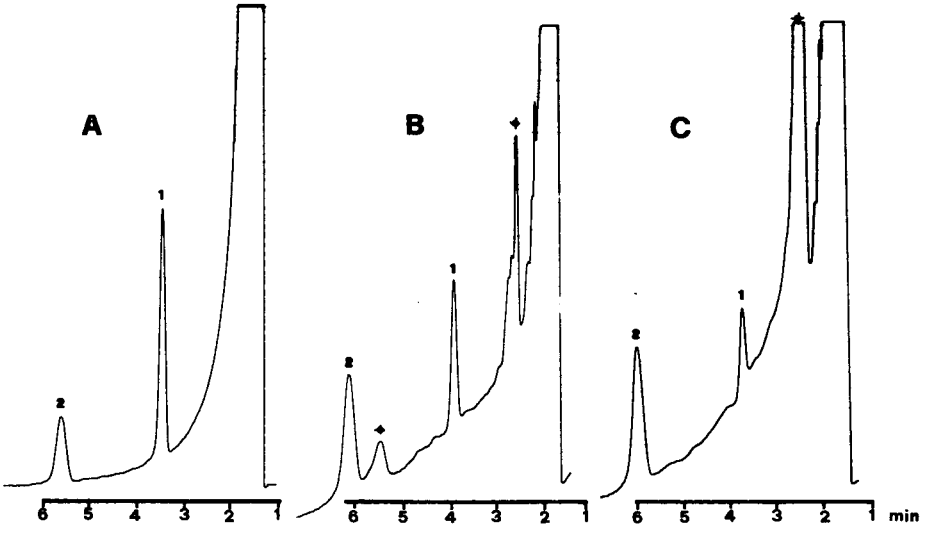

Fig. 1. GC response obtained with (A) extracted calibration sample, (B) extracted plasma of a patient undergoing therapy with clobazam, carbamazepine and phenytoin and (C) extracted plasma of a patient undergoing therapy with clobazam, phenobarbital and valproic acid. Peaks: (1) clobazam; (2) methylclonazepam (internal standard). Peaks marked with an asterisk are unidentified compounds in the extracted plasma of patients undergoing multiple drug therapy.

suitable GC stationary phase for the determination of CLB in the plasma of patients under multiple drug therapy. With other stationary phases commonly used in benzodiazepine analysis, multiple peaks interfering with the CLB peak were evident in chromatograms from the plasma of patients under co-medication. Unfortunately, N-desmethylclobazam (a basic compound) showed indications of irregular adsorption to this stationary phase (which is specific for acidic drugs), so that this metabolite cannot be measured concomitantly. For these reasons, we decided to determine only the concentration of CLB in the plasma of epileptic patients under multiple therapy, leaving aside the problem of its metabolite measurement.

The extracts from drug-free plasma showed no interference from endogenous plasma sustances. Fig. 1 shows a representative chromatogram of an extract from a plasma calibration graph (A) and two chromatograms obtained from epileptic patients taking other antiepileptic drugs (B, C). We did not observe any exogenous interferences in the chromatograms which might have altered the CLB analysis, even when drugs and substances listed above were examined. Fig. 2 shows the mass spectrum of CLB, obtained after the GC procedure. It has a molecular ion at m/e = 300, which is also the base peak. By comparing the CLB mass spectrum obtained after the GC inlet with the direct-inlet-system mass spectrum, it could be concluded that CLB leaves the GC column unchanged. Therefore, in our procedure, this substance was being measured in its intact form.

The concentrations of CLB in calibrator samples and their respective readings were seen to be linearly related; the line obtained by calculation with a

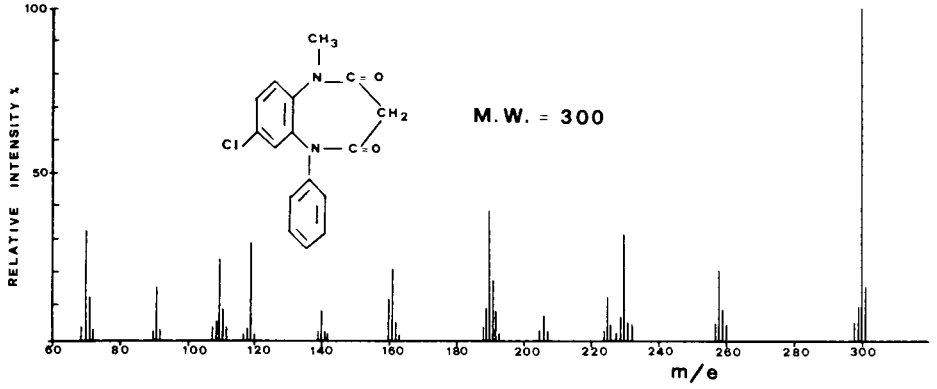

Fig. 2. Normalized electron-impact mass spectrum of clobazam obtained by injecting a plasma extract into an LKB-9000 gas chromatograph—mass spectrometer system.

TABLE I

RECOVERY OF CLOBAZAM FROM HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found* (ng ± S.D.)	Absolute recovery (% ± S.D.)
30	27.2 ± 1.48	90.66 ± 4.93
60	53.7 ± 3.34	89.50 ± 5.57
150	135.2 ± 6.42	90.13 ± 4.28
400	359.3 ± 15.54	89.82 ± 3.88
Mean recovery		90.12 ± 4.83

*Each value is the mean value of four determinations. These values have been corrected as described in the text.

TABLE II

REPRODUCIBILITY OF DETERMINATION OF CLOBAZAM IN HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found* (ng/ml)	S.D.	C.V. (%)
10	9.97	0.45	4.5
60	59.89	2.63	4.4
150	150.30	6.53	4.3
400	398.80	15.96	4.0

*Each value is the mean value of ten determinations.

least-squares linear regression method is $y = 0.0071x + 0.0066$; $r = 0.999$. The detection limit for CLB in plasma using the described procedure is about 5 ng/ml. The mean absolute recovery from four analyses of plasma samples containing four different CLB concentrations was 90.12 ± 4.83% (Table I). The results of the reproducibility study are illustrated in Table II; the mean coefficient of variation was 4.3%.

In a series of 16 patients, aged from 4 to 47 years, with a CLB dosage of 0.3—1.6 mg/kg/day (mean ± S.D. = 0.89 ± 0.38), we found concentrations in plasma of 20—197 ng/ml (mean ± S.D. = 81.0 ± 48.9) regardless of the co-medications. We did not believe it was useful to analyse these data statistically, as there are so many uninvestigated parameters (age, co-medications, etc.). The proposed method is, to the best of our knowledge, the most sensitive available for determining concentrations of CLB in human plasma when multiple drug therapy is used. The procedure is rapid, simple and reproducible. The chromatographic time for twenty analyses is about 2 h (6 min for each analysis), with an additional 40 min for samples preparation.

The drugs tested do not interfere in the analysis of CLB so that kinetic studies of CLB in patients undergoing multiple drug therapy can be performed.

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Note

High-performance liquid chromatographic assay for plasma dipyridamole monitoring

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In recent years there has been an increasing number of reports on dipyridamole's role in the treatment of various thromboembolic diseases [1—4]. Until recently, however, there was no rapid and simple method for the analysis of the plasma concentration of dipyridamole. The available methods at that time involved spectrophotofluorometric procedures described by Beizenherz et al. [5], Zak et al. [6], and Mellinger and Bohorfoush [7]. Fluorescence assays have as a drawback interference from plasma components and other fluorophores; furthermore, the required extraction procedures are tedious and complicated. While our studies were in progress, three additional methods were reported for the analysis of dipyridamole [8—10]. Pedersen [8] described the detection of dipyridamole by high-performance liquid chromatography (HPLC) using either ethanol to precipitate protein from serum (Procedure A) or a 1.0 *M* Tris buffer at pH 8.6 (Procedure B). However, in both cases if an internal standard was used, it was added after the extraction of the drug from the serum. These procedures allow only for detection of injection volume error, column efficiency and the detector response, but don't detect any drug loss or problems during the most critical step, the extraction step. Procedure A requires a 15-min waiting period after adding the ethanol to allow for the protein to precipitate, while Procedure B requires 1 ml of serum to be extracted twice with 8 ml of diethyl ether [8]. This means 16 ml of diethyl ether must be evaporated to 2 ml and re-extracted with 0.1 *N* hydrochloric acid. Therefore Procedure B is actually a three-step extraction method and requires a large volume of the extraction solvent.

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A newer technique [9] for the assay of dipyridamole in plasma added 100 ng of methoxy-dipyridamole as the internal standard in the extraction solvent. The extraction step, however, calls for 10 ml of dichloromethane for the separation of dipyridamole from plasma, and the amount of plasma extracted must be adjusted according to the concentration of dipyridamole in plasma. Furthermore, the internal standard used must be synthesized from dipyridamole, since it is not commercially available. The third report [10] used ion-pair chromatography with fluorescence detection. This method while extremely sensitive requires the availability of a fluorescence detector and the use of ion-pair chromatography which has the additional expense of buying PIC™ reagents.

The objectives of our study were to develop a method which: (1) employed a one-step extraction procedure; (2) used ultraviolet detection; and (3) did not involve paired-ion chromatography.

EXPERIMENTAL

Apparatus

The HPLC system uses a Waters Assoc. Model 6000A solvent delivery pump equipped with a U6K injector, a μBondapack C_{18} column (30 × 0.39 cm I.D.; particle size 10 μm) and a Model 440 absorbance detector. The signal from the detector was quantified using a Shimadzu Seisakusho Data Processor Chromatopac-E1A and Houston Instruments Omni-Scribe recorder.

Reagents

Dipyridamole, received from Rhodia (New York, NY, U.S.A.) was used as the standard throughout the study. Glass distilled acetonitrile and methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Any other reagents used were analytical grade. The mobile phase was a 50:50 mixture of acetonitrile and a 0.01 *M* solution of sodium phosphate in water adjusted to pH 7. Stock solutions of dipyridamole and lidocaine (the internal standard) in methanol (1 mg/ml, 10 μg/ml and 10 ng/ml) were stored at 4°C.

HPLC conditions

A flow-rate of 1.5 ml/min of the mobile phase produced a pressure of approximately 102 atm (1500 p.s.i.). The absorbance reading of dipyridamole in methanol was at 280 nm, since its maximum wavelength of absorption is at 285 nm [11].

Sample preparation

Standard plasma samples ranged from 0.01—2.0 μg/ml. The 1 and 2 μg/ml samples were prepared by first evaporating to dryness aliquots from a 10 μg/ml methanol stock solution of dipyridamole. The samples were then reconstituted with a 5-ml blank human plasma. The 0.5, 0.1, 0.05 and 0.01 μg/ml standards were prepared by dilution with human plasma of the 1.0 μg/ml standard.

Dipyridamole was extracted from the plasma standards and unknown samples by the following procedure. A 0.1-ml aliquot of 1.0 mg/ml methanol

solution of lidocaine (the internal standard) was added to a 15-ml culture tube. This solution was evaporated to dryness at 45°C by a stream of dry, filtered air. Then 1 ml of plasma and 0.5 ml of 0.1 *N* sodium hydroxide solution were added to the culture tube. This mixture was vigorously agitated for 15 sec on a vortex-type mixer. A 5-ml aliquot of ethyl acetate was added, and the plasma sample was agitated for 60 sec. Centrifugation at 1000 *g* for 6 min allowed the separation of the organic from the aqueous phase. A 4-ml aliquot of the organic phase was then transferred to a conical centrifuge tube. This aliquot was evaporated to dryness at 45°C as outlined above. The residue was reconstituted with 0.5 ml of the mobile phase. A 100-μl amount of each sample was then injected onto the column.

Linear regression analysis was performed on the results obtained from the standard plasma samples. The equation of the best fit for the standard curve was used to calculate the concentration of an unknown sample from the peak height ratio measured.

RESULTS AND DISCUSSION

Under the conditions described in the experimental section, dipyridamole and lidocaine have retention times of 5.5 ± 0.2 and 7.5 ± 0.2 min respectively. These conditions allow the detection in the range of 500 pg of dipyridamole injected onto the column. The extraction procedure permits the determination of plasma concentrations of dipyridamole as low as 5 ng/ml. Fig. 1 is a

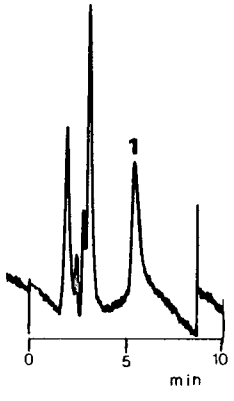

Fig. 1. Chromatogram obtained from a 50 μl injection of a 100 ng mobile phase solution of dipyridamole (1).

chromatogram of dipyridamole in the mobile phase at a concentration of 100 ng/ml; the amount of drug injected on the column was 5 ng. Fig. 2 illustrates the results obtained when plasma from a treated subject was assayed for dipyridamole. Fig. 2a is a plasma sample drawn before dosing of the subject with dipyridamole with the internal standard added. As may be seen from this figure, no interfering peaks occurred at the time corresponding to the retention time of dipyridamole or lidocaine. Fig. 2b is a chromatogram of a plasma sample obtained 1.33 h after the 50-mg dose of dipyridamole.

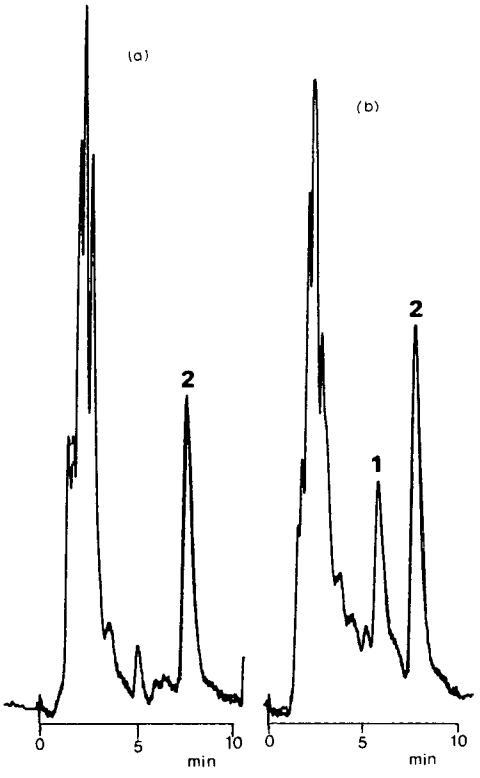

Fig. 2. Chromatograms obtained from human plasma samples. (a) Blank plasma sample obtained previous to dosing, with the internal standard, lidocaine (2), added. (b) Plasma sample drawn 1.33 h after a 50-mg oral dose of dipyridamole. The dipyridamole peak (1) corresponds to a plasma concentration of 101.7 ng/ml.

A standard curve for dipyridamole in plasma ranging from 0.01—2 μg/ml was prepared by plotting concentration against peak height ratio. A good linearity was obtained with a correlation coefficient of 0.9999 calculated by the least squares method. The slope of the line was calculated to be 0.0062, with a y-intercept of 0.1008.

The reproducibility of the assay was verified by extracting five plasma samples with a concentration of 0.1 μg/ml. The coefficient of variation of the peak height ratio of these samples was 4.84%. The percent recovery of dipyridamole from these standard plasma samples was 97.95 ± 2.36% with a coefficient of variation of 2.41%.

The preparation and extraction procedures of a sample require only 8 min, with 6 min of this being used in the centrifugation step. The longest time required in this assay, 15 min, was for the evaporation of the organic phase of the samples after the extraction step. A set of twelve samples, thus, can be prepared and is ready to inject onto the column within 30 min.

Dipyridamole is being considered as a possible antiplatelet agent in the treatment of thromboembolic diseases [8]. Dipyridamole plasma levels correlate well with the inhibition of platelet aggregation in patients after pros-

thetic heart valve replacement [12]. Thus it is desirable to monitor dipyridamole levels in a patient to attain proper dosing and maximum therapeutic efficacy.

The assay method was tested by monitoring the plasma concentration—time profile for dipyridamole after the administration of two intact 25-mg Persantine® tablets to two healthy male subjects. The volunteers fasted for 12 h prior to dosing and for the duration of the study. The range of the plasma levels observed over a 5-h period was 25—765 ng/ml (see Fig. 3). Subject 9A in Fig. 3 had a more erratic decay curve which may be indicative of enterohepatic recycling of the drug.

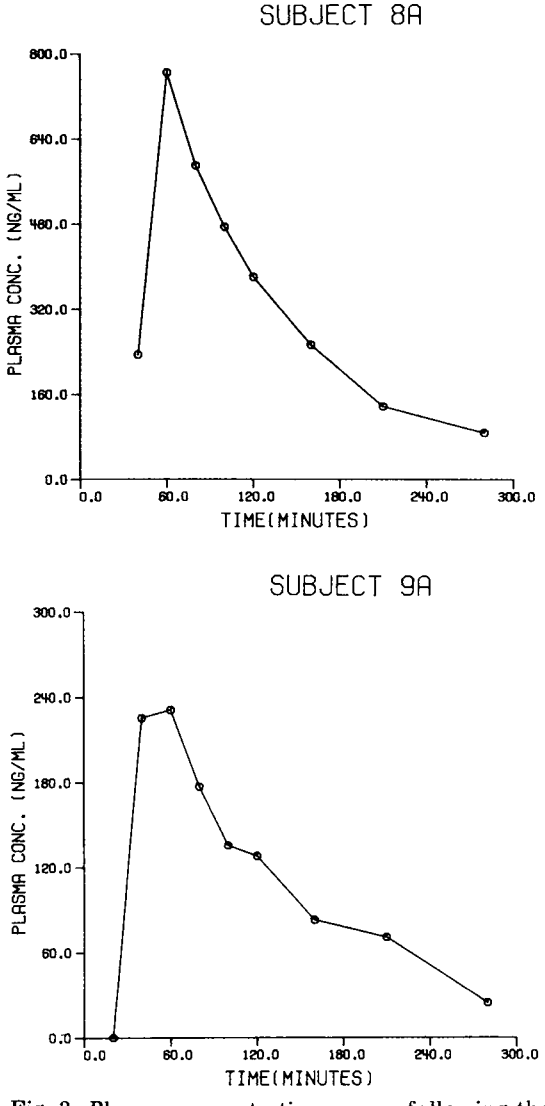

Fig. 3. Plasma concentration curves following the oral administration of two 25-mg tablets of dipyridamole to two healthy subjects.

The HPLC method discussed allows a specific, uncomplicated and rapid method of assaying for dipyridamole in plasma. This assay is currently in use in clinical pharmacokinetic studies designed to evaluate differences in dosage forms.

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Note

Efficient extraction and reversed-phase high-performance liquid chromatography—ultraviolet quantitation of acetazolamide in serum

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Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) is a carbonic anhydrase inhibitor which reduces the rate of aqueous humor formation and correspondingly decreases the intraocular pressure in patients with glaucoma. Recent methods for the quantitation of acetazolamide in biological fluids have included two high-performance liquid chromatography (HPLC) procedures [1, 2], an enzymatic method [3], and an electron-capture gas chromatography procedure [4]. The HPLC methods require extensive and time consuming extraction and evaporation steps [1, 2]. The carbonic anhydrase inhibition assay is plagued with insufficient precision [2, 3]. The method of Wallace et al. [4] is currently considered the method of choice, but Silber [5] has indicated that it would be desirable for a reversed-phase HPLC method to be developed that would only require a simple sample preparation procedure. The latter approach has been chosen. The method developed in our laboratories avoids time consuming evaporation steps, possesses excellent precision, adequate sensitivity and is well-suited for automation.

EXPERIMENTAL

Materials

Chlorothiazide and hydrochlorothiazide were supplied by Merck, Sharp and Dohme (West Point, PA, U.S.A.) and acetazolamide was obtained from Pfaltz and Bauer (Stamford, CT, U.S.A.). Acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) was UV-HPLC grade, citric acid monohydrate (J.T. Baker, Phillipsburg, NJ, U.S.A.) was a Baker analyzed reagent, water was house distilled, and all other chemicals were of reagent grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Serum was obtained from drug-free healthy

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male volunteers. SurfaSil was obtained from Pierce, Rockford, IL, U.S.A. for the purpose of siliconizing the surface of glassware.

Apparatus

A Waters Model 6000A pump and a 710B WISP autosampler were coupled to an Ultrasphere-ODS (5 μm, 4.6 × 250 mm) reversed-phase column (Altex Scientific, Berkeley, CA, U.S.A.). The analytical column was protected by use of a guard column (40 × 4.6 mm I.D.) packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). Absorbance of the eluate was monitored with a Waters 440 UV detector (254 nm). The absorbance output (1 a.u./V) of the detector was connected to both channels of a Houston Superscribe recorder (Houston Instrument, Austin, TX, U.S.A.). Full range recorder spans of 50, 200 and 500 mV were used to provide on-scale peaks.

Mobile phase

A mixture of acetonitrile—0.05 *M* acetate buffer, pH 4.5 (10:90, v/v) was filtered through a 0.45-μm nylon-66 membrane filter (Rainin Instrument, Woburn, MA, U.S.A.) and deaerated under vacuum. The mobile phase was pumped at a rate of 1 ml/min and developed an operating pressure of 2200 p.s.i. (ca. 152 bars).

Stock solutions

Aqueous acetazolamide solutions of 0.05, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0, 15.0 and 20.0 μg per 0.1 ml were stored in amber bottles at 4°C. The internal standard solution was 5 mg of chlorothiazide in a total volume of 2000 ml of ethyl acetate.

Procedures

Culture and tapered centrifuge tubes were treated with a 10% solution of SurfaSil in acetone. Serum (1 ml) was then placed in a 20-ml culture tube. Water (0.1 ml), 1.0 ml of citrate buffer (0.05 *M*, pH 4.6), about 700 mg of sodium chloride, and 10.0 ml of the internal standard solution were added to the sample. The tube was closed with a PTFE-faced screw-cap, vortexed for 1 min and centrifuged for 5 min. Inversion of the tube prior to placing it on the vortex apparatus was helpful in avoiding the formation of an emulsion. If an emulsion formed, it was possible to sonicate the contents of the tube in order to obtain a clear organic phase. The ethyl acetate layer was then transferred to a 13-ml tapered centrifuge tube. To the organic phase was added 0.4 ml of phosphate buffer (0.1 *M*, pH 11.9). The tube was capped, vortexed for 1 min, and centrifuged for 5 min. A 0.2-ml portion of the aqueous phase was transferred to a Waters low-volume insert for automated sample processing and injection of 35 μl.

Standards from 0.05—20 μg/ml were prepared by spiking 1.0 ml of blank serum with 0.1 ml of the appropriate acetazolamide stock solution. The standards were extracted in the same manner as the samples but without the addition of 0.1 ml water. Peak height ratios of acetazolamide to chlorothiazide were plotted versus acetazolamide concentration in μg/ml, and the resulting calibration curve was used to calculate the serum concentrations of the unknown samples.

Absolute recovery of 0.1 and 20.0 μg/ml spiked serum standards (n = 6) were determined from a calibration curve of acetazolamide peak height versus the amount on-column. Two 5.0 μg/ml spiked serum standards were processed without the addition of salt, and the absolute recovery evaluated as above.

An 8 μg/ml control standard was processed daily (n = 12) in order to evaluate the day-to-day reproducibility. Precision was assessed by processing spiked samples (n = 6) of 0.1, 1, 5 and 20 μg/ml and computing the peak height ratios.

Spiked serum samples of 1 and 20 μg/ml (n = 3) were processed and immediately injected. Injections were also made at 4, 8, 12 and 16 h after the initial injection. Peak height ratios were determined in order to validate the method of sample storage.

RESULTS AND DISCUSSION

Chromatograms of blank and spiked human serum are shown in Fig. 1.

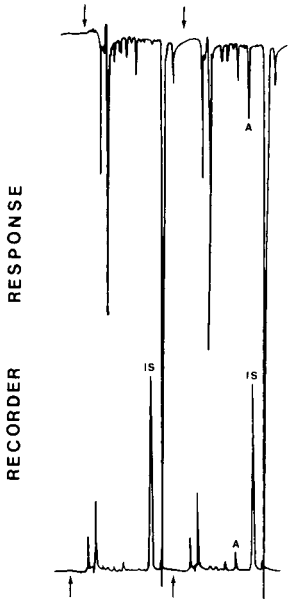

Fig. 1. Simultaneous recording of two full-scale absorbance ranges of 0.05 (upper) and 0.2 (lower) of blank serum and spiked serum (0.5 μg/ml) samples. Arrows mark injection; peaks: A = acetazolamide; IS = internal standard, chlorothiazide.

The retention volumes of acetazolamide and chlorothiazide are approximately 10 and 12 ml respectively and no interfering peaks are present. Hydrochlorothiazide was found to have a retention volume of about 14 ml, and therefore would not interfere with the chromatography.

Calibration curves were linear over the concentration range of 0.05—20 μg/ml and the intercept was essentially zero. A mean correlation coefficient of 0.999 ± 0.0003 was observed for the 12 calibration curves. The practical

limit for the detection of acetazolamide is 0.05 μg/ml.

Absolute recoveries for spiked serum samples (n = 6) of 0.1 and 20 μg/ml were 95.9 ± 1.82% and 97.6 ± 3.40% respectively. Processing of two 5 μg/ml samples without the addition of salt resulted in only a 65.0 ± 0.44% recovery. Previous HPLC assays for acetazolamide [1, 2] have used 10 ml of ethyl acetate as an extraction solvent, but these methods required that the process be repeated to provide almost complete recovery. The addition of sodium chloride to the sample provides 95% recovery of acetazolamide with one 10-ml ethyl acetate extraction.

The within-day precision was evaluated at concentrations of 0.1, 1.0, 5.0 and 20.0 μg/ml (n = 6) and the coefficients of variation (C.V.) were found to be 2.54, 1.12, 1.26 and 1.33%, respectively. Excellent reproducibility between days is also observed with a C.V. of 2.50% (n = 12) for an 8 μg/ml control standard.

During recent years there has been an increase in the usage of automated sample injectors for HPLC. This has allowed a greater number of samples to be processed daily, but also results in an increased period of time between extraction and injection of the sample. Therefore, stability of the sample while stored in the automatic injector should be evaluated. Furthermore, the pH 11.9 aqueous buffer that was used for back extraction and subsequent storage of the sample was of additional concern with regard to stability. It was observed that the pH of the buffer decreased to a pH of less than 9 after back extraction. Therefore, the problem of alkaline degradation of acetazolamide and chlorothiazide was diminished and resulted in essentially constant peak height ratios for up to 16 h (Table I). This period of time would allow the analysis of 64 samples. Beginning analysis of some samples in the middle of the day would provide time for up to 80 samples per day.

TABLE I

EVALUATION OF SAMPLE STORAGE FOR UP TO 16 h DURING AUTOMATED INJECTION

Concentration (μg/ml)	Peak height ratio (mean, n = 5)	C.V. (%)
1	0.1655	1.56
1	0.1682	1.27
1	0.1675	0.98
20	3.337	1.40
20	3.466	2.32
20	3.296	1.43

CONCLUSIONS

The described method is simple, rapid, sensitive and possesses excellent precision. The addition of sodium chloride to the sample obviates a second ethyl acetate extraction, and the back extraction step makes it unnecessary to evaporate the samples to dryness and reconstitute.

This assay is an improvement over previous HPLC acetazolamide assays and is a viable alternative to the assay of Wallace et al. [4] for serum levels of acetazolamide. It has been successfully applied to the analysis of over 500 samples from human volunteers who were administered various acetazolamide dosage forms.

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Note

Determination of salicylhydroxamic acid, a trypanocidal agent, by reversed-phase high-performance liquid chromatography

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Salicylhydroxamic acid (SHAM) specifically inhibits the respiration of bloodstream forms of Trypanosoma brucei [1—3] and has therefore been used in animals as a trypanocide [3, 4]. SHAM has been determined in plasma by several methods. A biological assay utilising inhibition of trypanosome respiration by SHAM has the advantage of only measuring the therapeutic SHAM concentration [3]. However, it does not distinguish between SHAM and active metabolites. A colorimetric method has been described [5], this being a modification of an assay for hydroxamic acids developed by Bergman and Segal [6].

In this note we describe a sensitive and reproducible method of estimating SHAM in whole blood using reversed-phase high-performance liquid chromatography (HPLC). This technique is able to distinguish SHAM and salicylamide, the major metabolite of SHAM [7, 8]. It is therefore suitable for the determination of the pharmacokinetic parameters of the drug in vivo. It has also been found suitable for the estimation of SHAM in homogenates of several animal organs.

EXPERIMENTAL

Reagents

Salicylhydroxamic acid was a kind gift of Professor T. Urbanski (Technical

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University, Warsaw, Poland) and phenacetin was obtained from Sigma (London) (Poole, Great Britain). All other chemicals used were of analytical grade and were supplied by BDH (Poole, Great Britain). These were used as supplied; aqueous solutions were prepared using glass-distilled water.

Extraction and chromatographic procedures

To 0.1 ml whole blood in a ground glass stoppered tube were added 30 μl internal standard solution (25 mg/ml phenacetin in methanol), 0.1 ml 0.02 *N* hydrochloric acid and 2 ml ethyl acetate. The mixture was roller mixed for 10 min and centrifuged at 1000 *g* for 5 min. A 1.5-ml aliquot of the supernatant organic phase was transferred to a conical tube and evaporated to dryness under a stream of air, in a water bath at 55°C. The residue was dissolved in 0.2 ml methanol and a 20-μl aliquot was injected onto the column.

SHAM standard samples were made up by adding aliquots of a 1 mg/ml SHAM solution (in methanol) to drug-free blood.

High-performance liquid chromatography

The chromatograph was a Pye-Unicam LC-XPS pump equipped with a 15 × 0.5 cm I.D. stainless steel column packed with Magnusphere (5 μm) C_{22} reversed-phase packing obtained from Magnus Scientific (Sandbach, Great Britain) and fitted with a Model 701 Rheodyne injection valve and a 20-μl injector loop. The apparatus was operated at ambient temperature. The eluting solvent was a degassed mixture of 0.043 *M* ammonium dihydrogen phosphate—methanol (70:30, v/v). The pH of this mixture was adjusted to 2.0 by the addition of approximately 5 ml orthophosphoric acid per litre of 0.043 *M* ammonium dihydrogen phosphate. The instrument was operated at a constant flow-rate of 1.2 ml/min and the absorption of eluent was monitored at 300 nm using a Pye-Unicam LC3 UV variable-wavelength absorbance detector.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1. The retention volumes for SHAM and phenacetin were 3.5 and 10.2 ml, respectively. A chromatogram of a blank blood sample is shown for comparison. No interfering peaks due to endogenous substances were present in the blank sample.

The linearity of the assay was determined by extracting five standard solutions in blood containing 0, 10, 20, 50 and 75 μg/ml SHAM. The correlation coefficient, *r*, was 0.991. Reproducibility was assessed at 1, 10, 20 and 50 μg/ml using six samples at each concentration. The results are shown in Table I.

The minimum level of detection for SHAM using this assay was 0.1 μg/ml from a 0.1-ml blood sample.

The accuracy of the assay was determined by analysis of twelve blood samples spiked at unknown concentrations. The mean percentage error between the known spiked concentration and that estimated was 8.9%.

The recovery of SHAM from the extraction procedure was 89% at 5 μg/ml, 83% at 30 μg/ml and 86% at 100 μg/ml. The principal metabolite of SHAM

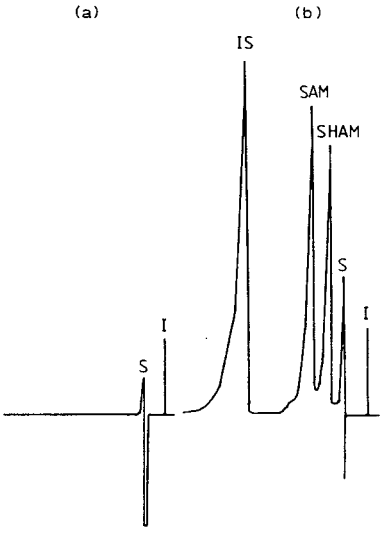

Fig. 1. Representative chromatograms for (a) blood containing no drug; (b) sample of a kidney homogenate taken following administration of 200 mg/kg SHAM to a mouse. Peaks: I = injection; S = solvent; SHAM = salicylhydroxamic acid; SAM = salicylamide; IS = internal standard. The concentrations of SHAM and SAM in this sample were 18 μg/g and 16.2 μg/g, respectively.

TABLE I

COEFFICIENTS OF VARIATION FOR SHAM DETERMINATIONS
n = 6 at each concentration.

SHAM concentration (μg/ml)	S.D.	C.V. (%)
1	0.065	6.2
10	0.045	4.0
20	0.126	6.6
50	0.200	4.0

is salicylamide [7, 8] and can be extracted in the same way as SHAM and estimated using this HPLC system. It has a retention volume of 7.4 ml and can therefore be separated from both SHAM and the internal standard.

Linearity of the method for salicylamide was confirmed by extracting samples containing 0, 40, 80 and 100 μg/ml. The correlation coefficient, r, was 0.999.

This assay provides a rapid and reproducible method for determining SHAM concentrations in small volumes of whole blood, plasma or tissue homogenates. It requires much smaller volumes of blood and is more accurate than methods for SHAM estimation described hitherto [3, 5]. The ability of this HPLC system to distinguish SHAM from salicylamide and other hydroxamic acids makes it suitable for determination of the pharmacokinetics of the parent compound which is known to be an active trypanocide [1—3]. The sensitiv-

ity is sufficient to measure SHAM in very small blood samples allowing for its use in the study of SHAM pharmacokinetics in the rodent models of the trypanosome infections of man and larger animals which are so widely used in the laboratory research into these infections (Fig. 2).

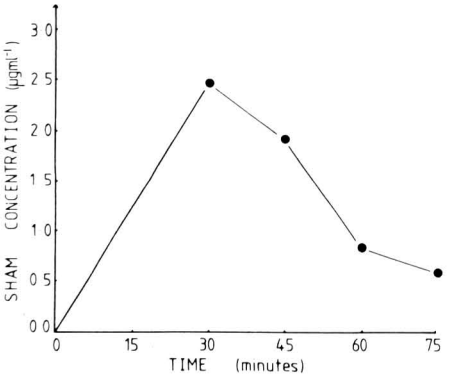

Fig. 2. Mean blood SHAM concentrations following oral administration of 200 mg/kg SHAM to five mice (mean elimination half-life of SHAM = 21 min).

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AB and Wv'tH held MRC student awards.

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Note

Determination of alcuronium chloride in biological fluids by high-performance liquid chromatography

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The neuromuscular blocking agent alcuronium chloride (diallyl-nortoxiferine, Alloferin®, Roche) has been in clinical use since 1961. Studies on its pharmacokinetics have been hampered by the lack of a specific method for its analysis in biological fluids.

Techniques that have been employed are bioassay [1], assay of radioactively labelled drug [2] and a fluorimetric method involving extraction with rose-bengal which, with modification has been shown to be suitable for a range of the available neuromuscular blocking agents [3—6]. A modification of this method using radioactively labelled rose-bengal has recently been reported [7]. These fluorimetric methods have the disadvantage in that they fail to differentiate between drug and quaternary base metabolites.

The application of reversed-phase high-performance liquid chromatography (HPLC) to the analysis of quaternary base drugs has received little attention. An HPLC method has been reported for tubocurarine and related alkaloids [8] and a method using paired-ion chromatography has been described for the determination of quaternary acetylcholinesterase inhibitors in biological fluids [9].

This paper describes an ion-pair HPLC method for the quantitation of alcuronium chloride in plasma and urine. The assay is suitable for pharmacokinetic studies on patients undergoing surgery.

EXPERIMENTAL

Reagents and materials

Dichloromethane and isopropyl alcohol (AR grade, Ajax Chemicals, Sydney, Australia) were redistilled prior to use. Disodium hydrogen phosphate was

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AnalaR grade from BDH (Poole, Great Britain).

Picric acid (BDH) was recrystallised from 20% ethanol in water and air-dried. Picric acid reagent was prepared by dissolving 2.5 g of picric acid in distilled water with the addition of 5.8 ml of anhydrous sodium carbonate (10%, w/v) and making up to 100 ml. The solution was then extracted with three equivalent volumes of 15% isopropyl alcohol in methylene chloride and the organic layers discarded.

Chromatographic solvent was made up from acetic acid (AnalaR grade BDH) sodium lauryl sulphate (BDH) and HPLC-grade methanol (Water Assoc., Milford, MA, U.S.A.).

Alcuronium chloride (Alloferin®) was a gift of Roche Products (Sydney, Australia); tubocurarine chloride stock solutions were prepared by dilution of tubocurarine injection [Tubarine®, Wellcome (Aust.), Sydney, Australia]. Stock solutions of alcuronium chloride (40 and 400 μg ml^{-1}) and tubocurarine chloride (7.5 and 50 μg ml^{-1}) were prepared in distilled water and stored at 4°C until required.

Chromatographic equipment

The liquid chromatograph consisted of a Waters Assoc. Model 6000A pump, Model U6K loop injector and Model 450 variable-wavelength detector.

Column temperatures were maintained by using a thermostatted water-bath.

Collection of biological samples

Blood samples were collected in heparinised tubes, the plasma collected following centrifugation and stored frozen until required. Urine samples were stored frozen until required.

Extraction procedure

To a 20-ml vial were added 1.0 ml of plasma or urine, 1.0 ml of 0.25 *M* disodium hydrogen phosphate, 2.0 ml of picrate reagent, 1.0 ml of tubocurarine internal standard (7.5 μg ml^{-1} for plasma and 50 μg ml^{-1} for urine) and 4.0 ml of 15% (v/v) isopropyl alcohol in methylene chloride. The vials were placed in a slowly rotating drum 150 mm diameter at 13 rpm and extracted for 5 h. Under these conditions equilibrium was achieved and no emulsification takes place. A 2.0-ml aliquot of organic phase was transferred to a glass vial and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 50 μl or volume as appropriate and submitted to HPLC analysis.

Chromatography of biological extracts

A Waters Assoc. μBondapak C_{18} column (30 cm × 6.4 mm I.D., 10 μm particle size) at 40°C was used using a mobile phase of methanol—water (80:20, v/v) containing 0.25% (v/v) acetic acid and 0.005 *M* sodium lauryl sulphate. The assays were performed at a flow-rate of 1.5 ml min^{-1}. Detection was at 292 nm. Absorbance range used was 0.02 a.u.f.s. for plasma and 0.1 a.u.f.s. for urine.

Quantitation

The procedure was standardized by analysing drug-free plasma and urine samples spiked with known quantities of alcuronium chloride solution (40 μg ml^{-1} for plasma and 400 μg ml^{-1} for urine). Peak height ratios of alcuronium chloride vs. tubocurarine chloride were used to establish calibration curves.

Reproducibility

Within-day reproducibility was determined by performing eight replicate analyses of spiked plasma and urine samples.

RESULTS AND DISCUSSION

The analytical method reported here is rapid and selective over the range 2.0—0.1 μg ml^{-1} and 20.0—0.2 μg ml^{-1} for alcuronium in plasma and urine, respectively. Linear calibration curves are derived over these concentration ranges being peak height ratio = 0.779 conc. + 0.0078 (n = 5, r = 0.999) for plasma and peak height ratio = 0.1189 conc. + 0.00165 (n = 6, r = 0.999) for urine. The intra-day variation is satisfactory, the coefficient of variation

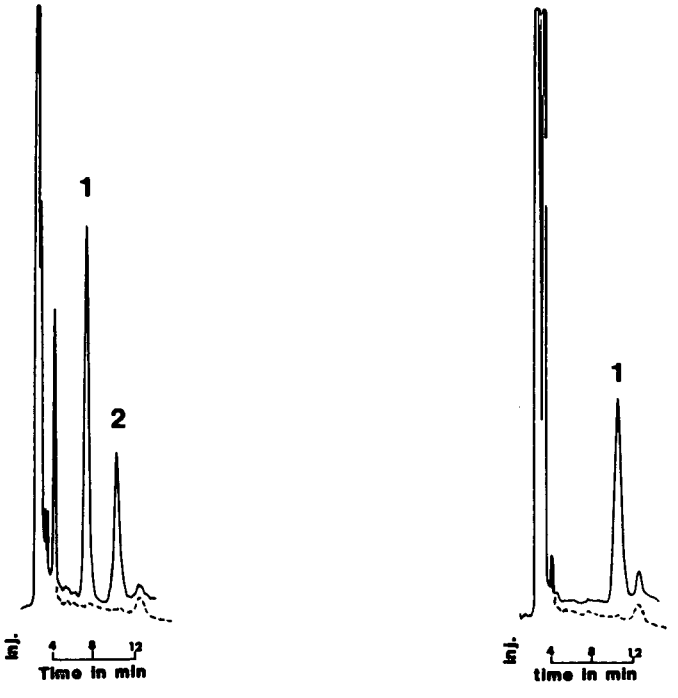

Fig. 1.——, Chromatogram derived from 1.0 ml of spiked plasma containing tubocurarine (1) (7.5 μg ml^{-1}, internal standard) and alcuronium (2) (0.5 μg ml^{-1}). — — —, Chromatogram derived from 1.0 ml of blank plasma. (0.02 a.u.f.s.).

Fig. 2.——, Chromatogram derived from 1.0 ml of plasma taken approximately 4 h after injection of alcuronium chloride during surgery showing alcuronium (1). Tubocurarine has been deleted. — — —, Chromatogram derived from 1.0 ml of blank plasma (0.02 a.u.f.s.).

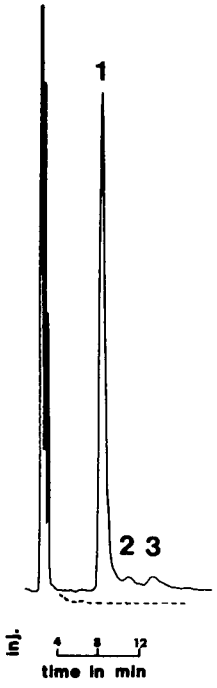

Fig. 3.——, Chromatogram derived from 1.0 ml of urine taken approximately 4 h after injection of alcuronium chloride during surgery showing alcuronium (1) and peaks due to minor metabolites (2) and (3). Tubocurarine has been deleted. — — —, Chromatogram derived from 1.0 ml of blank urine (0.1 a.u.f.s.). Flow-rate 1.8 ml min^{-1}.

based on eight replicate determinations being 2.8% for plasma and 1.6% for urine at concentrations of 1.0 and 10.0 $\mu g\ ml^{-1}$, respectively. Signal-to-noise ratios indicate that, with appropriate adjustment to internal standard concentrations, lower concentrations of alcuronium can be determined using this method.

Retention times for alcuronium and tubocurarine under the conditions were approximately 7 and 10 min respectively (Fig. 1). Blank plasma (Fig. 2) and urine (Fig. 3) show no interference arising from endogenous compounds. A minor peak due to an endogenous compound appears in the plasma and it is necessary to run the chromatogram at 40°C to complete resolve this peak from that due to the alcuronium. Picrate is eluted immediately and no interference is encountered. It was found to be necessary to pre-wash the picric acid reagent with extraction solvent to remove trace impurities which interfere with the baseline.

Experiments were performed to evaluate time to achieve extraction and the efficiency of extraction. Under the conditions employed, equilibration during extraction was achieved in approximately 2 h and resulted in quantitative extraction of the tubocurarine and 95% extraction of the alcuronium.

Figs. 2 and 3 show typical chromatograms of samples of plasma and urine respectively obtained 4 h after injection of alcuronium chloride during surgery.

The alcuronium peaks were shown to be homogeneous by monitoring at a range of wavelengths. There is some evidence in the sample (Fig. 3) that minor metabolites of alcuronium may occur. They possess longer retention times than alcuronium and do not interfere with either the alcuronium or tubocurarine peaks. Tests showed that they do not arise from any other medication given during or prior to surgery. This observation is at variance to that made by Raaflaub and Frey [2] who, using radioactively labelled drug, demonstrated that alcuronium is excreted unmetabolised.

Detailed pharmacokinetic studies are currently being undertaken using this assay procedure and will be published elsewhere.

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Note

High-performance liquid chromatographic determination of ethionamide and prothionamide in body fluids

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Ethionamide (2-ethyl thioisonicotinamide) was first used in the treatment of tuberculosis in the early fifties. Some ten years later the propyl analogue, prothionamide, was introduced since it was shown to be at least as active as ethionamide against *Mycobacterium tuberculosis* in vitro and against experimental tuberculosis in the mouse, but appeared to be better tolerated by patients. Recent experimental studies undertaken using the mouse foot-pad model have shown that both thioamides possess powerful antileprosy activity [1—3]. Both drugs display similar marked bactericidal action in the mouse and the minimal inhibition concentration (M.I.C.) of each drug against *M. leprae* was estimated to be about 0.05 μg/ml. These studies have encouraged considerable interest in the potential clinical use of ethionamide or prothionamide in combination with other established antileprosy drugs for the treatment of lepromatous leprosy.

Simple ultraviolet methods have been described [4, 5] that are capable of determining ethionamide and prothionamide concentrations of down to 0.3 μg/ml in 4—5 ml serum, while polarographic [6], quantitative thin-layer chromatographic [7] and gas—liquid chromatographic [2] methods have been reported for measuring concentrations of down to 0.2 μg/ml of the thioamides in 1 or 2 ml serum. However none of these methods is sufficiently sensitive to permit the measurement of serum levels approaching their M.I.C. against *M. leprae*. Such sensitivity is needed if the crucial pharmacological studies required to assess the role that ethionamide and prothionamide might eventual-

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ly play in the clinical treatment of leprosy are to be undertaken. Recently a radiochemical method of this sensitivity was described for determining serum concentrations of the two drugs in mice [2], but this method depends on the availability of radioactively-labelled ethionamide and prothionamide and is unsuitable for studies of their pharmacology in man.

In this paper, we describe a sensitive and specific high-performance liquid chromatographic (HPLC) method for the determination of ethionamide and prothionamide in plasma and urine and its application to pharmacokinetic studies of the two drugs in man.

EXPERIMENTAL

Chemicals

Ethionamide and prothionamide together with the ethionamide metabolite 2-ethyl-isonicotinamide were kindly donated by May and Baker (Dagenham, Great Britain) while the sulphoxide metabolites of the two drugs and 2-propyl-isonicotinamide were gifts from Dr. J.H. Peters and Professor J.K. Seydel. Stock solutions (1 mg/ml) of ethionamide and prothionamide were prepared by dissolving the drugs in methanol and could be stored at 4°C for many months without appreciable decomposition. The stock solutions were diluted with distilled water to give concentrations of either 15 or 60 μg/ml immediately prior to their use as internal standards, prothionamide being used as an internal standard for the determination of ethionamide and vice versa.

Collection of urine and plasma samples

In the first two studies after obtaining a pre-treatment urine sample, 500-mg doses of either ethionamide or prothionamide (Trescatyl and Trevintix, respectively; May and Baker) were ingested. Complete hourly urine collections were then obtained from 0—3 h, 2-hourly collections from 3—13 h while a pooled collection was made for the period from 13—24 h after dosage. In the third and fourth studies plasma samples were obtained pre-treatment, at hourly intervals up to 7 h, and 24 h after the ingestion of 500-mg doses of each drug, while urine collections were made from 0—0.5 h, at hourly intervals up to 7.5 h, from 7.5—23.5 h and from 23.5—24.5 h. The doses of ethionamide and prothionamide were taken on an empty stomach by a healthy volunteer (G.A.E.) weighing 65 kg. Each of the four doses was separated by an interval of at least a week. Urine samples were stored at −20°C and plasma samples over liquid nitrogen until analysis.

Extraction procedure

Aliquots (3 ml) of plasma/serum or urine were pipetted into stoppered centrifuge tubes together with 0.1 ml of a solution containing either 1.5 or 6 μg of the appropriate internal standard and extracted by shaking with 6 ml diethyl ether on a vortex mixer for 15 sec. After centrifugation, the organic phase was decanted and extracted with 1 ml 0.1 *M* hydrochloric acid. The acid extract was transferred to another centrifuge tube, 0.1 ml 1 *M* ammonium phosphate added and the pH adjusted to between 7 and 8 by the dropwise addition of 10% aqueous ammonia and the thioamides extracted by shaking

with 2 ml ethyl acetate. The ethyl acetate extract was transferred to a 10-ml tapered test tube, evaporated to dryness at 50°C under nitrogen, the residue dissolved in 1 ml dichloromethane, transferred to a 2-ml tapered vial and the solvent removed under nitrogen. The dried residue could then be kept at 4°C prior to chromatography.

Liquid chromatography

Analyses were performed using a Waters Assoc. (Northwich, Great Britain) Model M6000A pump, a Cecil CE 212 variable wavelength UV detector (Cambridge, Great Britain) set at 295 nm and a Waters U6K septumless universal injector. A normal-phase system was used consisting of a Waters μPorasil silica column (30 cm × 3.9 mm I.D., particle size 10 μm), which was eluted with a degassed glass microfibre filtered (GF/F, Whatman, Maidstone, Great Britain) mobile phase of diethyl ether—methanol (96 : 4) delivered at a flow-rate of 1.3 ml/min (ca. 6.2 MPa). The column was periodically purged with acetonitrile (BDH, Poole, Great Britain) that had been dried with magnesium sulphate and stored over a 3-Å potassium alumino-silicate molecular sieve, and re-activated with chloroform that had been dried over calcium hydride. The dried plasma and urine extracts were dissolved in 100 μl of the mobile phase, duplicate 25-μl aliquots injected and the mean ratio of the peak heights for the drug to that of the internal standard calculated.

Calibration curves

Calibration curves designed to encompass the highest levels of the thioamides expected in body fluids after the ingestion of therapeutic doses of the two drugs were prepared by spiking blank urine and horse serum (Gibco Bio-cult, Glasgow, Great Britain) with ethionamide (or prothionamide) to give concentrations of 0, 0.5, 1, 2.5 and 5 μg/ml, respectively. Duplicate 3-ml aliquots were then extracted and chromatographed as described above after the addition of 6 μg of the interal standard (prothionamide or ethionamide, respectively). Standard curves to cover lower thioamide concentrations were prepared using aliquots of urine and serum spiked with a tenth of the above concentrations and 1.5 μg of the internal standard. Calibration curves relating mean peak height ratio of duplicate injections to concentration of thioamide were shown to be linear, and the best straight lines and standard errors of slopes and intercepts were calculated by the least-squares regression method. Overall recoveries of both thioamides, calculated by comparing the peak heights of extracted samples with those when the drugs were injected directly, were between 70 and 80%.

Selectivity

The selectivity of the method with respect to the most widely used antituberculosis and antileprosy drugs and some of their principal metabolites was evaluated by applying the analytical procedure to solutions containing either 100 or 1000 μg/ml of the isonicotinamide and sulphoxide metabolites of ethionamide and prothionamide; clofazimine, dapsone, ethambutol, isoniazid and its metabolites acetylisoniazid and isonicotinic acid, *p*-aminosalicylic acid, pyrazinamide, rifampicin, streptomycin and thiacetazone in water or aqueous ethanol (9:1).

RESULTS

Analytical procedure

A representative chromatogram of the extract from the plasma sample obtained 2 h after the ingestion of 500 mg ethionamide is shown in Fig. 1. For this analysis 6 μg prothionamide was added as the internal standard. The retention times of prothionamide and ethionamide were 4.3 and 4.8 min respectively, giving near baseline separation with a resolution factor (R_s) of 1.21. For peaks with a resolution factor of greater than 1.0, peak height measurements should be accurate to within 3% [8]. Accordingly the concentrations of ethionamide or prothionamide in the samples were calculated from the peak-height ratio of the drug to that of the internal standard. The equations of the series of linear calibration curves for both drugs covering the two concentration ranges in urine and plasma/serum are given in Table I together with the standard errors of the slopes and intercepts. None of the intercepts was significantly different from zero and, as might have been expected, the slopes of the lines were inversely proportional to the amount of internal standard added. Replicate errors were similar for both drugs and averaged 2.2% and 3.6% for the higher and lower concentration ranges, respectively, whether determinations were carried out in serum or urine.

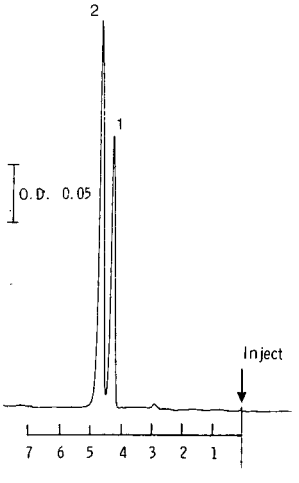

Fig. 1. Chromatogram of an extract of plasma from a volunteer 2 h after the ingestion of 500 mg ethionamide. Peaks: (1) prothionamide (the internal standard); and (2) ethionamide.

The response of the detector to both drugs was linear over a 500-fold range and injections of less than 5 ng could be quantitated giving a practical limit of detection of about 0.01 μg/ml of each thioamide in urine or plasma. Of the antituberculosis and antileprosy drugs and their metabolites that were tested, only dapsone and pyrazinamide interfered. Dapsone, whose retention time was similar to that of ethionamide, contributed about 50% to the method on a weight for weight basis, while pyrazinamide, which eluted with prothionamide, contributed to the extent of about 7%. Concomitant treatment with these

TABLE I

EQUATIONS OF CALIBRATION CURVES

Equation $y = mx + c$ where y is the ratio of the peak height of the drug to that of the internal standard, m the slope, x the concentration of ethionamide or prothionamide and c the intercept.

Drug	Biological fluid	Concentration range (μg/ml)	Slope ± S.E.*	Intercept ± S.E.
Ethionamide	Urine	0.5—5.0	0.435 ± 0.003	0.007 ± 0.012
	Urine	0.05—0.5	1.843 ± 0.009	0.015 ± 0.017
	Serum	0.5—5.0	0.450 ± 0.005	0.043 ± 0.026
	Serum	0.05—0.5	1.827 ± 0.049	0.018 ± 0.011
Prothionamide	Urine	0.5—5.0	0.526 ± 0.002	0.015 ± 0.006
	Urine	0.05—0.5	2.069 ± 0.074	0.064 ± 0.038
	Serum	0.5—5.0	0.523 ± 0.003	0.014 ± 0.009
	Serum	0.05—0.5	2.019 ± 0.031	0.014 ± 0.008

*Standard error.

drugs should therefore be avoided when determining ethionamide or prothionamide by this method.

Ethionamide and prothionamide plasma concentrations and urinary excretion after oral dosage in man

The plasma and urinary concentrations of ethionamide and prothionamide after the ingestion of 500-mg doses of each drug are illustrated in Figs. 2 and 3, respectively. The urinary concentrations of ethionamide or prothionamide correlated closely with the concomitant plasma concentrations of the two drugs (r = 0.94 for ethionamide from 2—7 h, and 0.99 for prothionamide from

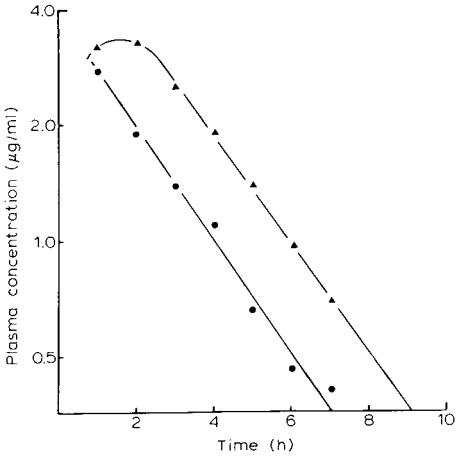

Fig. 2. Plasma concentrations of thioamides after oral dosage with 500 mg ethionamide (▲) or prothionamide (●).

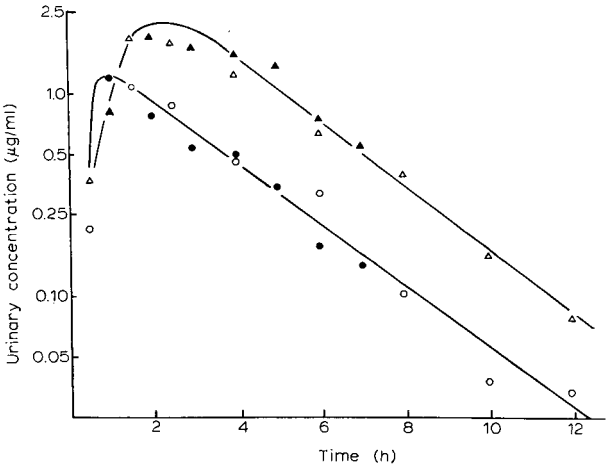

Fig. 3. Urinary concentrations of thioamides after oral dosage with 500 mg ethionamide (△ study I, ▲ study III) or prothionamide (○ study II, ● study IV).

1—7 h). During these time periods the ratio of urinary to plasma concentration averaged 0.75 for ethionamide and 0.42 for prothionamide. The urinary data for the pairs of studies on each drug have been combined in Fig. 3 since the results obtained on each occasion did not differ significantly. Both drugs were rapidly absorbed and maximal plasma and urinary concentrations were achieved within 2 h. Thereafter concentrations of both drugs in each body fluid fell exponentially at rates equivalent to half-lives of 2.23 ± 0.05 and 1.91 ± 0.06 h for ethionamide in plasma and urine, and 2.03 ± 0.07 and 1.94 ± 0.08 h for prothionamide. Although the peak plasma concentrations of both drugs were similar, from 2 h, ethionamide levels were nearly double those of prothionamide. By 24 h, the plasma concentrations of the two drugs had fallen to 0.06 and 0.03 μg/ml, respectively. The cumulative urinary excretion of ethionamide and prothionamide in the two pairs of studies were equivalent on average to 0.15% and 0.05%, respectively, of the administered doses.

DISCUSSION

The HPLC method described for the determination of ethionamide and prothionamide in plasma and urine is far more specific than the ultraviolet methods that were originally devised for their quantitation [4, 5] and some 40 times more sensitive. It is approximately ten times more sensitive than the polarographic [6], quantitative thin-layer chromatographic [7] and gas—liquid chromatographic [2] methods described for estimating the two drugs. Its sensitivity is similar to that of the method reported by Davidson and Smyth [9] using cathodic stripping voltammetry, but it is almost certainly more specific. As well as being sensitive and specific, this HPLC method is reasonably rapid and robust. Thus the average sample time is about 20 min, including extraction and chromatography, while the use of the internal standards that are closely related to the drug being measured and possess similar partition coeffi-

cients in the extraction systems employed, means that strict control over the volumes of organic and aqueous extracts recovered at each stage of the extraction procedure is unnecessary.

The ethionamide plasma concentrations determined after oral dosage with 500 mg of the drug (Fig. 2) confirm evidence from previous investigations utilising less sensitive polarographic or ultraviolet analytical methods that indicated that one might expect peak plasma/serum concentrations of about 3 μg/ml to be achieved within about 2 h of giving such a dose followed by an exponential decline equivalent to a half-life of about 2 h [10—12]. The pharmacokinetic studies have also shown that the rates of elimination of prothionamide and ethionamide from the body are virtually identical while the increased sensitivity of the HPLC method has enabled 24-h ethionamide and prothionamide plasma concentrations to be determined for the first time.

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Note

Determination of aspirin and its major metabolites in plasma by high-performance liquid chromatography without solvent extraction

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Aspirin (acetylsalicylic acid, ASA) is widely used for its analgesic, anti-inflammatory and antipyretic effects. It has been considered to be the drug of first choice in the treatment of rheumatoid arthritis [1, 2]. Aspirin has also been found to interfere with platelet aggregation and is now approved by the U.S. Food and Drug Administration as a therapeutic substance for reducing the risk of transient ischaemic attacks of stroke. In man, aspirin is rapidly hydrolyzed to salicylic acid (SA) which is further metabolized to salicyluric acid (SU), gentisic acid (GA), gentisuric acid (GU), salicyl acyl glucuronide (SAG), and salicyl phenolic glucuronide (SPG). Since aspirin, salicylic acid, salicyluric acid and gentisic acid have all been reported to show differing pharmacological effects as analgesics, antiplatelet agents and as inhibitors of prostaglandin synthetase in vitro [3, 4], it is desirable for analytical procedures to be able to quantify each substance in a blood sample from a subject after ingestion of aspirin.

The most frequently used analytical procedures for aspirin and its metabolites in biological fluids are colorimetry [5], fluorometry [6, 7] and gas—liquid chromatography [8]. Colorimetric procedures are limited by their poor specificity for salicylate and their dependence on a differential hydrolysis of aspirin to salicylic acid for quantification of aspirin. Fluorometric procedures have also depended on the hydrolysis of aspirin to salicylic acid for quantification. Gas—liquid chromatographic procedures have been

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found to be time consuming and are complicated by partial hydrolysis of aspirin during derivatization [9].

High-performance liquid chromatography (HPLC) is the most recent analytical technique used for the determination of salicylates in biological fluids [10—14]. In two of these methods aspirin has been determined [10, 14] but these involved time consuming extraction and evaporation processes. A specific, rapid and sensitive method for the determination of aspirin, salicylic acid, salicyluric acid and gentisic acid in plasma is now presented which requires only a simple protein precipitation step. In addition, the same chromatographic system can be used to quantify the total amount of salicylate excreted in the urine.

EXPERIMENTAL

Standards and reagents

Aspirin (acetylsalicylic acid), salicyluric acid, gentisic acid, phthalic acid and *o*-methoxybenzoic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Salicylic acid (AnalaR grade) and *p*-toluic acid were obtained from BDH Chemicals (Poole, Great Britain) and Hopkins and Williams (Essex, Great Britain), respectively.

Acetonitrile and methanol were specially purified for HPLC and supplied by Waters Assoc. (Milford, MA, U.S.A.). All other chemicals were analytical grade.

Instrumentation

Reversed-phase HPLC was performed using a Waters Model M6000A solvent delivery system and a U6K universal injector. A C_{18} μBondapak column (300 × 3.9 mm I.D., 10 μm average particle size) and a guard column (23 × 3.9 mm I.D.) packed with μBondapak C_{18}/Porasil B (also from Waters Assoc.) were used in all studies. The absorbance of the eluent was determined using a Waters Model 450 variable wavelength UV absorption detector. The absorbance was recorded on a dual-channel Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). Injections were made with a 50-μl Hamilton syringe.

Assay procedure for the measurement of aspirin and its metabolites in human plasma

Plasma samples were processed by transferring a 200-μl quantity into a glass tube (disposable borosilicate glass culture tubes, 6 × 50 mm, Kimble, IL, U.S.A.), and adding 20 μl perchloric acid (30%) solution containing the internal standard, *p*-toluic acid (0.02%). To this solution, 200 μl of methanol were then added. The sample was vortexed for 2 min and centrifuged at 1500 *g* for 5 min. An aliquot (20 μl) of clear supernatant was injected onto the column. The mobile phase consisted of acetonitrile—0.03% phosphoric acid, pH 2.5 ± 0.1 (30:70). The flow-rate was 1 ml/min. Absorbance was monitored at a wavelength of 237 nm.

Assay procedure for the measurement of total salicylate in urine

Total salicylate was estimated from the salicylic acid resulting from the hydrolysis of salicylate conjugates. For this hydrolysis process, 2 ml of urine were mixed with 2 ml of concentrated hydrochloric acid (10 *N*) in a 10-ml glass ampoule. The air inside the ampoule was removed by flushing with oxygen-free nitrogen immediately prior to sealing the ampoule. The ampoule and its contents were autoclaved at 120°C for 3 h. On cooling, the contents of the ampoule were vortexed for 10 sec and 20—100 μl of hydrolysate were made up to 300 μl with distilled water. To this solution, 200 μl acetonitrile were added. This solution was vortexed for 30 sec and centrifuged at 1500 *g* for 5 min. An aliquot (20 μl) of supernatant was injected onto the column. Chromatographic conditions used for the analysis of total salicylate in urine were identical to those used for the determination of aspirin and metabolites in plasma, except that the absorbance of the eluent was monitored at a wavelength of 313 nm.

Preparation of standard curves

Plasma standards were prepared by spiking drug-free plasma with known amounts of each analyte to produce concentrations of 0.5—200 μg/ml. The standards were then assayed in the described manner. Standard curves were then prepared by plotting the ratio of the peak height of analyte to peak height of internal standard versus concentration of analyte. Standards for salicylic acid, salicyluric acid and gentisic acid were prepared monthly and stored at −20°C between use. Standards for aspirin were prepared immediately prior to assay by spiking drug-free plasma with freshly prepared solutions of aspirin in methanol then vortexing vigorously for 30 sec. The strengths of the aspirin solutions were such that the amount of methanol added to the plasma never exceeded 1%.

To obtain standard curves for the hydrolysed urine, aqueous standard solutions of salicylic acid (2—500 μg/ml) were subjected to the same hydrolytic procedure as urine samples to be analyzed for total salicylate. Standard curves were prepared by plotting the peak height of salicylate versus concentration.

RESULTS AND DISCUSSION

Plasma samples

Typical chromatograms are shown in Fig. 1. The retention times for gentisic acid, salicyluric acid, aspirin, salicylic acid and *p*-toluic acid (internal standard) are 5.2, 6.2, 8.0, 11.2 and 13.6 min, respectively. With the present solvent system, a given analysis is completed in about 15 min.

The wavelength of 237 nm used in the present assay gave the best detector response for each salicylate component. This wavelength approximates to the optimal absorbance wavelength for gentisic acid, salicyluric acid and salicylic acid. Although the detector sensitivity is maximal for aspirin at 229 nm, the noise-to-signal ratio in the detector response is excessive at this wavelength. At the other absorbance maximum of 280 nm for aspirin, the detector response for aspirin is approximately one fifth the response obtained at 237 nm. At 237 nm, the limits of detection (with 20 μl supernatant) for gentisic

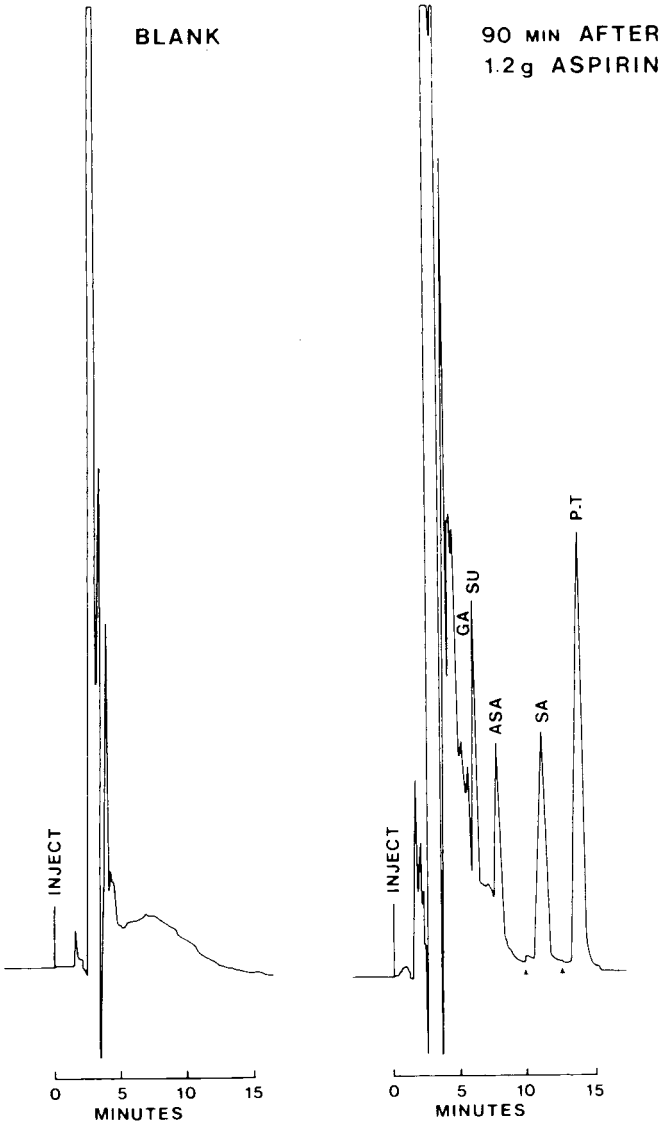

Fig. 1. Chromatograms of blank plasma and of plasma from the same subject 90 min after ingestion of 1.2 g soluble aspirin. The plasma concentrations of gentisic acid (GA), salicyluric acid (SU), aspirin (ASA) and salicylic acid (SA) are estimated to be 0.36, 2.4, 3.2 and 88.9 μg/ml, respectively; *p*-toluic acid (P-T) is the internal standard. Arrows indicate change in absorbance scale.

acid, salicyluric acid, aspirin and salicylic acid are 0.2, 0.5, 0.1 and 0.1 μg/ml, respectively.

As aspirin has been shown previously to exhibit weak fluorescence [15], an attempt was made to improve the sensitivity of the assay using a fluorescence detector (Schoeffel, FS970, Schoeffel, Westwood, NJ, U.S.A.). With an emission filter (KV320), the fluorescence of aspirin was found to

be optimal at an excitation wavelength of 229 nm but was insufficient to provide better detection sensitivity than found with the monitoring of eluent absorbance at 237 nm in the present assay system.

Linearity of response was found to be good (> 0.99) and consistently reproducible for standard curves based on both peak height ratio of salicylate compound to peak height of internal standard and for absolute peak height of a given salicylate compound following the injection of a known volume of aliquot. The reproducibility of the assay is given in Table I.

TABLE I

PRECISION OF ASSAY FOR ASPIRIN (ASA), SALICYLIC ACID (SA), SALICYLURIC ACID (SU) AND GENTISIC ACID (GA) IN PLASMA (n = 10)

	ASA	SA	SU	GA
Mean (μg/ml)	20.09	23.52	23.20	5.91
Standard deviation	0.914	0.595	0.736	0.132
Coefficient of variation (%)	4.55	2.53	3.17	2.23

Perchloric acid, used routinely for protein precipitation in other HPLC assays [16], was found to give complete protein precipitation. Only partial extraction of salicylate compounds from precipitated protein was achieved when methanol was not added. Addition of methanol and vortexing for 2 min was necessary for complete extraction of salicylate compounds from plasma protein. Complete extraction was indicated by standard curves (absolute peak height) of the salicylate compounds and *p*-toluic acid prepared in plasma being identical to standard curves for these compounds prepared in water. The supernatant of the present assay protein precipitation procedure gave a good, almost noise-free baseline after the initial plasma peaks, only one endogenous plasma peak was sometimes found at 8.8 min. This peak did not interfere with any of the compounds under investigation. Acetonitrile used as a protein precipitant in other HPLC assays for salicylate [13] was found to be unsuitable in the present assay. The supernatant of acetonitrile-extracted plasma resulted in a noisy baseline with interfering peaks being observed in the chromatogram.

The internal standard (*p*-toluic acid) chosen in the present plasma assay eluted after the other salicylate compounds and was not affected by endogenous plasma peaks. It was found, however, that recycling of the mobile phase resulted in an increase in the height of the *p*-toluic acid peak possibly due to some interaction with active binding sites on the column. No change in the peak height of *p*-toluic acid was found when the mobile phase was not recycled. Internal standards used in some other HPLC plasma salicylate assays were found to be unsuitable for the present plasma assay. Phthalic acid used by Peng et al. [10] overlapped with the gentisic acid peak. *o*-Methoxybenzoic acid has also been used as an internal standard in HPLC methods for the determination of salicylate [11—13], but was found to co-chromatograph with aspirin using the present system.

Interference by other drugs was studied by analyzing plasma samples from patients taking various drugs. Table II lists the drugs being taken by these patients. None of these drugs nor altered blood constituents, such as elevated bilirubin, interfered with the assay.

Since aspirin is rapidly hydrolyzed in whole blood to salicylic acid, it is essential to collect blood in a manner so as to minimize hydrolysis. Potassium fluoride (5 mg/ml) was found to slow aspirin hydrolysis in whole blood at 37°C more effectively than the cholinesterase inhibitor, ecothiopate (0.25 and 0.025 mg/ml). The rate of aspirin hydrolysis was halved by ecothiopate and reduced to a quarter with potassium fluoride. This extent of inhibition was also greater than that reported for physostigmine [17]. The greater effectiveness of the weak cholinesterase inhibitor potassium fluoride may result from its better penetration into red blood cells. The technique of collecting blood samples into chilled sample tubes containing potassium fluoride [8] was therefore adopted in the present assay system. As the half-life of aspirin in frozen plasma containing potassium fluoride is about 23.5 days [18], its is desirable to analyze plasma as quickly as possible after collection.

TABLE II

DRUGS TESTED FOR INTERFERENCE IN PLASMA

Allopurinol	Ibuprofen	Prazosin
Amiloride	Imipramine	Prochlorperazine
Ampicillin	Indomethacin	Quinidine
Chloral hydrate	Metamucil	Salbutamol
Chlormethiazole	Multivite	Spironolactone
Dextropropoxyphene	Nitrazepam	Sulindac
Diazepam	Paracetamol	Theophylline
Digoxin	Phytomenadione	Thiamine
Folic acid	Potassium chloride	Trimipramine
Frusemide		

The present assay system was used to monitor plasma aspirin levels with time from a healthy male adult given 1.2 g soluble aspirin in 100 ml of water. Blood samples from this subject were collected into chilled containers containing potassium fluoride [8]. The resultant plasma concentration—time profiles for aspirin, salicylic acid, salicyluric acid and gentisic acid are shown in Fig. 2. This plasma concentration—time profile is similar to those reported by other workers for comparable doses of aspirin [8, 13, 19].

Urine samples

As virtually all aspirin absorbed into the body can be accounted for by the recovery of salicylic acid in urine after hydrolysis of its conjugates [20, 21], the quantification of salicylic acid in urine using the HPLC conditions of the plasma aspirin assay permits a rapid evaluation of the extent of aspirin absorption. Fig. 3 shows chromatograms of hydrolyzed urine samples collected from a subject before and after a dose of aspirin. Salicylic acid is well separated from all urine peaks and can be quantified to as low as 1 μg/ml with an intra-assay coefficient of variation of 4.1%. Gentisic acid, which

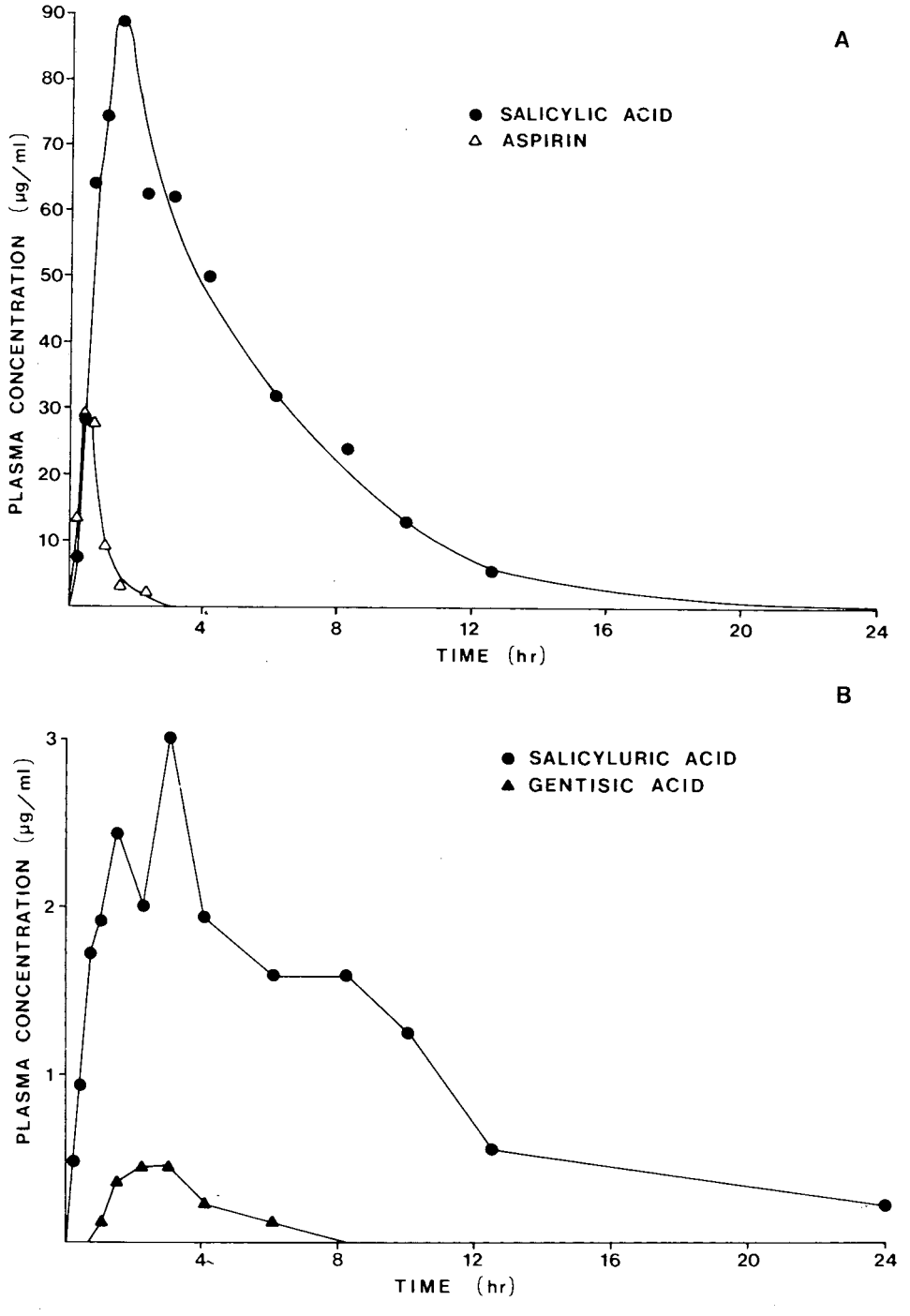

Fig. 2. Time course of (A) plasma aspirin and salicylic acid concentrations and (B) plasma salicyluric acid and gentisic acid concentrations found in a subject after ingestion of 1.2 g soluble aspirin.

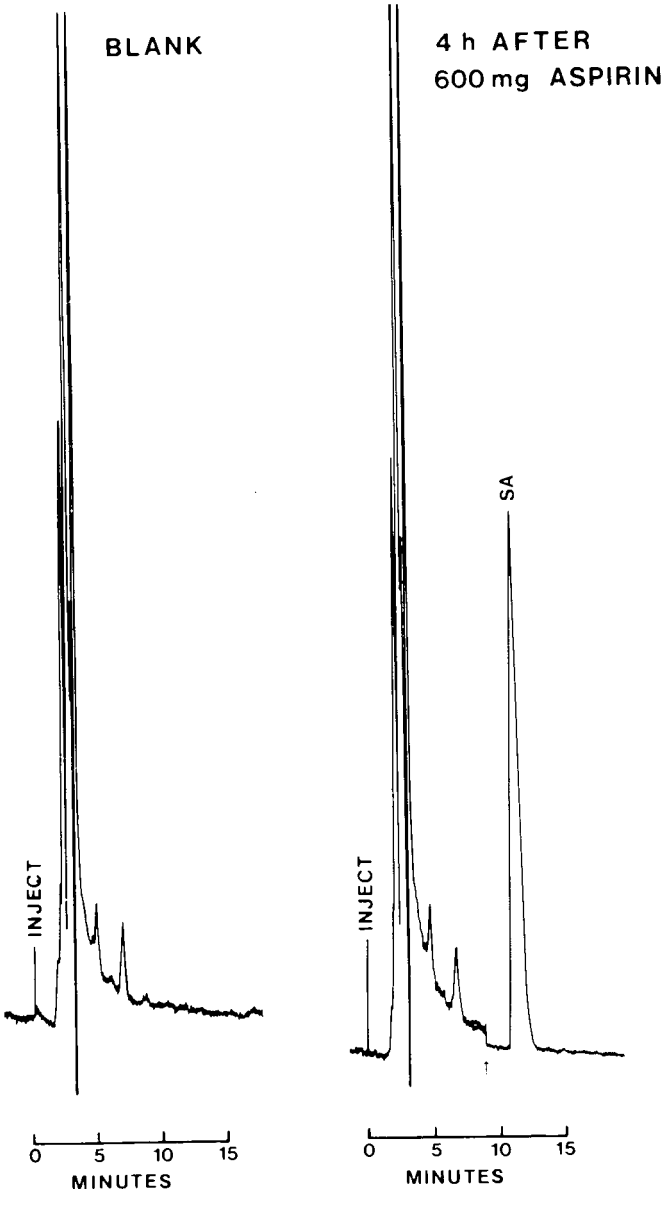

Fig. 3. Chromatograms of blank hydrolyzed urine and of hydrolyzed urine collected from the same subject 4 h after ingestion of 600 mg soluble aspirin. The concentration of salicylic acid (SA) in the hydrolyzed urine is estimated to be 186 μg/ml. Arrow indicates change in absorbance scale.

accounts for 1—3% of the dose of aspirin recovered in the urine after hydrolysis [20, 21], is poorly resolved from endogenous urine peaks and therefore not readily quantified with the present HPLC assay. Salicylic acid was completely recovered from urine samples and gave a linear standard curve of peak height versus salicylate concentration at the less intense but more

selective detector wavelength of 313 nm used to minimize interference in the urine assay. The total amounts of salicylate recovered in the urine of subjects after doses of aspirin were comparable to values obtained by other analytical procedures [6, 20—23].

CONCLUSION

The present procedure for quantifying aspirin in plasma differs from previous analytical procedures in that the time consuming extraction processes are replaced by a protein precipitation step. In addition, the use of the same chromatographic conditions for both plasma and hydrolyzed urine allows the pharmacokinetics of aspirin after oral administration to be readily followed.

ACKNOWLEDGEMENTS

This work was supported by grants from the Rheumatism and Arthritis Foundation of Tasmania (R.A.F.T.) and by the South Australian Association for Rheumatism and Arthritis (S.A.A.R.A.).

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CHROMBIO. 928

Note

Simultaneous determination of pentoxifylline and its hydroxy metabolite in plasma by high-performance liquid chromatography

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(First received January 20th, 1981; revised manuscript received March 13th, 1981)

Pentoxifylline [I; 3,7-dimethyl-1-(5-oxohexyl)-xanthine; Fig 1] is a drug used for the treatment of circulatory disorders. Although a number of procedures for the analysis of pentoxifylline and its major metabolite [II; 3,7-dimethyl-1-(5-hydroxyhexyl)-xanthine] in plasma have been developed for metabolic and pharmacokinetic studies, each of these has significant disadvantages. In particular, pentoxifylline has been quantitated spectrophotometrically [1] and colorimetrically [2] but these methods require multiple solvent extractions of plasma and preliminary separation of the drug by thin-layer chromatography. Such methods are inconvenient for processing large numbers of samples. Gas—liquid chromatographic conditions for the separation of pentoxifylline have been reported [1] but have only been applied qualitatively. Similarly, although a high-performance liquid chromatographic (HPLC) procedure for the analysis of pentoxifylline and its metabolite (II) in plasma has been used in bioavailability studies [3], the method requires a large sample volume, multiple solvent extraction of plasma is necessary, and few analytical details or precision data were reported.

As part of a clinical trial in progress at this Centre it was necessary to monitor plasma concentrations of pentoxifylline and its metabolite (II). In recent years, the convenience and versatility of HPLC has led to its acceptance as one of the most useful techniques available for the analysis of drugs in biological fluids [4]. Thus, an HPLC method suitable for the co-determination of pentoxifylline and its metabolite (II) in plasma has been developed which is simpler and more rapid than previously reported procedures. In addition, the method is sensitive, reproducible and readily applicable to pharmacokinetic studies.

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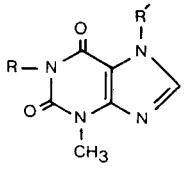

	R	R′
(I)	$-CH_2-(CH_2)_3-CO-CH_3$	$-CH_3$
(II)	$-CH_2-(CH_2)_3-CH(OH)-CH_3$	$-CH_3$
(III)	$-CH_2-(CH_2)_3-CO-CH_3$	$-CH_2CH_3$

Fig. 1. Structures of pentoxifylline (I), metabolite (II) and internal standard (III).

EXPERIMENTAL

Reagents and standards

Pure samples of pentoxifylline, its metabolite (II) and the internal standard [III; 7-ethyl-3-methyl-1-(5-oxohexyl)-xanthine] were supplied by Hoechst Roussel Pharmaceuticals (Melbourne, Australia). Other reagents and solvents were of analytical grade.

A stock solution was prepared by dissolving 20 mg of both pentoxifylline and its metabolite (II) in 1 l of distilled water. Standards were then prepared by diluting the appropriate volume of stock solution with drug-free plasma or distilled water to give final concentrations of pentoxifylline and metabolite (II) of 1000, 500, 250, 100 and 50 μg/l. The internal standard was prepared by dissolving 4 mg of the compound (III) in 1 l of distilled water. All solutions were stable for at least three months when stored at 4°C.

Chromatography

The high-performance liquid chromatograph used (Waters Assoc., Milford, MA, U.S.A.) consisted of a Model 45 solvent delivery system, a Model U6K universal injector, and a Model 450 ultraviolet absorbance detector operating at 275 nm. The instrument was fitted with a 30 cm × 3.9 mm I.D. reversed-phase μBondapak C_{18} column (10 μm, Waters Assoc.) and operated at ambient temperature. The mobile phase was methanol—phosphate buffer, 10 m*M*, pH 7.0 (38:62) used at a flow-rate of 2.0 ml/min.

Sample preparation

To 1 ml of plasma or aqueous standard in a 15-ml glass culture tube were added 0.1 ml of internal standard solution and 10 ml of dichloromethane. The solution was vortex-mixed for 1 min and then centrifuged at 1500 *g* for 3 min. After aspiration of the aqueous layer the organic phase was transferred to a conical-tipped glass tube and evaporated to dryness under vacuum at 40°C. The residue was redissolved in 0.1 ml of the mobile phase and injected into the chromatograph.

Unknown concentrations were determined by comparison of the pentoxifylline, metabolite (II)/internal standard peak height ratios with those of the calibration curves.

RESULTS AND DISCUSSION

Representative chromatograms of extracts obtained following this procedure are shown in Fig. 2. Sharp, symmetrical peaks with retention times of 5.0, 6.5 and 7.6 min are obtained for pentoxifylline, metabolite (II) and internal standard respectively. Fig. 2A shows the chromatogram of the extract of an aqueous standard containing 250 μg/l of both pentoxifylline and metabolite (II). Similarly, Fig. 2B shows the chromatogram of an extract of plasma, taken 3 h after a dose from a patient on chronic pentoxifylline treatment (400 mg, 12-hourly), containing 180 and 200 μg/l of pentoxifylline and metabolite (II) respectively. Drug-free plasma gave no interfering peaks under the chromatography conditions described (Fig. 2C).

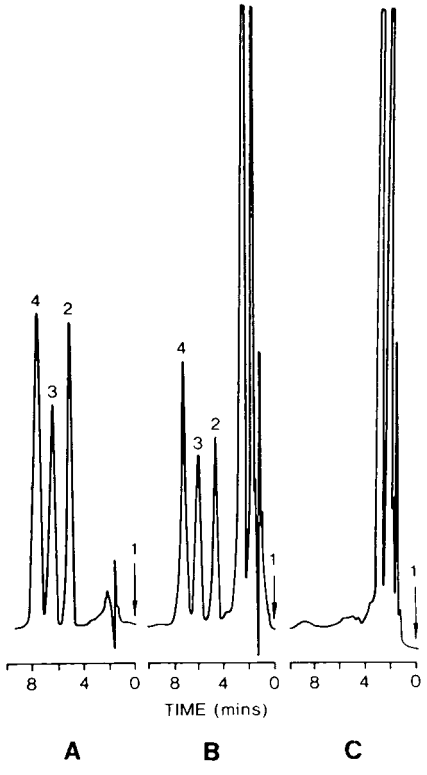

Fig. 2. Chromatograms of plasma or water extracts. (A) Aqueous standard containing 250 μg/l of both pentoxifylline and metabolite (II), 0.04 a.u.f.s.; (B) plasma sample containing 180 μg/l of pentoxifylline and 200 μg/l of metabolite (II), 0.04 a.u.f.s.; (C) blank plasma, 0.02 a.u.f.s. Peaks: 1, injection; 2, pentoxifylline; 3, metabolite (II); 4, internal standard.

Mean recoveries, calculated by comparing the peak height for extracted compound with that of an equal amount injected directly into the chromatograph, for samples containing 50—1000 μg/l of pentoxifylline and its metabolite (II) were 85.8 ± 2.9% and 75.8 ± 1.5% respectively. The recovery efficiency was essentially identical for samples extracted from equal volumes

of plasma or water thereby enabling the use of aqueous standards.

Calibration curves for pentoxifylline and its metabolite (II) were linear in the range 50—1000 μg/l and passed through the origin (Fig. 3). A total of twenty calibration curves were prepared from plasma or water over a period of approximately two months. The average coefficients of variation of the normalised peak height ratios were 5.8 ± 2.5% and 5.7 ± 1.4% for pentoxifylline and metabolite (II) respectively.

With photometric detection at 275 nm, the approximate absorption maximum for pentoxifylline and its metabolite (II) in the mobile phase, as little as 20 μg/l of both compounds may be quantitated. It should also be noted that detection at 280 nm may be used with little apparent loss of sensitivity. During several months of operation no other drugs, metabolites or endogenous plasma constituents have been found to interfere with the determination of both compounds. In addition, it has been shown that caffeine, salicylate and paracetamol do not co-chromatograph with either pentoxifylline, metabolite (II) or internal standard.

In summary, an HPLC procedure for the estimation of pentoxifylline and metabolite (II) in plasma has been developed. The method offers significant advantages in terms of rapidity, simplicity, reproducibility, and specificity over previously published procedures.

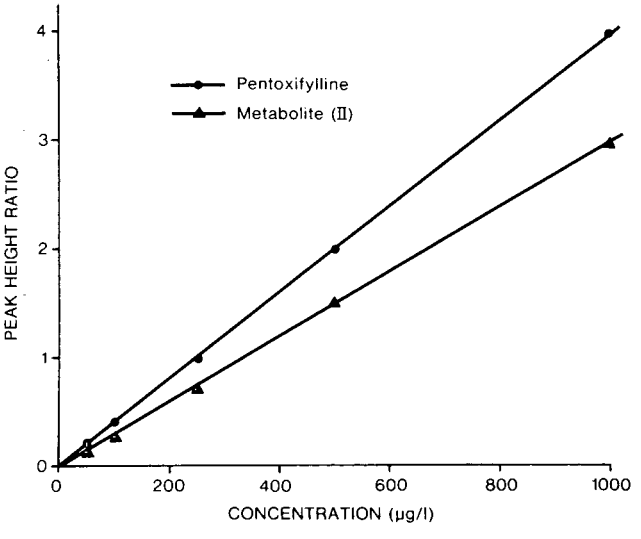

Fig. 3. Typical calibration curve for pentoxifylline and metabolite (II).

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CHROMBIO. 936

Note

Determination of midazolam by high-performance liquid chromatography

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(First received February 3rd, 1981; revised manuscript received April 17th, 1981)

In a previous communication [1] we reported a procedure for the serum determination of midazolam using gas chromatography (GC) with a nitrogen-selective detector. GC with nitrogen-selective detection resulted in a selective procedure for the direct determination of midazolam with a sensitivity of 50 ng/ml. We present, in this paper, a procedure for the direct determination of midazolam and its major metabolite (1-hydroxymethylmidazolam) using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. The sensitivity of the assay is increased by the use of HPLC and better linearity was observed in the lower (ng/ml) concentration range.

MATERIALS AND METHODS

For HPLC separations we used a Model 6000A solvent delivery system, Model 600 solvent programmer, Model 440 absorbance detector, Model 450 variable-wavelength detector, Model U6K universal liquid injector (Waters Assoc., Milford, MA, U.S.A.) and a dual-pen recorder (Houston Instruments, Austin, TX, U.S.A.). A prepacked 10-μm particle size μBondapak C_{18} (300 × 4 mm I.D.) column from Waters Assoc. was also used.

A Model 5840A gas chromatograph with dual nitrogen—phosphorus sensitive

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detectors and coiled glass columns, 1.2 m × 2 mm I.D., packed with 2% SP-2250 or 2% SP-2100 on Chromosorb W HP 100—120 mesh (Hewlett-Packard, Avondale, PA, U.S.A.) were used for GC separations.

Reagents

Heptane and isobutanol were analytical reagent (AR) grade. High-purity methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Sodium hydroxide, 0.05 mol/l, sulfuric acid, 1 mol/l and 0.05 mol/l, were prepared from concentrated solutions. Octanesulfonic acid, 0.05 and 0.005 mol/l, was prepared from its sodium salt, which was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.).

The HPLC mobile phase was octanesulfonic acid, 0.005 mol/l in methanol. Dilute 50 ml of 0.05 mol/l octanesulfonic acid with 150 ml of water and bring the volume to 500 ml with methanol. The pH was adjusted to 3.5 with sulfuric acid.

Standards

Midazolam maleate, flurazepam dihydrochloride and 1-hydroxymethylmidazolam were obtained from Hoffmann-LaRoche (Nutley, NJ, U.S.A.).

Standards of midazolam, its metabolite and the internal standard (flurazepam) were prepared at 1 mg/ml in absolute ethanol. Dilutions of the 1 mg/ml standards were used to make the appropriate working standards of midazolam and its 1-hydroxymethyl metabolite. A 5 mg/l flurazepam solution was also prepared.

Operating conditions

For HPLC we used a C_{18} reversed-phase column and a mixture of methanol—water (60:40, v/v) as the mobile phase, at a flow-rate of 1 ml/min. The eluent was monitored at 254 and 220 nm with the use of a constant- and variable-wavelength detector.

For GC analysis we used a column temperature of 260°C and a helium flow-rate of 40 ml/min.

Procedure

The extraction procedure has been reported [1]. Midazolam, 1-hydroxymethylmidazolam and flurazepam (0.2 ml of 5 mg/l) were extracted at basic pH (4 ml of 0.5 mol/l sodium hydroxide into *n*-heptane—isobutanol (96:4, v/v) and then back-extracted in 4 ml of 1 mol/l sulfuric acid. The solution was made basic with sodium hydroxide, the drugs were extracted into diethyl ether and the ether was evaporated. The residue was dissolved in 50 µl of absolute ethanol and 15 µl were injected for HPLC analysis. For GC, 1—2 µl was injected for analysis.

Peak height ratios of midazolam to that of flurazepam (the internal standard), were used to calculate the drug's concentration, by GC. For HPLC, peak height ratios of midazolam and 1-hydroxymethylmidazolam to that of flurazepam were determined at 254 and 220 nm.

RESULTS

Midazolam has a UV absorption maximum at 255 nm ($E^{1\%}_{1cm}$ = 650) in an acidic pH. A shoulder is observed at 220 nm. HPLC chromatograms at 254 nm of an unextracted standard mixture, and serum extracts of standards of 250, 500, and 750 ng/ml are given in Fig. 1. Similar results were observed at 220 nm. Blank sera assayed by this procedure showed no significant peaks that might interfere with the analysis (Fig. 2). Retention times for the 1-hydroxymethyl metabolite and midazolam are 0.73 and 0.86 relative to flurazepam, which elutes in 13 min. Fig. 2 illustrates the use of this procedure to analyze sera of patients who were induced for anesthesia with midazolam. When peak heights of sera containing 250, 500 and 750 ng/ml of midazolam were plotted against concentration, the resulting line had a slope of 0.0031, a y-intercept of −0.02, a standard error of estimate (Syx) of 0.02 and a correlation coefficient of 0.99. Within-run absolute analytical recovery determined with serum extracts (0.25—0.75 mg/l) averaged 78 ± 5% (n = 5). Relative within-run

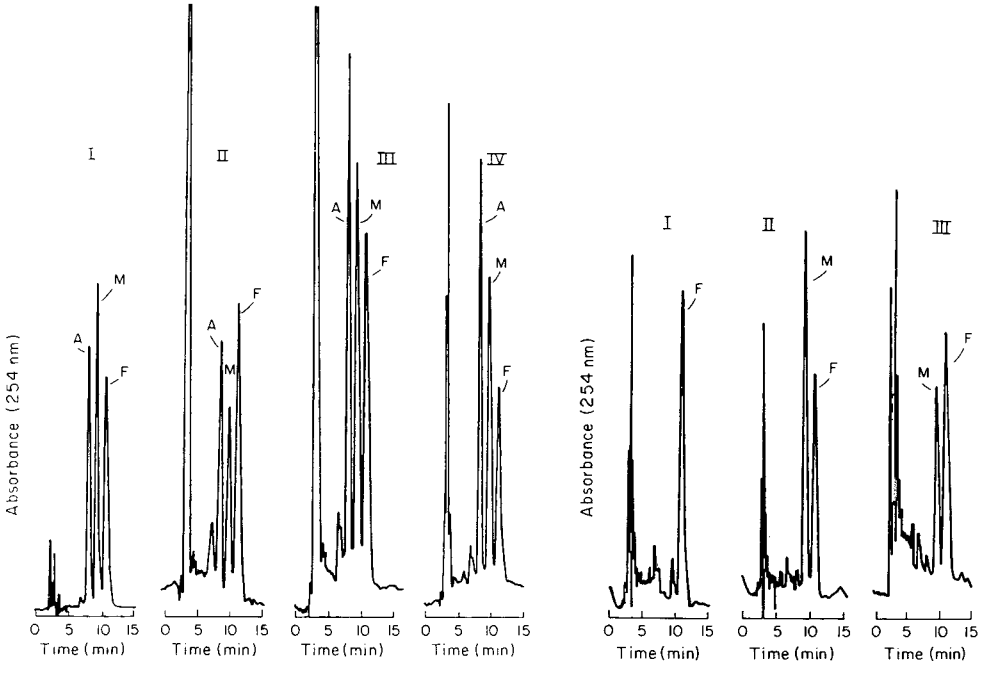

Fig. 1. Liquid chromatograms of (I) a mixture of pure unextracted standards 0.1 g/l, 1 μl injected; and serum standards containing II, 250; III, 500 and IV, 750 ng/ml of midazolam and its 1-hydroxymethyl metabolite. Peaks: A = 1-hydroxymethylmidazolam; M = midazolam; F = flurazepam, internal standard, 450 ng/ml. Eluting solvent methanol—water (60:40, v/v) with 5 mmol/l octanesulfonic acid, pH adjusted to 3.5. Absorbance scale is variable from 0.005 to 0.02 units.

Fig. 2. Liquid chromatograms of patients' sera extracts taken at times 0 (I), 1—2 (II), and 13—14 (III) min after the administration of midazolam for induction of anesthesia. Concentration of midazolam (M) is 676 and 320 μg/l in II and III respectively. F = Flurazepam (internal standard), 450 ng/ml. Notice the absence of 1-hydroxymethylmidazolam.

percentage recovery with serum extracts (0.25—0.75 mg/l) averaged 100 ± 11% (n = 5).

Within-run precisions (coefficients of variation) of serum-based controls containing 200 and 400 ng/ml of midazolam were 3 (n = 4) and 5% (n = 3), respectively. Between-run precision of a 500 ng/ml serum based standard was 10% (n = 5).

When peak heights of sera containing 250, 500, and 750 ng/ml of 1-hydroxymethylmidazolam were plotted against concentration, the resulting line had a slope of 0.0025, y-intercept of —0.02, Syx of 0.09, and a correlation coefficient of 0.99. The absolute recovery using serum standards (0.25—0.75 mg/l) averaged 97 ± 5% (n = 5) while the relative percent recovery averaged 100 ± 5% (n = 5). Within-run precision of serum-based controls containing 200 and 400 ng/ml of the 1-hydroxymethyl metabolite was 4% (n = 4 in each). Between-run precision of a 500 ng/ml serum standard was 11% (n = 9). Peak height ratios of sera extracts containing midazolam and 1-hydroxymethylmidazolam were linearly related to concentration up to 2 μg/ml. Diazepam interferes with midazolam while nordiazepam interferes with the 1-hydroxymethyl metabolite.

DISCUSSION

Midazolam maleate is an investigational benzodiazepine which is pharmacologically similar to diazepam. Its half-life, which is less than 3 h, is much shorter than the half-life of diazepam, which is greater than 20 h. Midazolam is, therefore, better suited for induction of anesthesia. Therapeutic and toxic ranges for midazolam have not yet been established for humans. Because 1-hydroxymethylmidazolam is the major reported metabolite of midazolam and since it does not chromatograph well by GC, we chose HPLC as an alternative means for performing patient serum determinations for midazolam and 1-hydroxymethylmidazolam. Our interest was to set up an assay for the determination of midazolam and its 1-hydroxymethyl metabolite, and then to determine parent drug and metabolite concentrations in patients' sera.

Parent drug and metabolite determinations are readily performed by HPLC as shown by Figs. 1 and 2. The same analysis performed by GC would require the formation of a derivative of 1-hydroxymethylmidazolam and would, therefore, be more complex than the present HPLC method. The relative within- and between-run recovery using serum standards averaged 100% for midazolam and 1-hydroxymethylmidazolam. In the case of the 1-hydroxymethyl metabolite the absolute recovery was close to 100%, which indicates that the 1-hydroxy metabolite is extracted better with the present procedure than midazolam. The absolute recovery of midazolam averaged 80%. Within-run precision for midazolam and 1-hydroxymethylmidazolam averaged 4% in the 250—750 ng/ml range by HPLC. In contrast the within-run precision for midazolam by GC in the 3 and 5 mg/l range was 6% [1]. The precision of the present method is 5% at serum midazolam concentrations of less than 50 ng/ml. This was established by running a 25 ng/ml serum standard in triplicate. It was not possible to obtain as good a sensitivity with the previously reported GC procedure [1] because of the high chromatographic background which was

observed at low concentrations in the vicinity of the midazolam peak. However, the previously reported GC procedure [1] gives results comparable to the present HPLC method at serum midazolam concentrations of greater than 100 ng/ml. Twenty-three serum samples analyzed first by GC and then HPLC gave a slope of 1.09, y-intercept of 120, a standard error of estimate (Syx) of 111, and a correlation coefficient of 0.98.

The use of flurazepam as internal standard is important in order to retain the flexibility of performing the chomatographic analysis by GC or HPLC. If one is interested in performing analysis only by HPLC, prazepam, which has a retention time of 1.5 relative to flurazepam, is a preferred internal standard. Prazepam could not be used as an internal standard for GC since it interferes with midazolam [1]. Of the commonly prescribed drugs diazepam interferes with midazolam while nordiazepam, quinidine, methaqualone and oxazepam interfere with the 1-hydroxymethyl metabolite. The following drugs did not interfere: amitriptyline, nortriptyline, doxepin, imipramine, desipramine, procainamide, loxapine, phenobarbital, secobarbital, salicylate, phenytoin, meprobamate, glutethimide, and disopyramide. A check for the presence of interfering substances with HPLC is to monitor the peaks at two wavelengths. A comparison of patient's midazolam determinations at 254 and 220 nm is given in Fig. 3. Least squares analysis gave a slope of 1.09, y-intercept of −46, a standard error of estimate (Syx) of 67 and a correlation coefficient of 0.99. The above results confirm that no detectable interference was observed with these sera midazolam determinations. In the case of an interfering substance such as diazepam the analysis could be performed by GC or GC—mass spectrometry as we have previously suggested [1, 2].

Patient samples analyzed for the presence of midazolam and 1-hydroxymethylmidazolam after dosing are given in Fig. 2. As can be seen, no detectable quantity of free 1-hydroxymethylmidazolam is observed in serum. Timed serum samples taken up to 8 h after dosing did not reveal the presence of the 1-hydroxymethyl metabolite. Serum midazolam concentrations decrease

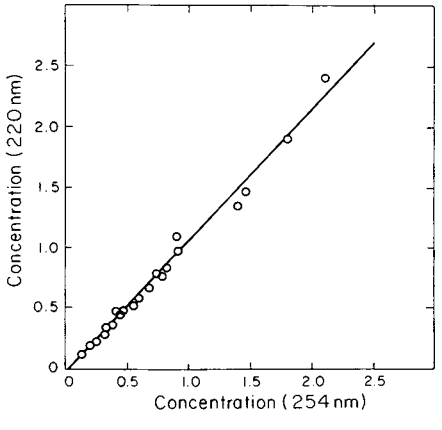

Fig. 3. Comparison of patients' sera midazolam determinations at 254 and 220 nm. See text for statistical analysis. Concentration in mg/l.

rapidly due to the distribution of the drug from the main (vascular) compartment into the peripheral (tissue) compartment. The half-life for the initial distribution (alpha-phase) from the present data is less than 10 min, with a half-life for the beta phase being 2—3 h. Thus at the end of 8 h, one would expect to see the presence of free 1-hydroxymetabolite if it is present in appreciable concentration in serum. A complete analysis of patient results as well as the possible presence of other metabolites, such as desmethyl midazolam, is currently in progress. The present HPLC method is a sensitive direct procedure for the determination of midazolam and 1-hydroxymethylmidazolam. Analysis can be performed with a sensitivity of 15 ng/ml and a precision of less than 5%.

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CHROMBIO. 906

Book Review

Sequencing of proteins and peptides (Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 9, edited by T.S. Work and R.H. Burdon), by G. Allen, Elsevier/North Holland Biomedical Press, Amsterdam, New York, Oxford, 1981, XVIII + 327 pp., price Dfl. 61.00, US$ 29.75 (paperback), Dfl. 163.00, US$ 79.50 (hardback), ISBN 0-444-80275-4 (paperback), 0-444-80254-1 (hardback).

This small book offers more than the title says: not only are the sequencing procedures described with many practical examples, e.g., the specific cleavage procedures are applied to a great number of proteins, but it also deals with many aspects of this topic that are frequently neglected. In addition to the general strategy for sequence determination it describes many types of preliminary characterization of proteins, including such specialized steps as haem and lipid removal. It is, perhaps, not surprising that one can find here a detailed description of advanced methods of amino acid analysis, but one is pleased to find a description of specific modification reactions, determination of molecular weight, determination of specific cleavage reactions (including specific enzymic cleavage) and a survey of the separation and purification of peptides. There is a very complete survey of detection methods for peptides, including detection methods specific for certain amino acid residues. The chapter on the determination of peptide sequences, which is the heart of the book, occupies about 70 pages and is superb by any criteria. The up-to-dateness of the volume is emphasized by about 20 pages devoted to the determination of structures introduced by post-translational modifications. Here the reader can obtain information on the determination of γ-carboxyglutamate, methylated lysines, identification of points of attachment of carbohydrate residues in proteins and cross-linking elements occurring within protein structures.

Proteins exhibiting specific properties, such as membrane proteins, are adequately described.

There are four appendices, all of them important. The first deals with manufacturers and suppliers of equipment and reagents for protein sequence analysis, the second describes the purification of solvents and reagents, the third deals with potential hazards in the protein chemistry laboratory and the last offers some notes on work with very small amounts of proteins and peptides.

In conclusion, for about 20 years Baily's classical book on techniques in

protein chemistry used to be on the shelf of every protein chemist ready for everyday use, but the present volume is likely to replace it in the 1980s.

Prague (Czechoslovakia) ZDENĚK DEYL

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Book Review

Protides of the biological fluids, Colloquium 28, edited by H. Peeters, Pergamon, Oxford, New York, 1980, XXI + 616 pp., price US$ 150.00, ISBN 0-08-026370-4.

The importance of and interest in biological fluid proteins is reflected in these lectures presented at the 28th Colloquium on Protides of the Biological Fluids. The structure of the volume is not strictly uniform, but nobody would expect it to be so. It contains almost 100 research papers which are likely to serve as a rich source of information not only for laboratory workers but also for those more clinically oriented. From the formal point of view most of the papers follow the routine publication scheme: Introduction, Materials and Methods, Results and Discussion, and a few others are based on poster presentations. All of them, however, have a useful abstract.

The Editor has grouped the contributions into three sections: (1) The Evolution of Proteins, (2) The Molecular Biology of Blood Coagulation and Fibrinolysis and (3) The Hybridomas. In many instances the means of obtaining the particular proteins is decisive and this aspect will be of interest to workers in separation science. It is surprising to realize the extent to which conventional procedures dominate this area. There have been virtually no attempts to use modern separation techniques, offering high resolution in spite of the fact that it is the application of these techniques that might help to extend our knowledge in this area. This does not mean that the book is not valuable for people in biomedical science. It simply gives a very clear image of what the situation in the separation of biopolymers is today. Generally, the book would be a good buy, but the contrast between the rather narrow topic and the high price is likely to decrease the number of copies actually sold.

Prague (Czechoslovakia) ZDENĚK DEYL

Vol. 225, No. 1

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY AND MASS SPECTROMETRY IN BIOMEDICAL SCIENCES

The Italian Group for Mass Sepctrometry in Biochemistry and Medicine, in co-operation with the International Scientific Center are organizing an "International Symposium on Chromatography and Mass Spectrometry in Biomedical Sciences" on June 21–23, 1982, in Bordighera, Italy.

The symposium will discuss all the latest aspects of chromatography, mass spectrometry and chromatography–mass spectrometry and their areas of applications, including biochemistry, medicine, toxicology, drug research, forensic sicence, clinical chemistry and pollution. The symposium will consist of presentations from eminent invited speakers and free communications. Facilities will be available for participants to display poster communications.

An instrument and publication exhibition will be held throughout the symposium

For further details, please contact: Dr. Albert Frigerio, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62 – 20157 Milan, Italy. Tel.: 35.43.546; Telex: 331268 NEGRI I.

INTERNATIONAL SYMPOSIUM ON THE SYNTHESIS AND APPLICATIONS OF ISOTOPICALLY LABELED COMPOUNDS

An international symposium on the synthesis and applications of isotopically labeled compounds will be held June 6–11, 1982, at the Hyatt-Regency Hotel, Kansas City, MO, U.S.A. The objective of the symposium is to provide a forum for the exchange of information between leading scientists involved in the synthesis and applications of isotopically (radio-active and stable) labeled compounds. The symposium will include a scientific exhibit. Topics will encompass synthesis, analysis, purification and storage of isotopically labeled compounds and their applications in biomedical, clinical and environmental studies as well as in metabolism, pharmacokinetics, and toxicology. The attendance will be limited to 750 participants.

For further information regarding submission of papers and registration, 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.

CALENDAR OF FORTHCOMING EVENTS

Sept. 20–25, 1981 Philadelphia, PA, U.S.A.	**8th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: Richard J. Knauer, Publicity Chairman, ARMCO INC., P.O. Box 1697, Baltimore, MD 21203, U.S.A.
Sept. 28–Oct. 1, 1981 Barcelona, Spain	**16th International Symposium Advances in Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel (713) 749-2623. (Further details published in Vol. 222, No. 2) (Complete program published in Vol. 209, No. 3)
Sept. 29–Oct. 2, 1981 Basle, Switzerland	**ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry** Contact: D. Gammeter, Secretariat ILMAC '81, Postfach. CH-4021 Basle, Switzerland. Tel. 061 26 20 20. (Further details published in Vol. 212, No. 2)
Oct. 22–23, 1981 Montreux, Switzerland	**Workshop on Liquid Chromatography – Mass Spectroscopy** Contact: Prof. Dr. R.W. Frei, Free University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands (Further details published in Vol. 207, No. 3)
Nov. 9–10, 1981 Berlin, G.F.R.	**Symposium on Practical Aspects of HPLC** Contact: Dr. I. Molnár, Wissenschaftliche Geratebau Dr. H. Knauer GmbH, Hegauer Weg 38, D-1000 Berlin 37, G.F.R. (Further details published in Vol. 207, No. 2)
Nov. 16–17, 1981 Washington, DC, U.S.A.	**The International Symposium on HPLC of Proteins and Peptides** Contact: Shirley E. Schlessinger, Symposium Manager, International Symposium on HPLC of Proteins and Peptides, 400 East Randolph, Chicago, IL 60601, U.S.A. (Further details published in Vol. 208, No. 2)
Nov. 23–25, 1981 Barcelona, Spain	**2nd International Congress on Analytical Techniques in Environmental Chemistry** Contact: Dr. J. Albaigés, General Secretary, Plaza de Espana, Barcelona–4, Spain. Tel: 223–31 01.
Dec. 1–5, 1981 Sãu Paulo, Brazil	**XI Pan American Congress of Pharmacy and Biochemistry** Contact: A.Ph.A. Division of Communications, 2215 Constitution Ave., N.W., Washington, DC 20037, U.S.A.
Dec. 2–3, 1981 Paris, France	**Journées de Chromatographie en Phase Liquide** Contact: H. Colin, Laboratoire C.A.P., Ecole Polytechnique, Route de Saclay, 91128 Palaiseau Cedex, France.
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 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.
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.
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 Mermaid Lane, Philadelphia, PA 19118, U.S.A. (Further details published in Vol. 211, No. 3).

June 21–23, 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.
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. 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. 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. 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)
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

NEW BOOKS

Introduction to high performance liquid chromatography, by R.J. Hamilton and P.A. Sewell, Chapman & Hall, London, **2nd ed.**, 1981, *ca.* 200 pp., price *ca.* £ 12.50, ISBN 0-142-13400-4.

Polymer catalysts and affinants – Polymers in chromatography, edited by B. Sedláček, C.G. Overberger and H.F. Mark, Wiley, Chichester, New York, 1981, 260 pp., price US$ 31.50, £ 14.50, ISBN 0-471-09014-X.

Pharmacological and chemical synonyms, compiled by E.E.J. Marler, Excerpta Medica, Amsterdam, New York, **7th ed.**, 1981, *ca.* 500 pp., price Dfl. 180.00, US$ 87.75, ISBN 0-444-90227-9.

Progress in clinical pharmacy III (Proc. 9th Eur. Symp. Clinical Pharmacy, Helsinki, August 13–16, 1980), edited by H. Turakka and E. van der Kleijn, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1981, XIV + 358 pp., price Dfl. 133.00, US$ 65.00, ISBN 0-444-80338-6.

Reviews in biochemical toxicology III, edited by E. Hodgson, J.R. Bend and R.M. Philpot, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1981, 384 pp., price Dfl. 123.00; US$ 60.00 (outside U.S.A. and Canada); US$ 49.95 (in U.S.A. and Canada), ISBN 0-444-00436-X.

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
Chromatographic Reviews							220/1					220/2		220/3
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2	224/3	225/1	225/2	226/1	226/2

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)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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