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BIOMEDICAL APPLICATIONS

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CHROMBIO. 1065

CHANGES OF ORGANIC ACIDS IN RAT HEART MUSCLE UNDER ISCHEMIC-LIKE CONDITIONS

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(First received May 27th, 1981; revised manuscript received August 12th, 1981)

SUMMARY

Gas chromatographic—mass spectrometric analysis demonstrated the presence of organic acids such as lactic acid, glycolic acid, compounds related to the tricarboxylic acid cycle, fatty acids, and deoxyaldonic acids in rat heart muscle. The variation of these organic acids was examined over a range of time elapsed after decapitation. The results showed that lactic acid, glycolic acid and deoxyaldonic acids of 3-deoxy-2-C-(hydroxymethyl)-tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythro-pentonic acid increased until 4 min after decapitation, but then decreased from 6 min after decapitation. On the other hand, 2-deoxytetronic acid and dideoxypentonic acid markedly increased and unknown peaks appeared on the gas chromatogram from 6 min after decapitation.

INTRODUCTION

Research into heart metabolism has primarily been concerned with energy metabolism. Organic acids, such as lactic acid, pyruvic acid, the tricarboxylic acid cycle-related compounds α-ketoglutaric acid or citric acid, and fatty acids, have been determined in the coronary blood or in the heart muscle of the rat or in patients with ischemic heart disease [1—4]. Research on organic acids that are not related to energy metabolism has been relatively uncommon, which is due, in part, to the difficulty in developing satisfactory quantitative techniques.

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With recent developments in the application of gas chromatography—mass spectrometry (GC—MS) to the medical sciences, it is now possible to analyze simultaneously many compounds in a metabolic system, thus providing an efficient method for investigating the whole metabolic system in question.

We have therefore examined the constituents of organic acids and their related metabolites in normal rat heart muscle after decapitation, using GC—MS. The variation of the organic acids, particularly deoxyaldonic acids, in rat heart muscle under pathological conditions was investigated.

MATERIALS AND METHODS

Chemicals

Lactic acid, glycolic acid, glycerol, succinic acid, fumaric acid, palmitic acid and stearic acid were commercial products. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Tokyo Kasei Co. (Tokyo, Japan). All other reagents were of the highest purity available commercially.

Gas chromatography and gas chromatography—mass spectrometry

A Shimadzu GC-6A gas chromatograph with dual flame ionization detectors was used. A glass coiled column (2 m × 3 mm I.D.) was packed with 3% OV-17 on Gas-Chrom Q (80—100 mesh). The column oven was maintained isothermally at 80°C for 2 min and then programmed at 6°C/min until 290°C. Peak areas and retention times were determined with an on-line Shimadzu Chromatopac 4-B computer equipped with a printer. For identification of the compounds, a JEOL JMS-D 100 GC—MS system with an on-line JMA 2000 data acquisition system was used.

Mass spectra were recorded at an ionizing voltage of 75 eV with a 300-μA trap current, and ion source temperature of 280°C. The magnet of the mass spectrometer was scanned repetitively over field strengths from m/z 50 to m/z 700 every 5 sec.

Sample preparation

Rats (250—300 g) of the Sprague—Dawley strain bred with commercial foods and water ad libitum were decapitated, and their hearts were immediately excised and frozen in dry ice—acetone. In order to examine the variation of the organic acids with time elapsed after decapitation, hearts were obtained at 2, 4, 6, 10, 15 and 30 min, and then frozen. One hundred milligrams were removed from each specimen, minced with scissors in cold saline solution and homogenized with 500 μl of the saline solution using a Potter homogenizer. Prior to deproteinization with 3 ml of 99% cold ethanol, protein in the heart muscle was determined by the Bio-Rad protein assay method. Heptadecanoic acid (20 μg/mg of protein) was added to the homogenate as an internal standard. The samples were centrifuged at 25,000 g for 10 min. The precipitate was washed once with cold ethanol to extract any remaining soluble material and centrifuged again. The second supernatant was combined with the first supernatant.

The supernatant of each ethanol-treated specimen was concentrated to 0.5 ml to remove the ethanol. Distilled water, 3 ml, was added to the residue.

The solution was acidified to pH 1 with 2 *N* HCl. Organic acid fractions were obtained by extraction with equal volumes of diethyl ether and ethyl acetate twice. Organic solvent extracts were dried over anhydrous sodium sulphate for 1—2 h. The extracts were concentrated to 1 ml using an evaporator and then dried under a stream of nitrogen.

The samples were trimethylsilylated by adding 200 μl of BSTFA to the residue; the mixtures were then heated to 60°C for 1 h in glass tubes with PTFE-covered screw caps. Aliquots of the samples were subjected to GC and GC—MS analysis.

RESULTS

The profile of organic acids in 100 mg of rat heart muscle is shown in Fig. 1. Over 40 peaks were detected on the gas chromatogram. The peak

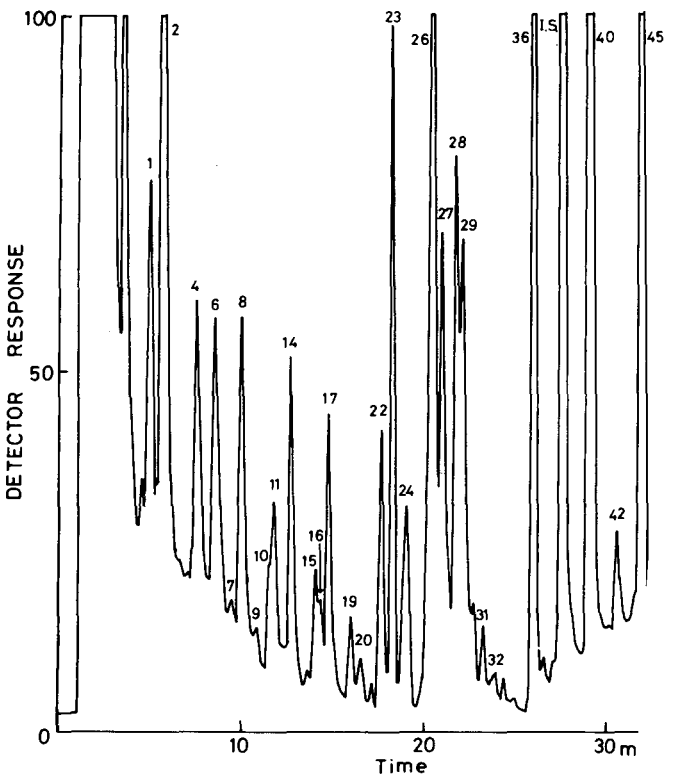

Fig. 1. Gas chromatogram of trimethylsilyl (TMS) derivatives of organic acids obtained from 100 mg of rat heart muscle, excised and frozen within 30 sec. The peaks were identified as follows: (1) lactic acid, (2) glycolic acid, (4) 3-hydroxypropionic acid, (7) glycerol, (9) 2-methylglyceric acid, (10) phosphoric acid, (11) glyceric acid, (14) succinic acid + fumaric acid, (15) 3-deoxytetronic acid, (16) 2-deoxytetronic acid, (17) 3-deoxy-2-C-(hydroxymethyl)tetrono-1,4-lactone, (19) malic acid, (20) dideoxypentonic acid, (22) 3-deoxy-2-C-(hydroxymethyl)tetronic acid, (23) 3-deoxypentono-1,4-lactone, (24) 3-deoxy-erythropentonic acid, (26) 3-deoxy-2-C-(hydroxymethyl)pentono-1,4-lactone, (27) β-glycerophosphoric acid, (28) α-glycerophosphoric acid + 3-deoxy-2-C-(hydrocymethyl)-erythropentonic acid, (36) palmitic acid, (40) stearic acid.

which appears between peaks 36 and 40 is the internal standard, heptadecanoic acid. The ratio of each peak area to that of the internal standard was calculated, and the deviations of each peak from ten rat heart muscles were examined. The deviations of all peaks except peaks 6 and 27 were within 100% (data not shown). The profile of organic acids in rat heart muscle is therefore relatively stable.

Identification of these peaks was performed by comparing their mass spectra and retention times with those of laboratory samples or from literature references. A mass spectrum obtained from peak 24 is shown as an example (Fig. 2). The molecular ion at *m/z* 438 was not detected but ion at *m/z* 423 (M — 15) was found as a relatively small peak. Other fragment ions, ion at *m/z* 333 (M — 15 — 90), ion at *m/z* 245 (335 — 90) and at *m/z* 231 (321 — 90), were detected. The base peak was observed at *m/z* 73. This mass spectrum and the retention time were consistent with that previously reported [5]. Peak 24 was, therefore, identified as 3-deoxyerythropentonic acid tetra-trimethylsilyl derivative. In this way, each peak was identified and detection of various deoxyaldonic acids were made. The compounds identified are shown in the legend of Fig. 1.

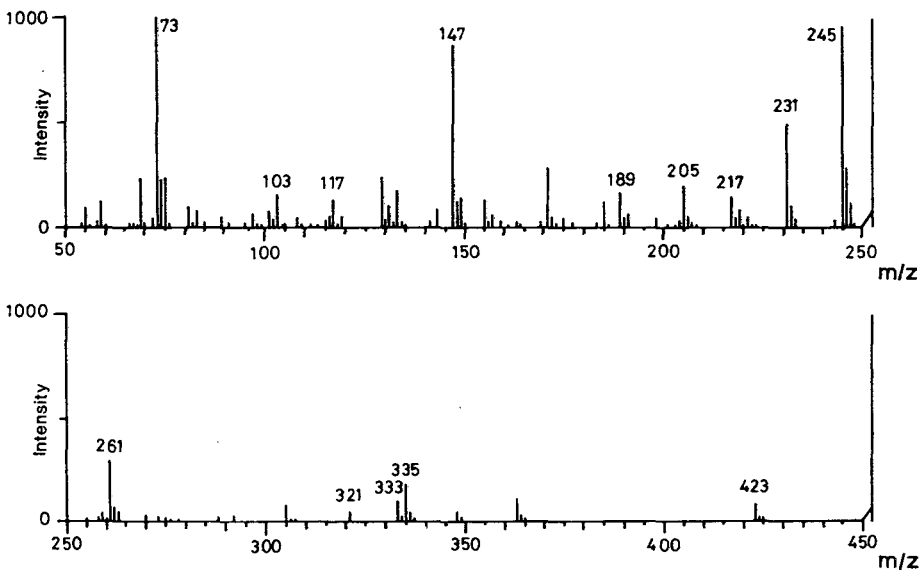

Fig. 2. Mass spectrum of the TMS derivative of 3-deoxyerythropentonic acid, which was obtained from peak 24 in Fig. 1.

The variation of these organic acids detected in rat heart muscle was examined by the time elapsed after decapitation to determine the metabolic change. The gas chromatogram of organic acids detected in heart muscle obtained 4 min after decapitation is shown in Fig. 3. Almost all peaks were increased in intensity compared with the control (analyzed within 30 sec of decapitation), especially glycolic acid, 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid and fatty acids (as indicated by arrows).

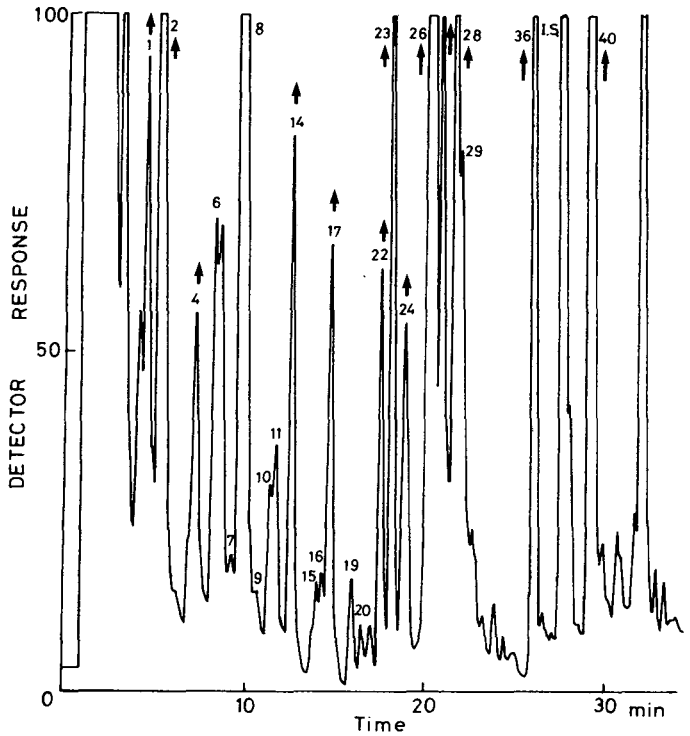

Fig. 3. Gas chromatogram of TMS derivatives of organic acids from 100 mg of rat heart muscle excised and frozen at 4 min after decapitation. The peak numbers in the figure correspond to those in Fig. 1.

It was observed that the heart had virtually stopped beating and coronary flow was at a standstill 6 min after decapitation.

The gas chromatogram obtained from the experiment of 10 min elapsed is shown in Fig. 4. Most peaks including 3-deoxy-2-C-(hydroxymethyl)tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid (as indicated by arrows) had decreased when compared to Fig. 1. Only two deoxyaldonic acids, 2-deoxytetronic acid (peak 16) and dideoxypentonic acid (peak 20), however, had markedly increased; in addition, unknown peaks appeared at the position of peaks 24, 28, 32 and 36. The mass spectra of these unknown peaks have almost the same fragment ions but different molecular ions with differences of 74 mass units, i.e. M^+ = 444, 518, 592 and 666. These data suggest that the unknown peaks may be homologous compounds.

The variation of these deoxyaldonic acids with time elapsed after decapitation is tabulated in Fig. 5. It can be seen that the three deoxyaldonic acids — 3-deoxy-2-C-(hydroxymethyl)tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid — increased in heart muscle until 4 min, but decreased markedly from 6 min after decapitation. Both 2-deoxytetronic acid and dideoxypentonic acid, however, increased from 6 min elapsed after decapitation.

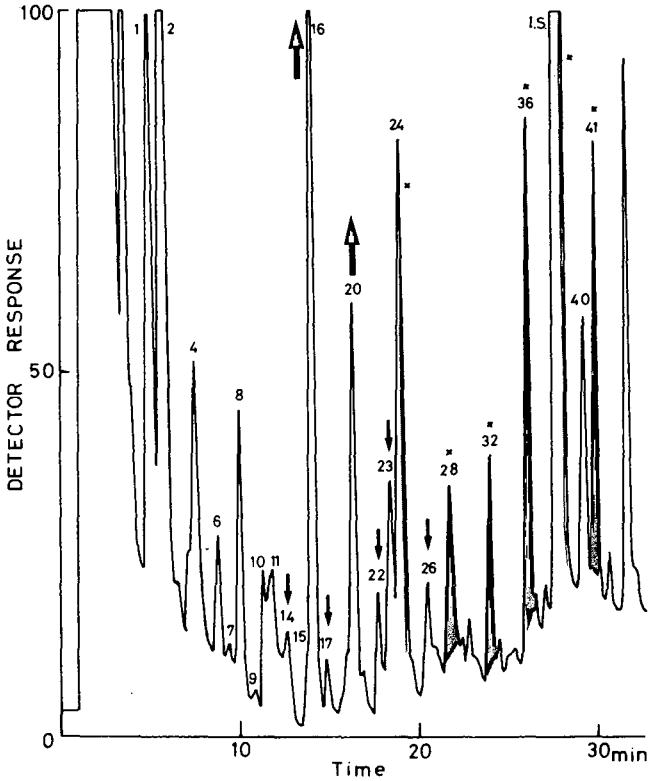

Fig. 4. Gas chromatogram of TMS derivatives of organic acids from 100 mg of rat heart muscle excised and frozen at 10 min after decapitation. The peak numbers in this figure correspond to those in Figs. 1 and 3.

DISCUSSION

GC—MS has been used successfully in profiling organic acids in various fluids and tissues (brain and heart muscle), and to identify compounds which are well separated by a packed column. More peaks would be separated by using a capillary column, but it is not suitable for profiling because of poor reproducibility. Previously profiling of organic acids has been successfully performed using a packed column [6—8]. The packed column was therefore used here for the separation of organic acids in heart muscle.

It is well known that heart muscle utilizes fatty acids in preference to glucose as an energy source. The evidence that palmitic acid and stearic acid were detected in large amounts on the gas chromatogram, further confirms the importance of fatty acids in the energy metabolism of heart muscle.

Deoxyaldonic acids have been detected by GC—MS analysis in biological fluids such as serum, urine and amniotic fluid, as well as in rat brain tissue [9—11]. The detection of the deoxyaldonic acids 3-deoxytetronic acid, 2-deoxytetronic acid, dideoxypentonic acid, 3-deoxy-2-C-(hydroxymethyl)-tetronic acid, 3-deoxyerythropentonic acid, 3-deoxy-2-C-(hydroxymethyl)-erythropentonic acid and their lactones in heart muscle has been reported

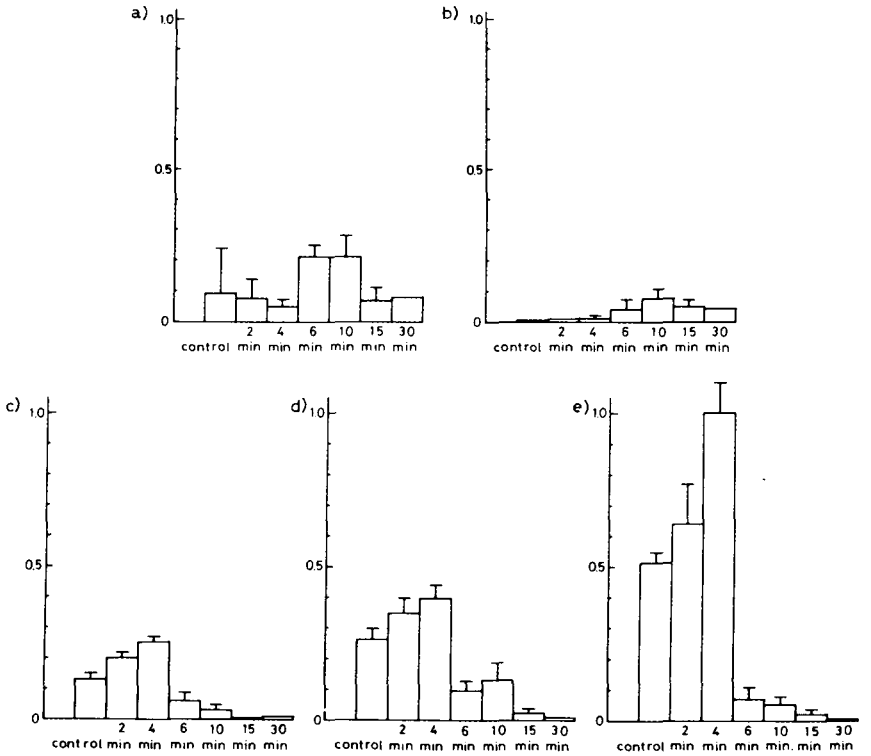

Fig. 5. The changes in deoxyaldonic acids after decapitation. Each bar represents data averaged from three specimens. The abscissa represents 2 min, 4 min, 6 min, 10 min, 15 min and 30 min time elapsed, and the ordinate represents peak area ratio with respect to an internal standard. (a) 2-Deoxytetronic acid, (b) dideoxypentonic acid, (c) 3-deoxy-2-C-(hydroxymethyl)tetronic acid, (d) 3-deoxyerythropentonic acid, (e) 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid.

[12]. Neither the function nor the metabolism of deoxyaldonic acids, however, is clearly understood. Fell et al. [13] have recently determined 2-deoxytetronic acid in urine and plasma. They recommended that alkaline conditions should be avoided during sample preparation because 2-deoxytetronic acid can be formed by the action of alkali on certain sugars such as maltose. As our extraction procedure was not performed under basic conditions, we believe that 2-deoxytetronic acid in heart muscle is a bona fide metabolite and not an artifact of sample preparation.

Lawson et al. [6] noted that urinary excretion of 2-deoxytetronic acid changed more than that of the other deoxytetronic acids in an experiment in which a high glucose diet was given. They therefore concluded that the immediate metabolic precursors of this compound are more closely related to glucose than are those of the other deoxytetronic acids. Fell et al. also indicated that 2-deoxytetronic acid in urine may largely result from chemical degradation of body carbohydrate and may possibly be a metabolite of γ-hydroxybutyric acid by β-oxidation. Lawson et al. [6] considered that as the amounts of aldonic acid and deoxyaldonic acid found in urine, other

than 2-deoxytetronic acid, change little in association with large fluctuations in the composition of the diet, they may be mainly endogenous. It is known that oxidation of pentoses and deoxypentoses may yield pentonic and 2-deoxypentonic acids, and ascorbic acid is metabolized via threonic acid as well as via L-lyxonic acid and L-xylonic acid (pentonic acid). It is therefore presumed that certain deoxyaldonic acids detected in rat heart muscle may be derived from carbohydrate or be concerned with glucose metabolism, especially from the pentose phosphate shunt, although this has not been elucidated. However, little is known about the origin of 3-deoxy-2-C-(hydroxymethyl)aldonic groups at present.

Lactonization is a potential problem when measuring acids that contain a hydroxy group at the 4 carbon position. As Horning and Horning [7] identified the tetronic acid lactones in solvent (ethyl acetate and ether) extracts of acidified urine and Thompson et al. [8] noted that lactones were formed under acidic conditions, it is believed that lactones detected in heart muscle are also formed under acidic conditions, with which the extraction of organic acids was performed.

It is presumed that the supply of oxygen and coronary blood flow are severely reduced at 4 min after decapitation, although specific measurements were not made. Lactic acid and glycolic acid were increased, and it was observed that the heart was remarkably bradycardic at the time. It is reasonable to assume, therefore, that the whole heart muscle was in an ischemic condition at this time lapse, although this experimental ischemic condition is different from regional ischemia, so-called "ischemic heart disease". The peak of glycolic acid is always detected as a larger peak than that of lactic acid, which is regarded as one of the indexes of ischemia. This suggests that glycolic acid may also be an index of ischemic heart, as it changes in parallel to lactic acid. It is presumed that glycolic acid may be formed from glyoxylic acid by the same kind of enzymatic reaction as that of lactate formation. It was observed that peaks of deoxyaldonic acids of 3-deoxy-2-C-(hydroxymethyl)tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid were increased at 4 min time lapse. These results suggest that these three deoxyaldonic acids may also accumulate in ischemic heart muscle, although the degree of hypoxia and ischemia was not precisely determined.

It was observed that the heart stopped beating and coronary flow was at a standstill 6 min after decapitation. Two aldonic acids — 2-deoxytetronic acid and dideoxypentonic acid — had clearly increased, the three deoxyaldonic acids mentioned above decreased significantly and unknown peaks appeared. Although the results can not be fully explained at present, it may be of value in the future to understand why 2-deoxytetronic acid and dideoxypentonic acid temporarily accumulate, and to determine the unidentified compounds produced in early dead (infarcted) heart muscle.

It is presumed, however, that the cause of the variation in these deoxyaldonic acids resulting from reduced coronary blood flow might be related to glucose metabolism. As the metabolism of deoxyaldonic acid has not yet been elucidated, we await future research in this field.

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CHROMBIO. 1067

SEPARATION OF SOME NATURAL AND SYNTHETIC CORTICOSTEROIDS IN BIOLOGICAL FLUIDS AND TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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(First received May 20th, 1981; revised manuscript received August 17th, 1981)

SUMMARY

A high-performance liquid chromatography (HPLC) technique was developed for the determination of radiolabeled triamcinolone acetonide (TAC), cortisol and their metabolites in rhesus monkey plasma, urine and tissue samples. After protein precipitation, the parent compounds and metabolites were simultaneously resolved using a single-column reversed-phase HPLC system. TAC was subsequently verified by mass spectrometry and TAC glucuronide was tentatively identified by enzymatic hydrolysis and mass spectrometry of the hydrolysis product. The endogenous hormones, cortisol and cortisone were presumptively identified by cochromatography with authentic standards on two different HPLC systems and positively identified by reverse-isotope recrystallization. Other metabolites of both compounds were detected by selective enzymatic hydrolysis and HPLC. This method is rapid and reproducible with a total recovery >80%.

*This work was presented in part to the Teratology Society on June 11, 1980, in Portsmouth, NH, U.S.A.

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INTRODUCTION

Cleft palate has been produced in the offspring of a number of species by administration of either cortisol or triamcinolone acetonide (TAC) to the pregnant female [1—9]. In addition, a characteristic pattern of craniofacial malformation results from maternal TAC treatment during early gestation plus thymic involution and growth retardation from treatment during middle and late gestation in nonhuman primates [9—11]. The mechanisms by which these teratogens manifest themselves are unknown. It has been suggested that the unmetabolized drug may be the actual teratogen [12] and that embryo exposure to parent glucocorticoid may be altered due to maternal metabolism and detoxification as demonstrated in corticosteroid-resistant mouse strains [13].

To gain a better understanding of the mechanism of action of this class of hormones, metabolic studies were initiated in order to elucidate the complete metabolic profile of the compounds in question. Sensitive, specific and reproducible analytical techniques are a prerequisite to solving these problems.

Recently, high-performance liquid chromatography (HPLC) techniques have been developed for corticosteroid determination in biological samples [14—20]. However, these methods are neither specific nor useful for separation of the more polar metabolites along with the non-polar compounds. This report describes an HPLC method for separation of cortisol, cortisol glucuronide, 6β-hydroxycortisol, cortisone, cortexolone, triamcinolone (TA) and triamcinolone acetonide (TAC) and demonstrates the use of this method for the simultaneous assay of radiolabeled conjugated and non-conjugated corticosteroids in urine, plasma, and tissue.

EXPERIMENTAL

Instrumentation

The Waters Assoc. (Milford, MA, U.S.A.) HPLC system consisted of a continuous flow, constant volume Model 6000A solvent delivery system equipped with a U6K universal loop injector governed by a Model 660 solvent programmer. UV-absorbing materials passed through the 8-μl flow-cell of a Model 440 UV spectrophotometer set at a fixed wavelength of 254 nm and absorption was traced with a Fisher Recordall Series 5000 (Fisher Scientific, Springfield, NJ, U.S.A.). Fractions were collected with an ISCO Golden Retriever (Instrumentation Specialties, Lincoln, NE, U.S.A.). All radioactivity was counted in a Tracor Analytic Mark III liquid scintillation system Model 6881 (Tracor Analytic, Atlanta, GA, U.S.A.).

Chemicals

Radioisotopes [4-^{14}C]cortisol (2032.04 MBq or 54.92 mCi/m*M*) and [6,7-^{3}H(N)]triamcinolone acetonide (1339.4 GBq or 36.20 Ci/m*M*) were purchased from New England Nuclear (Boston, MA, U.S.A.) and purified on a 5-μm LiChrosorb RP_{18} column using the system described in Methods. After purification they were considered to be > 98% pure based on radioactivity.

The unlabeled triamcinolone acetonide (Kenalog®) for injection was ob-

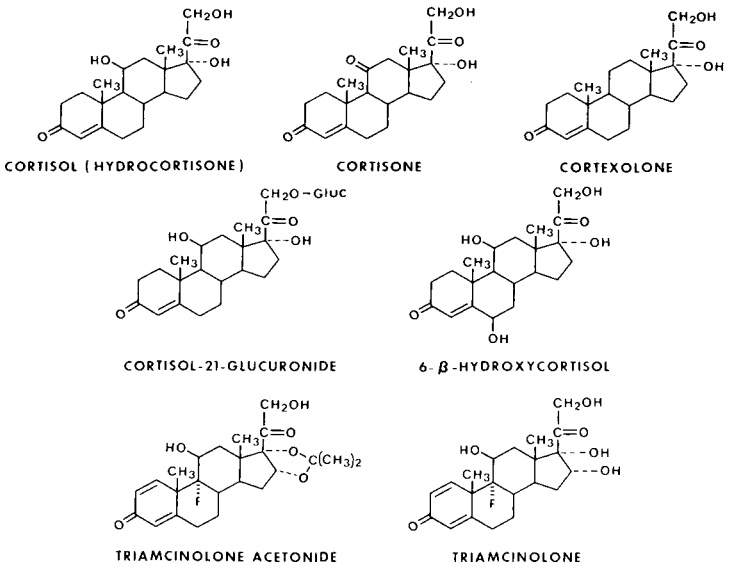

Fig. 1. Structures of cortisol, triamcinolone acetonide and analogues separated by HPLC.

tained from Squibb (Princeton, NJ, U.S.A.) and used as received. No UV (254 nm) absorbing contaminants were found with HPLC analysis.

The other unlabeled derivatives were purchased as listed: triamcinolone acetonide (TAC), triamcinolone (TA) and cortisol (hydrocortisone) from Sigma (St. Louis, MO, U.S.A.); cortisone and cortexolone (11-desoxycortisol) from Research Plus Steroid Labs. (Denville, NJ, U.S.A.) and cortisol-21-glucuronide and 6β-hydroxycortisol from Steraloids (Wilton, NH, U.S.A.). Conformation was verified by mass spectrometry. The structures of these compounds are shown in Fig. 1.

Bovine liver β-glucuronidase Type I and *Helix pomatia* β-glucuronidase with sulfatase activity Type H-1 were obtained from Sigma.

HPLC systems

HPLC System A consisted of a 250 × 10 mm I.D. stainless-steel column commercially packed with 5 μm LiChrosorb RP-18 purchased from Chrompac (Whittier, CA, U.S.A.). A 70 × 6 mm stainless-steel guard column packed with 35—50 μm Bondapak C_{18} Corasil (Waters Assoc.) was connected between the injector and the column. The guard column did not diminish the efficiency or resolution of the main column. At ambient temperature, a convex gradient (No. 5 Waters 660 programmer) was set at a flow-rate of 1.5 ml/min. The initial conditions were methanol—0.01 *M* ammonium acetate, pH 6.9 (10:90) and the final conditions were 100% methanol. Pump pressure never exceeded 136.05 bar.

HPLC System B consisted of two μBondapak C_{18} columns (Waters Assoc.) connected in series. They were each 300 × 3.9 mm, 10 μm particle size, and run isocratically with methanol—water (45:55) at 1 ml/min.

HPLC solvents and chemicals

The organic solvents used were distilled-in-glass quality from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was purified through a Millipore Milli-Q-system (Bedford, MA, U.S.A.) fed by a deionized water source. Ultrapure ammonium acetate (Mallinckrodt, St. Louis, MO, U.S.A.) was used as a solvent buffer. All aqueous solvents were filtered through a Millipore BDWP 0.6-μm filter and degassed prior to use.

METHODS

Urine collection and preparation

A 23 μg/kg dose of [4-^{14}C]cortisol (740 kBq) and a 0.085 μg/kg dose of [6,7-^{3}H]TAC (1480 kBq) plus 10 mg/kg unlabeled TAC in suspension was administered intramuscularly to a pregnant rhesus monkey gestational age 134 days. Urine was collected via a Foley catheter for 4 h after drug administration. Proteins and salts were precipitated by the addition of 1 volume of methanol—ethanol (1:1) to 1-ml aliquots of urine. The samples were centrifuged for 10 min at 4000 *g*. The supernatant was transferred to a 5-ml conical test tube and reduced under a stream of nitrogen at 37°C to approximately 0.2 ml for subsequent HPLC analysis.

Blood collection and preparation

Blood samples were collected through a femoral artery catheter and immediately centrifuged to separate the plasma from the red blood cells. The plasma

TABLE I

RETENTION TIME AND RECOVERY OF STANDARDS ON HPLC SYSTEM A

Standard	Amount injected* (μg)	Retention time (min)	Recovery (%)
6β-Hydroxycortisol	5 (5 μl)	17.98	96.6
Cortisol glucuronide	20 (20 μl)	23.31	100.0
TA	10 (20 μl)	25.95	80.35
Cortisone	5 (10 μl)	29.39	92.8
Cortisol	10 (10 μl)	31.07	100.0
TAC	10 (20 μl)	33.99	93.1
Cortexolone	5 (10 μl)	34.75	84.8

*The same amounts were added to urine, plasma and tissue samples.

was transferred to a 10-ml graduated test tube and the standards (Table I) were added. An equal volume of methanol—ethanol (1:1) was introduced to precipitate the protein and the sample was then refrigerated (−20°C) for 30 min or overnight. After refrigeration, the samples were centrifuged in a table top centrifuge for 10 min at 1500 *g*. The precipitate was washed two more times with an equal volume of methanol—ethanol (1:1). The pooled supernatants were transferred to a 5-ml conical test tube and reduced to dryness under nitrogen at 37°C. The residue was resuspended in 0.2 ml methanol—water (65:35) for HPLC analysis.

Tissue extraction and preparation

Fetal tissue samples were collected 5 h after administration of the dose to the maternal monkey. Tissue samples were weighed and placed in wide mouthed screw top plastic bottles; to these, 10—15 ml methanol—dimethoxymethane (1:1) plus standards (Table I) were added. The samples were then homogenized with a Polytron (Brinkman, Westbury, NY, U.S.A.) for 1 min or until breakup was complete. They were then placed in a 37°C shaker bath and the extraction allowed to proceed overnight. The suspension was then centrifuged for 5 min at 4000 *g* in a Damon IEC Model PR6000 centrifuge (Curtin Matheson, Houston, TX, U.S.A.) with a four place horizontal rotor IEC 284 pin type. The supernatant was then filtered using Whatman No. 1 filter paper. The tissue precipitate was resuspended with methanol—dimethoxymethane (1:1) and refiltered. Washing of the precipitate was done on the filter at this time with methanol. The collected filtrate was measured and 10% of the volume removed to a scintillation vial, taken to dryness and counted to determine the amount of radioactive compounds present. The remaining filtrate was taken to dryness under nitrogen and the residue resuspended with up to 0.5 ml methanol—water (65:35). The precipitate, if any, was removed by centrifugation and the resulting supernatant was ready for HPLC analysis. The tissue residue left on the filter paper was allowed to dry. A portion was later combusted in an Oxymat (Intertechnique, Plaisir, France) and the amount of non-extractable radioactivity left in the tissue residue was determined to be between 2 and 4%.

Chemical identification

Mass spectrometric identification of underivatized components separated by HPLC was performed on a Finnigan 4023 mass spectrometer combined with an Incos data system. Samples were deposited into glass sample cups (5 μl volume), evaporated to dryness with a stream of dry helium, and introduced into the mass spectrometer via the solid probe. The probe was ballistically heated to 400°C. Data were collected throughout the heating cycle. For greater sensitivity, the mass spectrometer was operated in the multiple specific ion mode. Ionization was accomplished in conventional electron-impact (EI) mode.

Sample compounds to be recrystallized were brought to constant specific activity with 50 mg of authentic carrier steroid using the solvent pair methylene chloride—isooctane. After the consecutive crystallizations, the specific activity of the crystals was ±5% of the average of the three final values [21].

The peaks considered to be metabolites of cortisol and TAC were resuspended in aqueous 0.2 *M* sodium acetate buffer, pH 5. They were incubated with bovine enzyme and *Helix pomatia* (HP) by dividing the peaks into three aliquots (20% for control, i.e. no enzyme, 40% bovine and 40% HP). One ml of enzyme solution (10,000 units/ml 0.2 *M* sodium acetate buffer, pH 5) was added to the appropriate aliquot and the samples were incubated at 37°C in a shaker bath for 4 h. An equal volume of methanol—ethanol (1:1) was introduced to stop the reaction and the samples were immediately frozen. After thawing, any precipitate was centrifuged out and the supernatant taken to dryness under nitrogen. The residue was resuspended in 400 μl methanol—water (65:35) and analyzed on the described HPLC system A.

RESULTS

Corticosteroid standards

Reversed-phase chromatography on HPLC system A provided a high degree of resolution and separation for the corticosteroid standards (cortisol, cortisone, cortexolone, cortisol glucuronide, 6β-hydroxycortisol, TAC and TA) even though they encompass a wide polarity range. The standards were initially injected individually to establish characteristic retention times. A chromatogram of the standards injected simultaneously is shown in Fig. 2. In order to determine column efficiency, the standards were injected on the column, collected, reduced in volume and reinjected on the same column. The percent recovery was determined by comparing the UV peak areas for each standard. The retention times and percent recovery on the LiChrosorb column are shown in Table I for the standards.

To remove highly pigmented material from samples such as urine or tissue, the sample was injected onto the column at initial conditions but the program was not initiated for a period of 20 min, thus allowing much of the water-soluble pigments to be eluted even before starting the program. This method provided cleaner peak fractions and allowed peak character to be demonstrated on the UV trace.

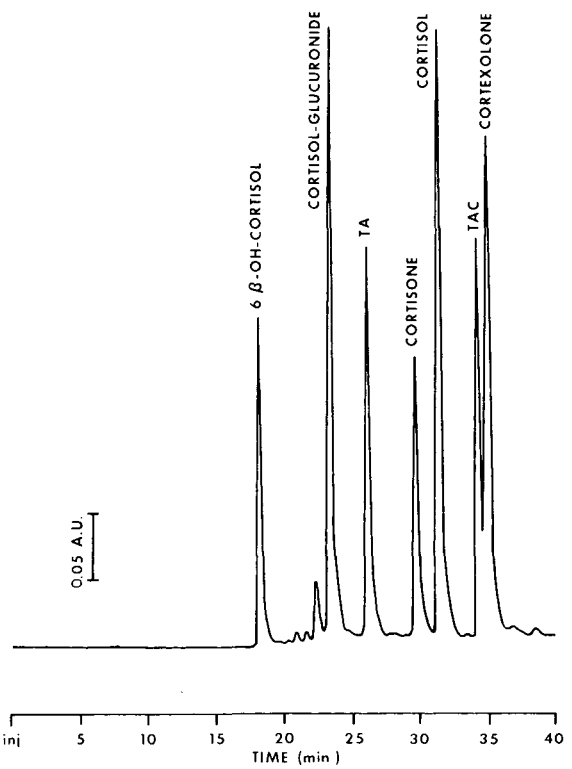

Fig. 2. HPLC profile of corticosteroid standards (HPLC system A). Chromatographic conditions: column, LiChrosorb RP-18, 5 μm, 250 × 10 mm. Mobile phase: methanol—water (0.01 *M* ammonium acetate) (10:90) to 100% methanol, convex gradient elution (Waters program No. 5) in 50 min; flow-rate 1.5 ml/min; sample size, as in Table I.

It was noticed that continued application of highly pigmented, lipid-containing samples to the column resulted in poor resolution of standards. It was discovered that if chloroform was pumped through the column at 0.3 ml/min overnight or preferably, over a weekend, the ability of the column to resolve the standards returned. Transition to and from chloroform was made directly from final conditions (100% methanol).

Urinary metabolites

The urine collected from the maternal monkey at the 60 min time point was used for analysis because of the high concentration of radioactivity. Five aliquots of 1 ml each were processed as described under Methods and applied to HPLC system A. The five separate injections were made in order to prevent overloading the column and to estimate the reproducibility of the method. Multiple injections were necessary to collect enough compound per peak for further analysis. Aliquots (50 μl) from the 0.75-ml fractions were counted by liquid scintillation and as Fig. 3 shows, the parent compounds and metabolites are designated according to retention time and radioactivity. These peaks were pooled individually and the percent radioactivity in each peak as compared to total radioactivity in all peaks was calculated (Table II).

The peaks which cochromatographed with cortisone, cortisol and TAC were resuspended in 400 μl methanol—water (65:35) and rechromatographed on HPLC system A for further cleanup. Finally they were collected, pooled and taken to dryness. The identity of TAC was verified by mass spectrometric com-

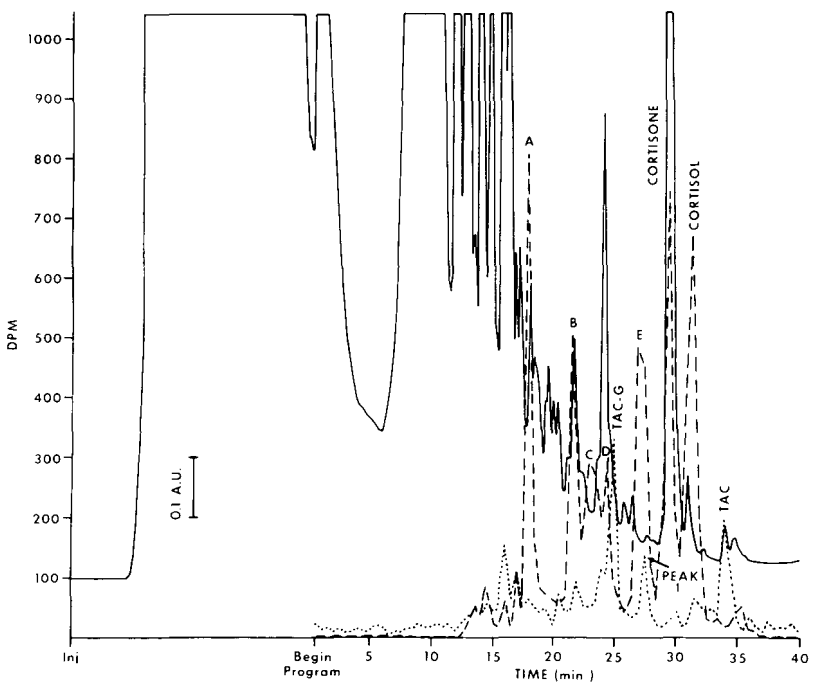

Fig. 3. HPLC profile of urinary metabolites. Chromatographic conditions: same as in Fig. 2, except that standards were not added. · · · , ^{3}H; — — —, ^{14}C; ——, UV at 254 nm.

TABLE II

COMPARISON OF RETENTION TIMES AND RECOVERY OF INDIVIDUAL HPLC-RESOLVED RADIOLABELED COMPOUNDS

Data compiled from five separate aliquots of urine at 60 min. Values are means ± S.E.

Compound	Retention time (min)	Percentage*	Coefficient of variation
^{3}H			
TAC-G	25.1 ± 0.10	52.98 ± 0.94	3.96
TAC-peak 1	27.5 ± 0.16	12.97 ± 0.38	6.55
TAC	34.2 ± 0.12	34.05 ± 0.88	5.78
^{14}C			
Peak A	18.2 ± 0.12	15.71 ± 0.57	8.21
Peak B	21.6 ± 0.19	11.47 ± 0.70	13.68
Peak C	23.2 ± 0.12	6.37 ± 1.08	37.99
Peak D	24.3 ± 0.12	9.54 ± 1.40	32.91
Peak E	27.4 ± 0.19	17.54 ± 0.99	12.65
Cortisone	29.7 ± 0.12	18.12 ± 0.44	5.40
Cortisol	31.2 ± 0.12	21.23 ± 0.60	6.35

*Percentage compound of total compounds resolved.

parison to a TAC authentic standard, both having ions at 434 (molecular ion), 413 (M–HF), and 375 (base peak).

Because cortisol and cortisone are endogenous compounds, mass spectrometry was not used as a means for further verification. Alternatively, they were chromatographed on HPLC system B. One milliliter fractions were collected and aliquots counted. Radiolabeled compound thought to be cortisol comigrated with the standard. Radioactivity thought to be cortisone clearly showed two peaks, one of which comigrated with the cortisone standard. These peaks were collected and subjected to reverse-isotope recrystallization. Over 95% of the cortisol was identified as such by this method. Sixty percent of the initial HPLC resolved cortisone peak was identified as cortisone coinciding with the results obtained from HPLC system B which indicated two peaks. Therefore, the original peak on HPLC system A, which comigrated with the cortisone standard contained two compounds, cortisone and an unknown metabolite.

The polar metabolite peaks were subjected to enzyme hydrolysis as described. Although the results varied between peaks, the profiles for each peak by both enzymes were the same suggesting that none of the metabolites were sulfates. The evidence for only glucuronidation was strengthened by the percent hydrolysis data shown in Table III. The percent conversion for each enzyme is almost the same indicating that the sulfatase in the *Helix pomatia* enzyme provided no additional liberation of free steroid over what was liberated by β-glucuronidase alone.

The peak labeled TAC-G was presumed to be a glucuronide of TAC because it had the same retention time as the TAC-G produced and isolated from a liver microsomal enzyme preparation using [^{3}H]TAC and UDPGA [22]. TAC-G was predominantly hydrolyzed to TAC (Table III), and the liberated TAC was identified by mass spectrometry. TAC peak 1 exhibited virtually no conversion back to TAC. Its relative retention time is consistent with the TAC metabolite,

TABLE III

COMPARISON OF THE HYDROLYZING CAPABILITY OF TWO DIFFERENT ENZYMES ON URINARY METABOLITES

Percent conversion to less polar metabolites.

Metabolite peak	Treatment		
	Control	Bovine enzyme	*Helix pomatia*
TAC-G	0	66.9	76
TAC-peak 1	0	12.5	11
Peak A	67	–	67
Peak B	0	84.5	83.7
Peak C	0	62.2	62.9
Peak D	0	46.1	48.7
Peak E	0	100	94.5

6β-hydroxy-TAC (6β-OH-TAC) which has been cited as a major metabolite of TAC [23—27]. Mass spectrometric analysis revealed the base peak at 391 *m/z* (375+16) which is consistent with the addition of one hydroxyl group to TAC.

The enzymatic hydrolysis products of cortisol-derived material were analysed by HPLC. The controls except for peak A, showed no conversion and appeared as well defined parent peaks. Peak A control had the same profile as with enzyme added, indicating that spontaneous breakdown had occurred in the buffer system alone. Neither peak A nor its degradation products comigrated with any standard. Among the substances liberated from peaks B and C were compounds comigrating with cortisone and cortisol. Intact B and C did not migrate with available standards. Peak D was tentatively identified as cortisol-21-glucuronide because it comigrated with the authentic standard in HPLC System A and upon β-glucuronidase hydrolysis, liberated a compound comigrating with cortisol. Peak E was converted to a much less polar metabolite which chromatographed in the same region as cortexolone.

From these data, it can be said that the major urinary metabolites of TAC and cortisol are glucuronides with one of the cortisol metabolites tentatively identified as cortisol-21-glucuronide. TAC metabolism, although limited, produces a glucuronide of TAC (TAC-21-glucuronide) and probably 6β-OH-TAC.

Plasma metabolites

A 10-min plasma sample was processed as described and its HPLC metabolite profile was compared with that of a 60-min urine sample. Fig. 4 shows that the plasma profile compared favorably with the urinary profile. It consisted of radiolabeled peaks comigrating with cortisol, cortisone, TAC and several conjugates. The cortisone peak produced only one definitive peak which comigrated with the cortisone standard in both HPLC systems. A new peak was noted having a retention time of 35 min, the same as cortexolone. This nonpolar ^{14}C-peak was collected and chromatographed on HPLC system B, but failed to migrate with either cortexolone or corticosterone thereby eliminating the possibility of it being either of those compounds. Insufficient material was available for further analysis.

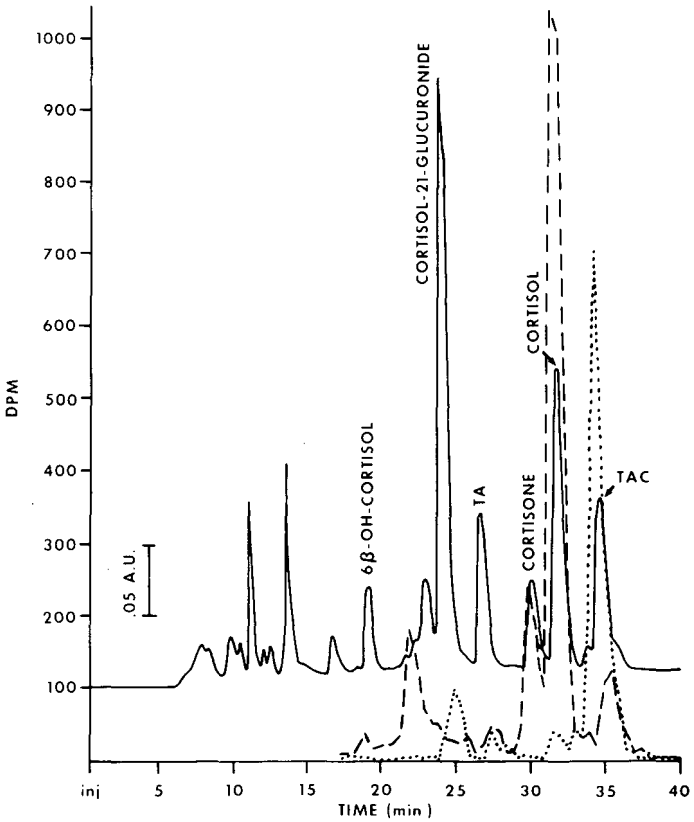

Fig. 4. HPLC profile of plasma metabolites. Chromatographic conditions: as in Fig. 2. · · ·, ^{3}H; — — —, ^{14}C; ——, UV at 254 nm.

Tissue metabolites

Five hours after the [^{14}C]-cortisol—[^{3}H]-triamcinolone acetonide dose was administered, the fetal liver tissue sample was collected and analyzed as described. The resulting chromatogram, Fig. 5, defines the TAC peak and at the same time indicates that cortisol has been metabolized to a variety of products, some of which chromatograph in the same areas as the urinary peaks B, C, E and cortisone. As with the plasma samples, there was not sufficient compound present for further analysis.

DISCUSSION

These results demonstrate that this HPLC methodology is capable of reproducibly separating both natural and synthetic corticosteroids and their metabolites from urine, plasma, and tissue. TAC was definitively identified via mass spectrometry as were cortisol and cortisone by reverse-isotope recrystallization. Evidence was obtained for the tentative identification of TAC glucuronide, 6β-OH-TAC, cortisol-21-glucuronide and several other cortisol derived glucuronide metabolites. Previous methods [23,28] utilized various extraction

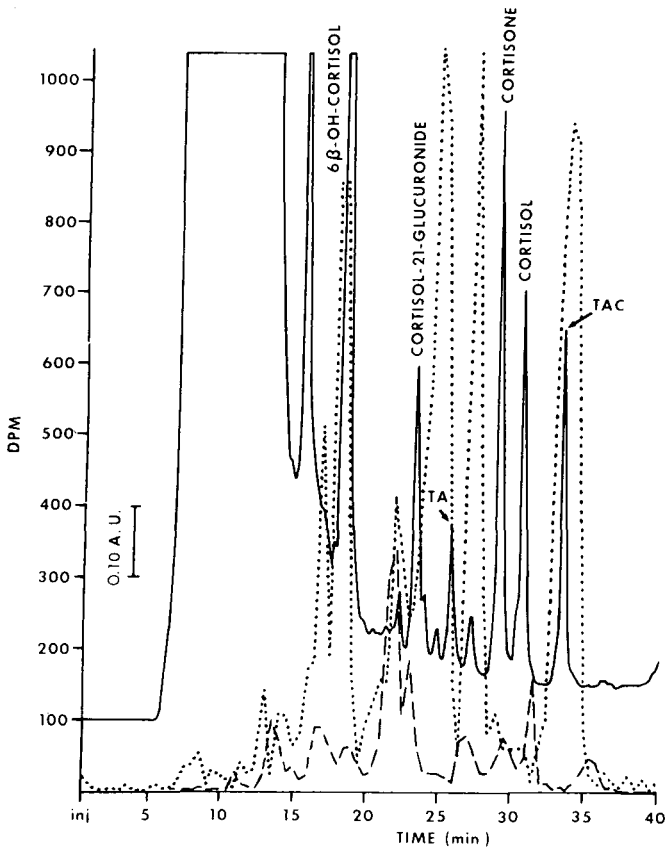

Fig. 5. HPLC profile of fetal liver metabolites 5 h after maternal administration of dose. Chromatographic conditions: as in Fig. 2. · · · , ^{3}H; — — —, ^{14}C; ——, UV at 254 nm.

procedures and several chromatography systems (column, paper, and thin-layer). In this study, a single HPLC system (A) is reported for biological samples which is fast, easy and reproducible with the added advantage that this preparative technique resolves conjugated and nonconjugated metabolites simultaneously. We have previously reported that this single-column HPLC approach provides excellent resolution of synthetic [29] and endogenous [30] estrogens.

In the present report, quantification of the various corticosteroid metabolites is based on radioactivity. An advantage of radioisotope studies is that the sensitivity of the method is determined by the specific activity of the radioisotope in question. In the case of [^{14}C]-cortisol (the lowest specific activity isotope used in this study), the isotope is available commercially and has a specific activity of over 1850 MBq/mmole or 50 mCi/mmole. Therefore, based on the ability to quantify 200 dps of radiolabeled corticosteroid (signal-to-noise ratio of 8:1) the sensitivity of the present radioisotope method is greater than 1.0 ng per 0.5 ml biological fluid (2.0 ppb).

Previous studies [23,24,27] indicate 6β-OH-TAC as being the major metabolite of TAC with little or no conjugation. Kriplani et al. [23] found gluc-

uronide conjugates accounted for 21, 6, and 4% of the radioactivity in the urine of dogs, monkeys and rats respectively. Sulfate conjugates were mentioned only with regard to dog urine and accounted for 11% of the radioactivity. Florini et al. [24] found other metabolites in the water-soluble urine constituents but could not identify them. It has been suggested [23] that the Δ^1, 9α-fluoro and the 16,17-acetonide substituents may block normal reduction reactions prerequisite to conjugation. Our studies agree with this hypothesis to the extent that a major portion of TAC radioactivity excreted is the parent compound. However, these preliminary studies indicate that a glucuronide conjugate of TAC (probably TAC-21-glucuronide) is the major metabolite with 6β-OH-TAC and other more polar metabolites constituting the remainder of the metabolic profile. The large percentage of TAC glucuronide identified in this study may be due to the mild clean-up procedure and the ability of the HPLC system to separate the more polar compounds. Unlike TAC, cortisol is largely metabolized to cortisone and other polar compounds, most of which are glucuronide conjugates as evidenced by their recovery after hydrolysis with β-glucuronidase. This is in agreement with previous findings [31—33] regarding cortisol metabolism in primates. Glucuronidation, rather than sulfation, appears to be the predominant metabolic pathway for the subhuman primate.

In conclusion, the described sample preparation and HPLC procedures are capable of an efficient and rapid separation of free and conjugated metabolites of radiolabeled corticosteroids from biological fluids and tissues.

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IMPROVED PROCEDURE FOR THE SEPARATION OF PHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We describe a rapid and efficient high-performance liquid chromatography procedure for the separation of phospholipids. The separation is accomplished on a microparticulate silica gel column using isocratic elution and UV detection at 203 nm. The solvent mixture is acetonitrile—methanol—85% phosphoric acid(130:5:1.5, v/v). Complete separation is achieved within 30 min of phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidyldimethylethanolamine, lysophosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine and sphingomyelin. The method is suitable for the analysis of phospholipids in tissue extracts.

INTRODUCTION

Currently the use of high-performance liquid chromatography (HPLC) for the analysis of phospholipid constituents present in biological membranes is still limited. A major obstacle to the development of a satisfactory method for this application has been the problem of detection. Refractive index and flame ionization detection methods are insensitive [1]. UV detection is sensitive and non-destructive, but the 200-nm range of phospholipid absorbance limits the choice of eluting solvents to those which do not absorb in that region. The solvent systems described in previous reports [2—5] provide the separation of only a few phospholipids. These methods are inadequate for the analysis of phospholipids in tissue extracts. It is well recognized that in the separation of phospholipids by silica gel thin-layer plates the presence of either acids or bases in chloroform—methanol—water solvent mixtures greatly improves the resolution. By analogy with thin-layer chromatography (TLC) we have developed an HPLC procedure using a silica gel column and a solvent mixture of ace-

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tonitrile—methanol—85% phosphoric acid. With this procedure all the major phospholipid components in tissue lipid extracts can be separated in a single run.

EXPERIMENTAL

Materials

Soybean phosphatidylinositol, bovine brain phosphatidylserine and lysophosphatidylserine, egg yolk phosphatidylethanolamine and lysophosphatidylethanolamine, egg yolk phosphatidylcholine, bovine liver lysophosphatidylcholine, egg yolk sphingomyelin, dipalmitoyl phosphatidic acid and dipalmitoyl phosphatidylglycerol were purchased from Sigma (St. Louis, MO, U.S.A.). Phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine were obtained from Gibco (Grand Island, NY, U.S.A.). Acetonitrile and methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Phosphoric acid, 85%, was of analytical grade from Mallinckrodt (St. Louis, MO, U.S.A.). [^{14}C-Methyl]phosphatidylcholine was obtained from New England Nuclear (Boston, MA, U.S.A.).

Tissue lipid extracts

Sprague-Dawley male rats weighing 150 g were used. They had access to the diet up to the time of sacrifice. Immediately after decapitation, heads and livers were placed in liquid nitrogen. Erythrocytes and serum were obtained from a healthy human donor. A 1-g amount of rat tissue, 1 ml of erythrocytes or 1 ml of serum was homogenized in 30 ml of chloroform—methanol (2:1, v/v). After filtration the lipid extract was separated into two phases according to the procedure of Folch et al. [6]. The lower phase was dried under nitrogen and redissolved in chloroform before HPLC analysis.

Chromatographic conditions

We used a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatographic system consisting of a Model 6000 solvent delivery system, a Model U6K injector, a Model 450 variable-wavelength detector and a strip chart recorder. The chromatographic column was a 30 cm × 4 mm I.D. prepacked stainless-steel Micro-Pak SI-10 column (Varian Assoc., Palo Alto, CA, U.S.A.), which contained silica gel, particle size 10 μm. The acetonitrile—methanol—85% phosphoric acid (130:5:1.5, v/v) solvent was delivered to the column at a flow-rate of 1 ml/min at a pressure of approximately 34 bar (500 p.s.i.) at room temperature (21°C). The detection was at 203 nm. The reference cell contained air. Phospholipid standards and tissue lipid extracts were dissolved in chloroform. Sample volumes and the recorder response are indicated in figure legends. Each day after the analysis the column was washed successively with 30 ml each of methanol—water (1:1, v/v), methanol and dichloromethane before storing it overnight in *n*-hexane.

RESULTS

Several solvent systems, made up of acetonitrile—methanol—85% phosphoric acid in various proportions, were tested for their ability to separate mixtures of phospholipid standards. An isocratic mobile phase containing acetonitrile—methanol—85% phosphoric acid (130:5:1.5, v/v) was found to be successful in separating the six major phospholipid components present in tissue extracts: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SPH). Fig. 1 shows a representative chromatogram. Most of the minor phospholipids, except for phosphatidyldimethylethanolamine (PDME) and lysophosphatidylethanolamine (LPE), co-eluted with other phospholipids and could not be resolved. Cardiolipin and neutral lipids were eluted with the solvent front. Phosphatidic acid (PA) and phosphatidylglycerol (PG) had the same retention time and were partially co-eluted with PC. Their distinctive

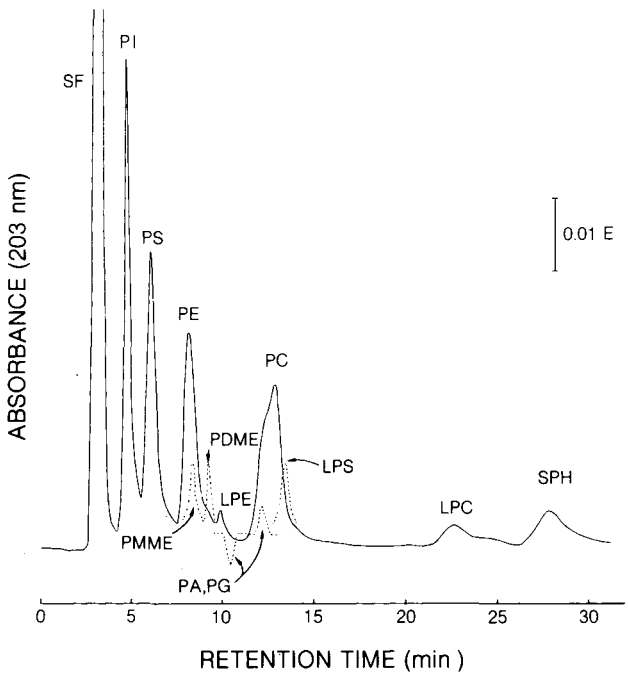

Fig. 1. Chromatogram of phospholipid standards. The amount injected was 1.5 μl of chloroform containing 0.5 μg each of PS, PE and PC, 2.5 μg each of PI and SPH, and 5 μg each of LPC and LPE. Retention times of minor phospholipid classes were determined by separate injections and are indicated by dotted lines. Chromatographic conditions: flow-rate, 1 ml/min; mobile phase, acetonitrile—methanol—85% phosphoric acid (130:5:1.5, v/v); UV detection at 203 nm; recorder response 0.1 a.u.f.s.; and ambient temperature. Peaks: LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PMME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine; SF, solvent front; and SPH, sphingomyelin.

pattern, characterized by having both a trough and a peak, was also observed by Kiuchi et al. [1] using a flame ionization detector. The mechanism for this pattern is not known. The identity of peaks on the chromatogram was established by injecting into the chromatograph the individual phospholipid standard as well as mixtures of standards. The recovery of phospholipid applied to the column was determined with [^{14}C]-phosphatidylcholine (approximately 9000 dpm per injection), and was found to be greater than 95%.

Before sample analysis the silica gel column was stored in *n*-hexane (see Experimental). Upon changing to a new solvent mixture, sufficient time was allowed for the column to become re-equilibrated. Fig. 2 illustrates that the resolution of phospholipids improved as the time of pumping the solvent through the column increased. If a complete separation of LPC and SPH is desired, we recommend that the column be equilibrated for more than 5 h.

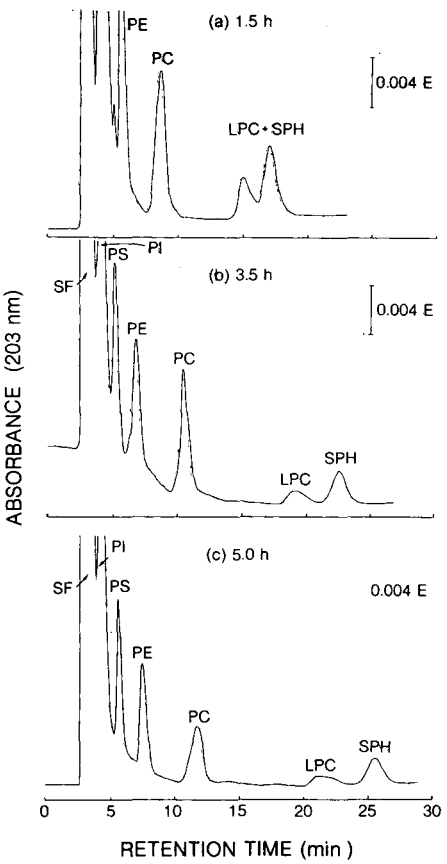

Fig. 2. Influence of equilibration time on the separation of phospholipids. The column absorbant was originally stored in *n*-hexane. The solvent, acetonitrile—methanol—85% phosphoric acid (130:5:1.5), was pumped through the column for (a) 1.5 h, (b) 3.5 h, or (c) 5 h before the injection of samples. Sample volumes were 7 μl and recorder response 0.04 a.u.f.s. Other conditions were the same as in the legend to Fig. 1.

Aliquots of lipid extracts from rat brain, rat liver, human erythrocytes and human serum were injected directly into the chromatograph for analysis. In rat liver (Fig. 3a, b) and brain (Fig. 3c, d) PI, PS, PE and PC peaks were readily detected. The peak of SPH was detectable only when a larger aliquot was injected or when the sensitivity of the recorder was increased. PS, PE, PC, LPC, and SPH were major constituents in erythrocytes (Fig. 3e), while PI was not detectable under the analytical condition. It is noteworthy that the Folch procedure is not well suited for the extraction of erythrocyte lipids [7]. Despite the use of crude Folch extracts, the HPLC method revealed phospholipid peaks free of interferences by other materials. In the serum (Fig. 3f) PC was the largest component. PE, LPC and SPH were present, although in much smaller amounts than PC. PI and PS were not detectable. These patterns are consistent with the published results on the quantitative analysis of rat liver [8], rat brain [9], human erythrocytes [7] and human serum [10].

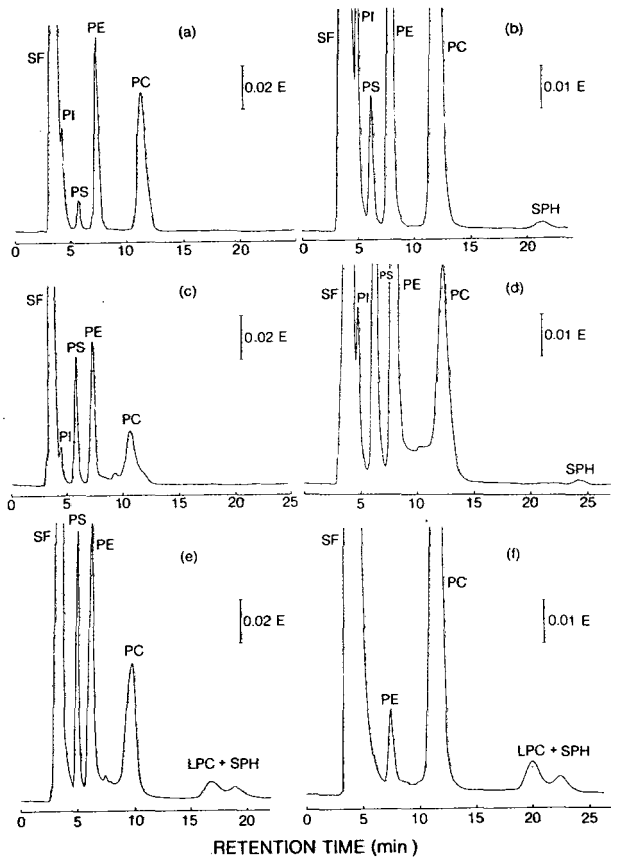

Fig. 3. HPLC analysis of lipid extracts. Lipids were extracted from tissues as described under Experimental. (a) Rat liver, 3.8 μg lipid P; sensitivity, 0.2 a.u.f.s.; (b) rat liver, 7.0 μg lipid P; sensitivity, 0.1 a.u.f.s.; (c) rat brain, 3.6 μg lipid P; sensitivity, 0.2 a.u.f.s.; (d) rat brain, 5.6 μg lipid P; sensitivity, 0.1 a.u.f.s.; (e) human erythrocytes, 1.0 μg lipid P; sensitivity 0.2 a.u.f.s.; and (f) human serum, 2.0 μg lipid P; sensitivity, 0.1 a.u.f.s. Other conditions were the same as in the legend to Fig. 1. Lipid P is phosphorus in lipid extracts.

DISCUSSION

The uniqueness of the method described in this report is the solvent mixture of acetonitrile—methanol—85% phosphoric acid (130:5:1.5, v/v). Compared with the method of Jungalwala et al. [2], which used a silica gel column and a solvent mixture of acetonitrile—methanol—water (65:21:14, v/v), the presence of phosphoric acid greatly improves the resolution. It permits the separation of all the major membrane phospholipid constituents in a single run without using gradient elution. Jungalwala's method is effective in separating PC, SPH, and LPC. However, PS co-elutes with PE. PI is not resolved. The methods reported by other investigators also have various limitations for the analysis of phospholipids in tissue extracts. With a silica-based cation-exchange column and an isocratic mobile phase of acetonitrile—methanol—water, Gross and Sobel [4] were able to separate PC, LPC, LPE and SPH. But, they failed to resolve PS and PE. The method of Geurts van Kessel et al. [3] employed a silica gel column and gradient elution using hexane—isopropanol—water mixtures. They reported separating cholesterol, PA, PE, PI, PS, LPC and LPE, whereas PC and SPH were only partially resolved. Hanson et al. [5] utilizing a silica-based anion-exchange column and gradient elution separated the lipid extract from egg yolk into neutral lipid, PC, SPH, LPC and PE fractions. PI and PS could not be eluted.

UV absorption by lipids at the 200-nm region is due largely to the presence of double bonds [2]. The absorption by other functional groups, such as ester carbonyl and amino, also occurs, but it is small in extent. Being sensitive, convenient and non-destructive, UV detection is ideal for monitoring the separation of lipids by HPLC. However, it is complicated to use UV detection for lipid quantitation, because the area under a given absorbance peak reflects the number of double bonds rather than the number of molecules. Previous investigators [2] suggested two methods for the quantitation of phospholipid fractions. First, the quantity is calculated from the apparent molecular extinction coefficients (ϵ] of the material analyzed and the peak area. The apparent ϵ is determined by performing HPLC of a representative sample, the concentration of which is already known, and measuring the UV response. Second, the effluents from specific peaks are collected and quantified by independent methods. It should be noted that our solvent mixture contains phosphoric acid and may interfere with phospholipid quantitation, since most chemical methods employ acid digestion to liberate phosphorus from phospholipid, which is then measured. It is necessary to use only those methods which do not involve acid digestion [11,12]. Another disadvantage of our method is that the use of acidic solvents may lead to a degradation of plasmalogens.

Jungalwala et al. [13], and we [14] previously used a different approach for the quantitative analysis of phospholipids that contain primary amino groups, i.e., PE, LPE, PS and LPS. Before HPLC analysis these phospholipids are converted into either UV-absorbing biphenylcarbonyl derivatives [13] or fluorescent dansyl derivatives [14]. In these instances, the peak area reflects the amount of phospholipid eluted.

In biochemical research it is a common procedure to measure the radioactivity in the phospholipid fraction following the administration of isotope-labeled lipid precursors to cell cultures. Examples are the measurements of PI turnover

and methylation of PE. These are crucial events occurring on the cell surface in the stimulus–receptor interactions [15, 16]. Being efficient in separating PI, PDME and PC, the HPLC procedure described in this report is well suited for this application. Compared to conventional TLC methods, the HPLC method should be less laborious and more accurate.

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RAPID PURIFICATION OF PROTEOLIPIDS FROM RAT BRAIN SUBCELLULAR FRACTIONS BY CHROMATOGRAPHY ON A LIPOPHILIC DEXTRAN GEL

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SUMMARY

Proteolipids from adult rat brain subcellular fractions were purified by a one-step procedure involving chromatography through Sephadex LH-60 eluted with an acidified chloroform—methanol mixture.

The protein peak was eluted with the void volume and was free of adventitious lipids. The degree of purification was similar to that attained with the neutral—acidified chloroform—methanol dialysis method with the advantage that this new procedure can be carried out in only 3 h, with a recovery of proteins of 95—100%. Samples containing different lipid/protein ratios passed through the gel gave similar elution profiles.

When labeled amino acids or palmitic acid were added to myelin total lipid extracts, no radioactivity was eluted with the protein, indicating that the proteolipid apoproteins purified by this method do not adsorb hydrophobic low-molecular-weight compounds.

INTRODUCTION

Proteolipids, first described by Folch and Lees [1], are hydrophobic proteins present in different types of cellular membranes. They are operationally defined as hydrophobic lipoprotein complexes soluble in chloroform—methanol (2 : 1) and insoluble in water. Their apoproteins are also soluble in organic solvent mixtures and can be transferred to aqueous solutions by appropriate procedures.

They are abundant in white matter of the central nervous system, constituting one of the major protein components of the myelin membranes [2]. Peripheral nerve myelin, on the other hand, contains small amounts of proteolipids while grey matter and non-neural tissues contain much smaller amounts than white matter.

Non-myelin proteolipids have been implicated in calcium transport in

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the sarcoplasmic reticulum [3], and in proton transport in bacteria [4] and in mitochondria [5]. Nerve ending proteolipids have been extensively studied by the group of De Robertis [6].

The purification of these hydrophobic proteins, especially the purification of myelin proteolipid, has been the subject of numerous publications describing several procedures such as emulsion-centrifugation [7], precipitation with organic solvents [8], dialysis against neutral chloroform—methanol and acidic chloroform—methanol [9], and chromatographic methods using silicic acid [10], polystyrene gels [11], Sephadex LH-20 [12], Bio-Gel P-10 [13], DEAE-cellulose [14], Dowex resins [15], etc. All these procedures have some drawbacks and only by using neutral and acid dialysis can highly delipidized preparations be obtained. This procedure, however, is time consuming and produces loss of protein material [9]. Effective, rapid and quantitative methods are, up to now, almost absent in the literature.

In this paper, we present a method of purification that involves the use of gel chromatography through Sephadex LH-60, which is very quick (2—3 h), permits almost 100% recovery of the protein material, and furnishes a final product of similar purity to that obtained with the method of Tennenbaum and Folch-Pi [9].

MATERIALS AND METHODS

Preparation of subcellular fractions

Wistar rats, 35—40 days old, of either sex were used throughout. Animals were killed by decapitation and the forebrains rapidly removed and placed in the cold. Subcellular fractions were obtained using the method of De Robertis et al [16] except that the crude mitochondria were subfractionated on a discontinuous gradient of 0.8 *M*, 1.0 *M* and 1.25 *M* sucrose. The layer floating at the 0.32—0.8 *M* interface (fraction A) is myelin; the 0.8—1.0 *M* interface is fraction B; the 1.0—1.25 *M* layer contains synaptosomes (C) and the pellet (D) mitochondria. The layers were carefully removed, diluted with 0.25 *M* sucrose to approximately 0.3—0.4 *M* sucrose and centrifuged at 100,000 *g* for 30 min. The pellet from fraction A (myelin) was further purified according to the procedure of Norton and Poduslo [17]. Microsomes were obtained by centrifugation of the post-mitochondrial supernatant at 100,000 *g* for 60 min, and purified nuclei were prepared from an aliquot of the initial homogenate as described by Krawiec et al. [18]. All the pellets were resuspended in a suitable volume of water for lipid extraction and chemical determinations.

Preparation of proteolipids

Crude total lipid extracts were obtained from an aliquot of the initial homogenate and from the resuspended pellets of the subcellular fractions by the procedure of Folch and Lees [1]. In order to precipitate the basic proteins, 0.05 volume of 0.1 *M* KCl was added to the crude lipid extract, the mixture being left in the cold overnight [19]. After the addition of a few drops of methanol, the precipitate was removed by filtration and the lipid extracts, free of basic proteins, were washed once with water and twice with

the theoretical upper phase (chloroform—methanol—water, 3 : 48 : 47) according to the description of Folch et al. [20]. The washed lower phases (TLE) were concentrated about ten-fold, after the addition of 0.5 volume of chloroform, by evaporating at 30°C under vacuum. To precipitate proteolipids, 5 volumes of acetone were added to the concentrated solutions and the mixture was left at −15°C for 2 h. The precipitate formed (crude proteolipid) was separated by centrifugation in the cold at 4000 *g* for 20 min. The pellets were dried under a stream of nitrogen and dissolved in chloroform—methanol (1 : 1, v/v) containing 5% 0.1 *N* HCl to obtain a protein concentration between 2 and 4 mg/ml. In some experiments acetone precipitation was omitted; instead, the TLEs were carefully concentrated under vacuum and chloroform—methanol (1 : 1, v/v) containing 5% 0.1 *N* HCl (approximately 3 ml per ml of concentrated TLE) was added. The results obtained using either procedure were similar.

Preparation of Sephadex columns

Prior to column packing the Sephadex LH-60 was washed two times with distilled water and two times with acetone through a fritted flass filter under vacuum to remove fines. The gel was then suspended in chloroform—methanol (1 : 1) containing 5% 0.1 *N* HCl and washed three or four times with this mixture. This type of Sephadex must remain in the solvent mixture for at least 3 h at room temperature for complete swelling. Glass columns measuring 40 × 1.8 cm, fitted with a porous glass plate were used in all cases. The gel slurry was carefully poured into the column and the solvent allowed to drain slowly. Four volumes of the solvent mixture were passed through the column, prior to chromatography, in order to eliminate traces of acetone. A similar procedure and column design were used to prepare the Sephadex LH-20 columns.

Chromatography

Crude proteolipid solutions with a volume smaller than 5 ml and containing between 2 and 12 mg of protein were applied to the Sephadex LH-60 or LH-20 column and eluted with chloroform—methanol (1 : 1) containing 5% 0.1 *N* HCl at room temperature (16—22°C) at a flow-rate of 0.3 ml/min; 3-ml fractions were collected, aliquots of which were used for chemical determinations or radioactivity counting.

Chemical procedures and radioactivity counting

Protein was assayed by the method of Lowry et al. [21] as modified by Hess and Lewin [22] using bovine serum albumin as standard. Total cholesterol, lipid phosphorus and galactose were measured by the procedures described by Searcy and Bergquist [23], Chen et al. [24] and Hess and Lewin [22], respectively.

Suitable aliquots from each eluted fraction were evaporated to dryness and counted in a Packard Tri Carb scintillation counter using 10 ml of counting solution containing 5.5% of 2,5-diphenyloxazole and 0.1% of 1,4-bis(5-phenyl-oxazol-2-yl)benzene and Triton—toluene (80 : 20, v/v). Thin-layer chromatography (TLC) of lipids was carried out on 20 × 20 cm silica gel H glass plates [25]. Individual lipids were detected with iodine vapour.

Chemicals and reagents

All the chemicals were reagent grade and the solvents were redistilled before use. Sephadex LH-20 and LH-60 were purchased from Pharmacia (Uppsala, Sweden). L-[4,5-^{3}H]Leucine (55 Ci/mmol), [U-^{14}C]L-phenylalanine (406 mCi/mmol), and [9,10-^{3}H]palmitic acid (17 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.).

RESULTS

As shown in Table I, proteolipid proteins are present in all subcellular membranes, except in the soluble fraction. Very small amounts are found in purified nuclei, in agreement with results previously reported for mouse brain by Nussbaum and Mandel [26], while the microsomal fraction contains a higher amount of these proteins. Proteolipid proteins are concentrated in myelin and in fraction B, which together with those present in mitochondria and in the synaptosomal fraction represent 90% of all the proteolipid proteins found in the brain.

TABLE I

SUBCELLULAR DISTRIBUTION OF PROTEOLIPID PROTEINS

Fraction	Morphological constitution	Total protein (mg/g FT)*	Proteolipid proteins		
			mg/g FT*	SC**	RSC***
Total homogenate	—	117.4	3.610	0.031	1
Purified nuclei	—	1.2	0.051	0.042	1.35
A	Myelin fragments	4.6	1.760	0.383	12.35
B	Curved membranes, vesicles and myelin fragments	7.8	0.510	0.065	2.10
C	Nerve endings	7.3	0.410	0.056	1.80
D	Mitochondria	11.5	0.480	0.042	1.35
Microsomes	Curved membranes, vesicles, rough and smooth ribosomes	19.4	0.300	0.016	0.51
Soluble	—	16.9	ND§	ND	ND

*FT = fresh tissue.
**SC (specific concentration) = mg proteolipid protein per mg total protein.
***RSC (relative specific concentration) was estimated as the ratio between tne specific concentration in the fraction and that in the total homogenate.
§ND = not detectable.

Fig. 1 shows the chromatographic separation of the protein from the contaminating lipids obtained with a column of Sephadex LH-20. Although an acceptable separation is obtained, traces of lipids are eluted together with the proteins, one of the main lipid contaminants being sphingomyelin, as shown by TLC of fractions 13—14 (Fig. 1B). Similar results were presented by Gagnon et al. [12], who purified human myelin proteolipids by gel chromatography in a similar column using acidified chloroform—methanol. Only

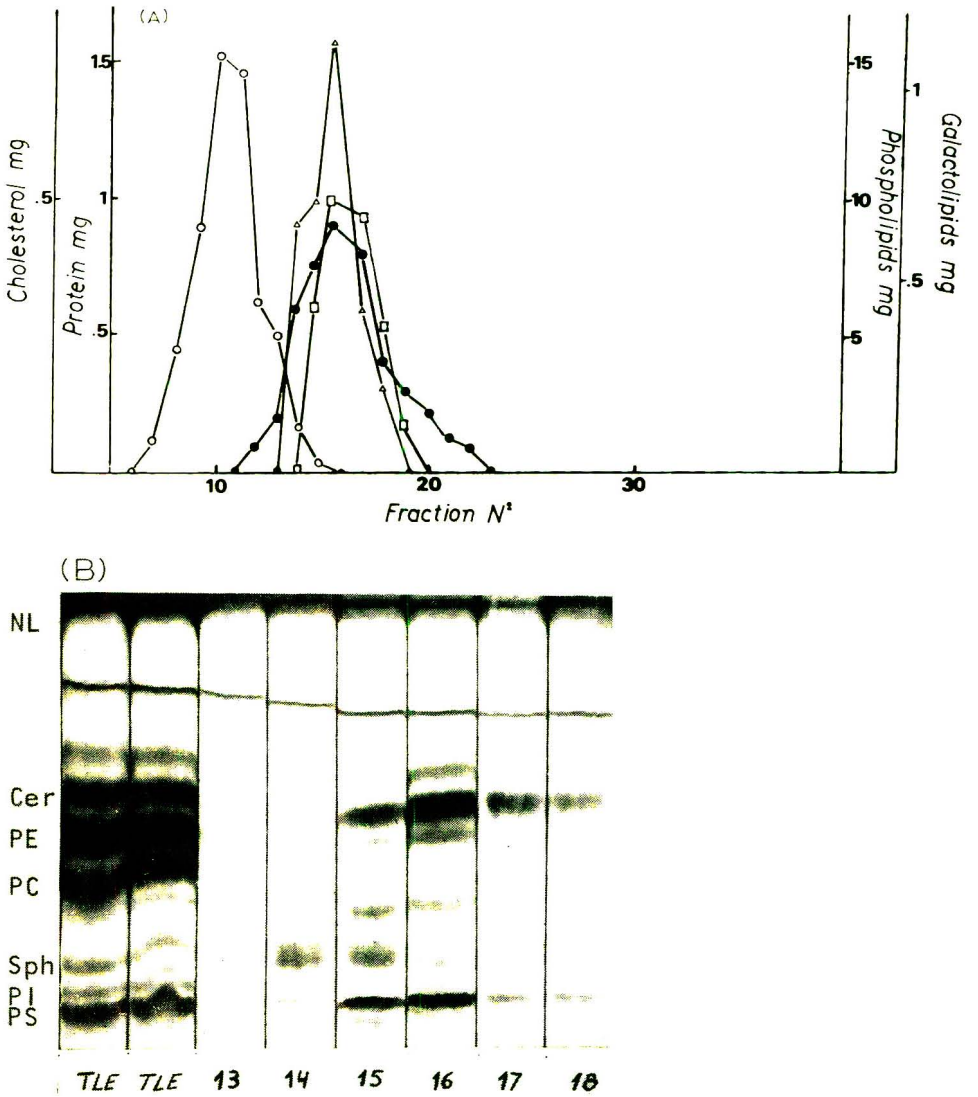

Fig. 1. (A) Purification of total rat brain proteolipids by chromatography on Sephadex LH-20. (○), Protein; (●), phospholipids; (^), cholesterol; (□), galactolipids. (B) Thin-layer chromatograms of the fractions containing lipids. PS = phosphatidyl serine; PI = phosphatidylinositol; Sph = sphingomyelin; PC = phosphatidylcholine; PE = phosphatidylethanolamine; Cer = cerebrosides; NL = neutral lipids.

by repeated chromatography was it possible to obtain a pure material, and protein losses could also occur during this time-consuming procedure. In view of these results, we decided to try a similar methodology but using Sephadex LH-60.

The pattern obtained with rat brain TLE as well as with TLE from different subcellular fractions eluted through Sephadex LH-60 are shown in Fig. 2. The profiles are similar both for lipids and proteins in all membrane frac-

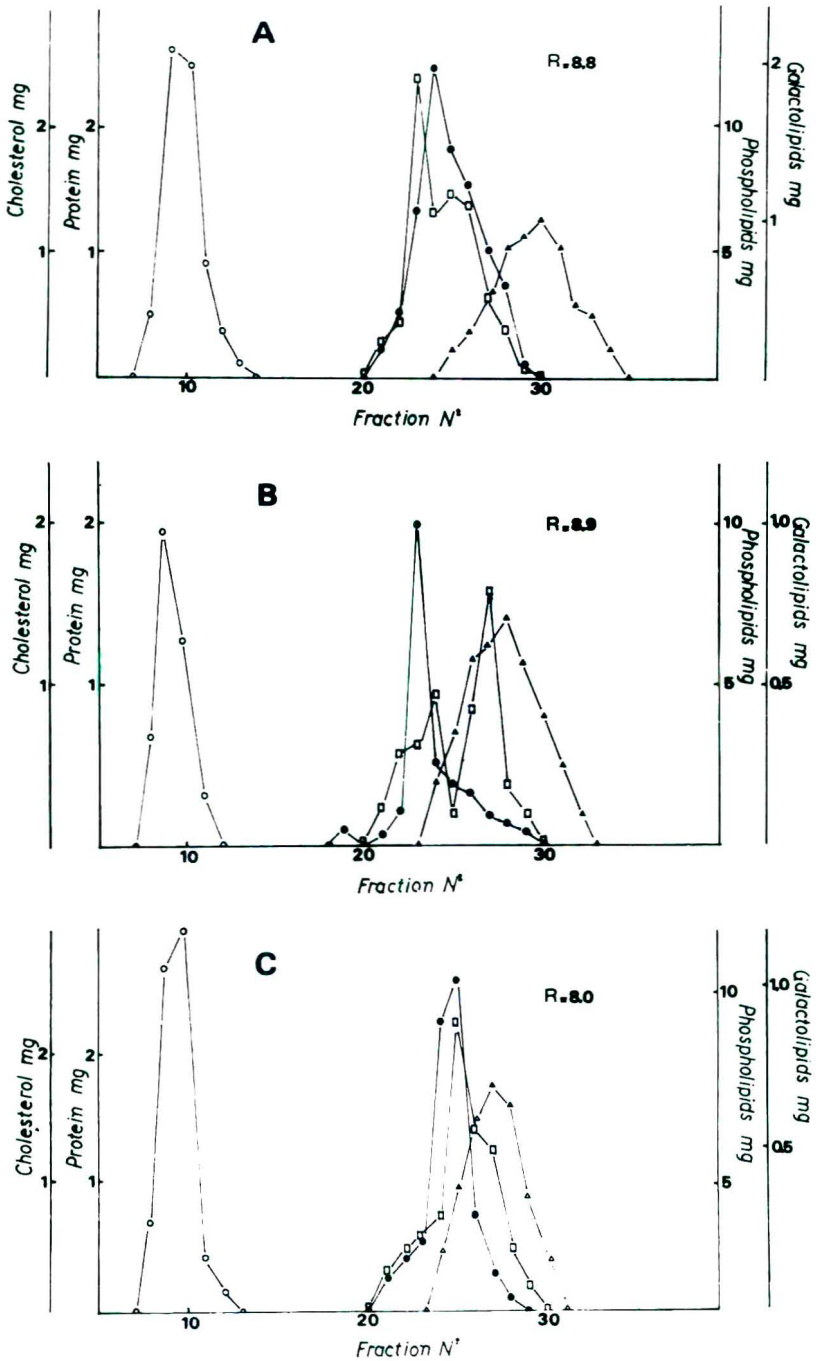
A
R.8.8
Cholesterol mg
Protein mg
Phospholipids mg
Galactolipids mg
Fraction N°
B
R.8.9
Cholesterol mg
Protein mg
Phospholipids mg
Galactolipids mg
Fraction N°
C
R.8.0
Cholesterol mg
Protein mg
Phospholipids mg
Galactolipids mg
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Fig. 2.

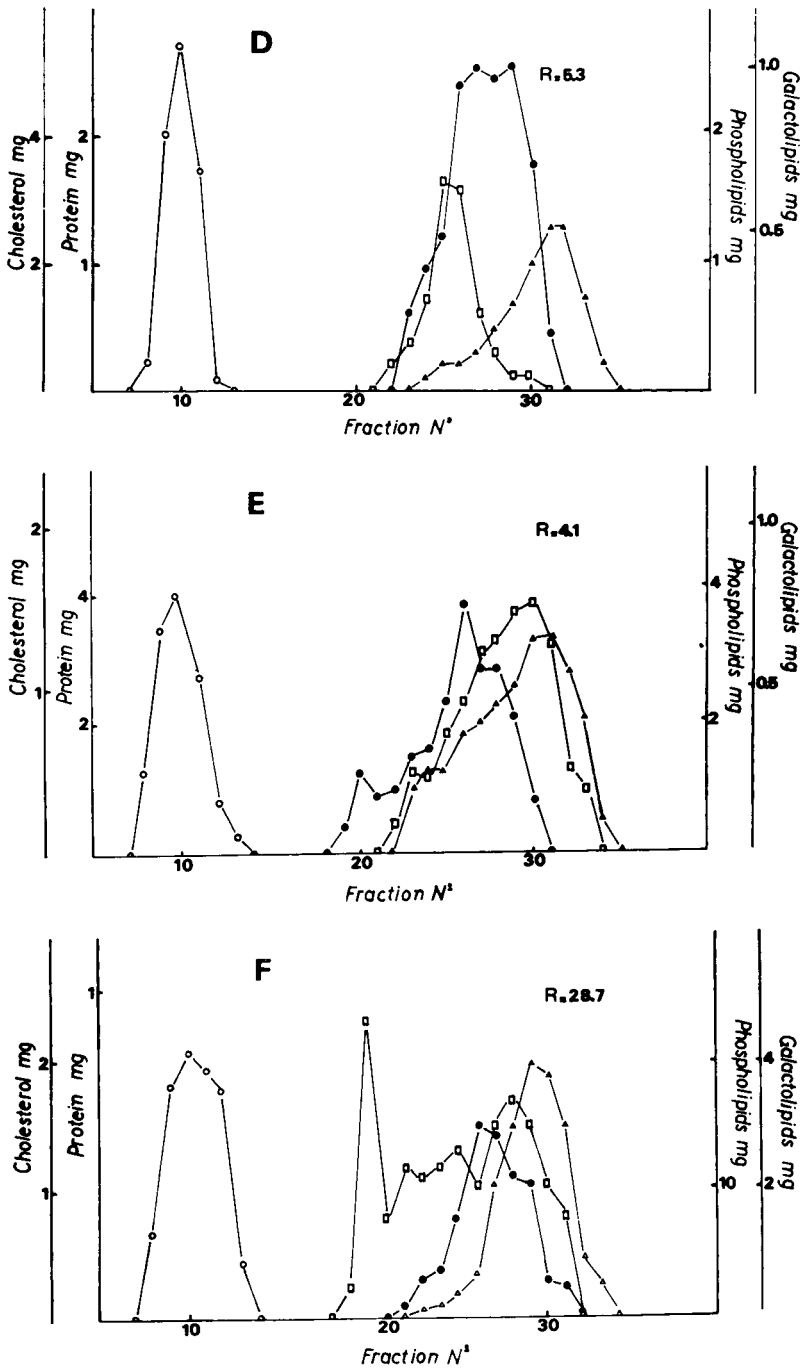

Fig. 2. Purification of proteolipids from rat brain subcellular fractions by chromatography on Sephadex LH-60: (A) whole brain; (B) myelin; (C) fraction B; (D) synaptosomes; (E) mitochondria; (F) microsomes. (○), Protein; (△), cholesterol; (□), galactolipids; (●), phospholipids. R represents the ratio between total lipids and total proteolipid proteins loaded on to the column.

tions. Furthermore they are independent of the lipid/protein ratio present in the samples applied to the column. The protein peak, representing 90—95% of the protein present in the original sample, is eluted within the theoretical void volume and in a very small volume (4—5 tubes). Contrary to what was found using chromatography on LH-20 no lipids co-eluting with the proteins were detected by chemical analysis. Five or six tubes containing no detectable material were collected at the end of the protein peak and before the appearance of the peak containing various lipids, which are eluted in a wide range of tubes, cholesterol being the last to appear.

When the peaks containing the proteins or the lipids were independently re-chromatographed through Sephadex LH-60 using the same solvent mixture, the corresponding materials appeared in their original elution positions. The tubes containing proteins were pooled and concentrated by evaporation under nitrogen in order to perform chemical studies to determine the amount of lipids with reference to protein present in this purified preparation. Lipid phosphorus was found to be 0.035% by weight while galactose and cholesterol were absent. For purposes of comparison, we include in Table II results obtained by other investigators using well-known procedures for the preparation of proteolipid apoproteins. The degree of purification with our method can be compared only with that obtained by extensive dialysis against neutral and acid chloroform—methanol as described by Folch-Pi and Stoffyn [27]. Furthermore, the purified apoproteins can be obtained in only 2—3 h, while the procedure of Folch-Pi and Stoffyn takes at least 14 days. Another important point to be stressed is that there is very little loss of protein material while, as shown by Folch-Pi [28], small peptides are lost during dialysis.

TABLE II

AVERAGE COMPOSITION OF PURIFIED PROTEOLIPID APOPROTEINS OBTAINED FROM BOVINE WHITE MATTER AND MYELIN BY DIFFERENT PROCEDURES

All data are expressed as percentage by weight.

Procedure	Protein	Lipid phosphorus	Galactolipids	cholesterol	$E^{278}_{1\%}$
Emulsion-centrifugation [7]					
Crude proteolipid	30—35	1.0—1.2	5—13	7	6—8
Concentrated	55—60	1.0	3—7	1	9—10
Sephadex LH-20 [15]	75	0.68	10.6	—	13.2
Sephadex LH-20 + Dowex 1-X2 [15]	94	0.074	2	—	16.3
Dialysis against chloroform—methanol [36]	70—85	0.4—1	2.5	0.2	10.5—13.5
Dialysis against chloroform—methanol + acidified chloroform—methanol [27]	99—100	0.04	ND*	ND	16—22
Our preparation (for myelin proteolipid apoprotein)	99—100	0.035	ND	ND	13—14

*ND = not detectable.

It is well known that proteolipid proteins in neutral chloroform—methanol adsorb hydrophobic and aromatic amino acids [29], particularly phenylalanine, and that these amino acids are retained by the protein, even after extensive dialysis. In order to ascertain if free amino acids were co-eluted together with the protein, 20 μCi of [^{3}H]leucine and 5 μCi of [^{14}C]phenylalanine were added to the TLEs, which were subsequently chromatographed as described above. Neither ^{3}H nor ^{14}C was detected in the peak corresponding to protein (Fig. 3A). Recovery of the amino acids was approximately 100%.

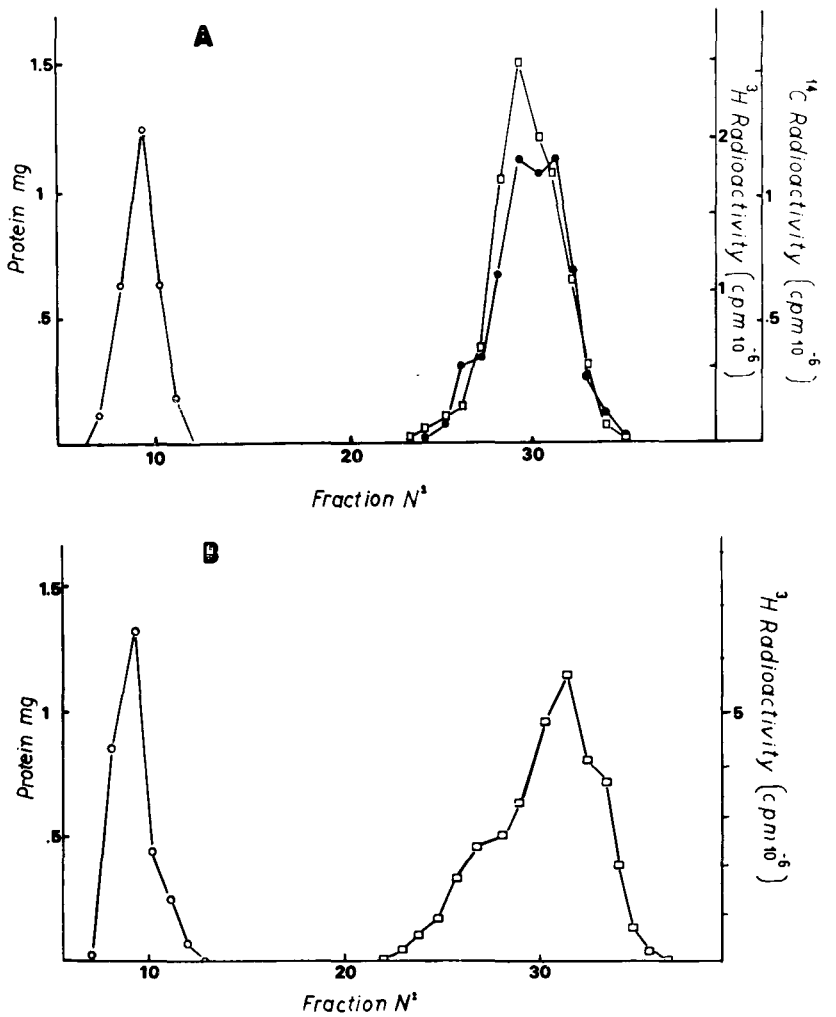

Fig. 3. Column chromatography of myelin TLE on Sephadex LH-60 in the presence of externally added radioactive amino acids or palmitic acid. (A) [^{14}C]Phenylalanine and [^{3}H]leucine were added to TLE containing myelin proteolipids prior to chromatography (see Results). (○), Protein; (□), ^{3}H radioactivity; (●), ^{14}C radioactivity. (B) [^{3}H]Palmitic acid was added to TLE containing myelin proteolipids prior to chromatography (see Results). (○), Protein; (□), ^{3}H radioactivity.

Adsorption of fatty acids to proteolipid apoproteins was also measured as described above, 50 μCi of [^{3}H]palmitic acid being added prior to chromatography (Fig. 3B). The results were similar to those obtained with amino acids.

Using a column of the size mentioned above, the flow-rate can be increased to 0.7 ml/min (if the sample volume and the amount of protein loaded are not changed) without changing the effectivity of the method. If the flow-rate is kept at 0.3 ml/min, the amount of protein loaded can be increased to 30 mg, provided that the sample volume does not exceed 5 ml. This volume, however, can be increased to 8 ml if the flow-rate and amount of protein loaded are maintained within the values mentioned under Materials and Methods.

DISCUSSION

Mokrasch [15] was the first to use hydroxypropyl derivatives of Sephadex G-25 (Sephadex LH-20) for the chromatographic purification of proteolipids. The protein, eluted with the void volume of the column, was re-chromatographed through another column containing Dowex 1-X2, in order to obtain maximal delipidization. Recovery, however, was low and the final product was unstable. In 1969, Soto et al. [30] used a combination of gel chromatography and partition chromatography in Sephadex LH-20 for the fractionation and partial purification of grey and white matter proteolipids. A complete purification of proteolipids from human myelin was obtained by repeated gel chromatography on Sephadex LH-20 eluted with acidified chloroform—methanol [12]. This method has been widely used for the purification of proteolipids by several investigators [31]. Using this solvent, the lipids bound by strong electrostatic interactions are separated from the apoprotein and are eluted according to their molecular weights. If the acid is omitted, the protein eluted from the column is contaminated with acidic lipids while zwitterionic and neutral lipids are separated, the product obtained being very similar to that obtained after extensive neutral dialysis.

If water is omitted from the solvent mixture, or if the chloroform/methanol ratio is increased, recovery of the protein diminishes and partition effects occur.

We thought that Sephadex LH-60 could be a better matrix than Sephadex LH-20 for the separation of the proteins and the lipids and decided to use an acid mixture of organic solvents in order to be sure that gel filtration would occur. Only two problems could invalidate our method: (1) that the matrix of Sephadex LH-60 would adsorb proteolipids, and (2) that the protein could be enclosed within the fractionation range, since Sephadex LH-60, using organic solvents, has an exclusion limit of 15,000. None of these problems occurred since 90—95% of the protein was recovered in a single peak eluted with the void volume.

Fillingame [5] was the first to use hydroxypropyl Sephadex G-50 eluted with chloroform—methanol (2 : 1) for the purification of dicyclohexylcarbodiimide-proteolipid subunits of *Escherichia coli*, obtained a low-molecular-weight protein of 8400 in the void volume. Lees and Macklin [32] in 1972 used Sephadex LH-60 for the separation of the low-molceular-weight hydro-

phobic peptides obtained from bovine white matter proteolipids after digestion with elastase. Lees et al. [33] recently communicated that bovine white matter proteolipids could be fractionated into two peaks by chromatography through Sephadex LH-60 eluted with chloroform—methanol—acetic acid. The protein peak appearing at the void volume contained 25,000 and 20,000 proteolipids, and the peak enclosed within the gel fractionation range 13,000 proteolipid. With our method, and using the solvent mixture proposed, this fractionation does not occur. Gel electrophoresis of the protein peak obtained from a TLE from rat brain shows several bands corresponding to molecular weights ranging between 8000 and 60,000. It is evident that the range of fractionation of Sephadex LH-60, as happens with Sephadex LH-20, is reduced in organic media as compared to aqueous media. Also, bovine white matter proteolipid apoproteins have been shown to be asymmetric [34]. The combination of these two factors is a possible explanation of why proteolipid proteins with relatively low molecular weights (> 8000) are eluted at the void volume.

We have used this procedure for the preparation of the proteolipid apoproteins from different subcellular fractions, which we have analyzed by sodium dodecyl sulphate (SDS) and SDS—urea polyacrylamide gel electrophoresis system, in order to calculate the correct molecular weights using the Ferguson relationship [35], with excellent results.

Since we have demonstrated that the apoproteins obtained by this simple method do not adsorb amino acids, fatty acids or any other lipids, we are currently using the method described above for sample purification of proteolipid proteins from myelin, total homogenate and brain microsomes to study turnover rates of proteolipid apoproteins and of their covalently bound fatty acids.

ACKNOWLEDGEMENTS

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CHROMBIO. 1066

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR IDENTIFICATION AND QUANTITATION OF NUCLEOTIDES IN LYMPHOCYTES AND MALIGNANT LYMPHOBLASTS

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SUMMARY

A method for the identification and quantitation of nucleotide pools in lymphocytes and leukemic blasts is described. Separation of these metabolites was performed by anion-exchange high-performance liquid chromatography using a pH and concentration gradient consisting of several linear steps.

The mono-, di- and triphosphates of adenosine, cytidine, guanosine, inosine, uridine and xanthosine could conveniently be separated together with NAD^+, cyclic AMP, $NADP^+$ and uridinediphosphoglucose (UDPG).

In addition, data on the accuracy and precision of the method are given and its potentials for use in the analysis of nucleotide pools in leukemic lymphoblasts are illustrated.

INTRODUCTION

Purine and pyrimidine metabolites are essential for human cells: they play an important role in the transfer of chemical energy for metabolic processes, in DNA and RNA synthesis, in cell proliferation and in cell regulation. Although more and more information is accumulating about purine and py-

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rimidine metabolism in relation to leukemia, our present insights are far from complete. Recent studies have demonstrated marked differences in adenosine deaminase, purine nucleoside phosphorylase and 5′-nucleotidase activities in leukemic blasts of various immunologically characterized subsets of acute and chronic lymphatic leukemia [1—4]. Selective toxicity of deoxyribonucleosides towards malignant human T-cell lines has been reported [5—7]. Determination of the levels of purine and pyrimidine metabolites in leukemic cells may contribute to a better understanding of the purine and pyrimidine metabolism in these cells.

In the present investigation an anion-exchange high-performance liquid chromatographic (HPLC) method was developed in order to determine nucleotide pools in these cells. The described method might also be used to measure nucleotide pools in lymphoid cells from patients with immune dysfunctions where deficient activities of enzymes of the purine interconversion pathway were observed [8—10].

Previously published methods [11—13] did not seem satisfactory for our purposes. To optimize the separation of the various nucleotides a multi-step gradient was chosen.

EXPERIMENTAL

Chemicals

The nucleotides cyclic AMP, NAD^+ and UDPG, used for standardization of the method, were obtained from Sigma, St. Louis, MO, U.S.A., and from Boehringer, Mannheim, G.F.R. The other chemicals were obtained from E. Merck, Darmstadt, G.F.R. Water used for all buffers was purified in a Milli-Q System (Millipore, Bedford, MA, U.S.A.).

Apparatus

A Spectra-Physics Model SP 8000B (Spectra-Physics, Santa Clara, CA, U.S.A.) with automated data system and a two-channel printer-plotter was used. A variable- and a fixed-wavelength UV detector (Spectra-Physics) were used for monitoring the peaks. Chromatographic columns were operated at 40°C. The columns were pre-packed Partisil-10 SAX columns (250 × 4.6 mm I.D., particle size 10 μm; Whatman, Maidstone, Great Britain). Injections were made using a 50-μl high-pressure Valco Valve loop injector.

Chromatographic procedure

The separation is based in principle on that described by Brown [11]. However, we used a modified system, changing the pH of the buffers and using a multi-step gradient.

Chromatography was carried out with two potassium dihydrogen phosphate buffers and water in a ternary system. The buffers and water contained 2% acetonitrile. The gradient is given in Table I. The low-concentration buffer consisting of 0.05 *M* potassium dihydrogen phosphate and 2% acetonitrile was adjusted to pH 3.35 with phosphoric acid. The high concentration buffer of 0.25 *M* potassium dihydrogen phosphate, 0.50 *M* potassium chloride and 2% acetonitrile was adjusted to pH 5.25 with potassium hydroxide. During

TABLE I

MOBILE PHASE SEQUENCE USED FOR THE SEPARATION OF NUCLEOTIDES IN THE CHROMATOGRAPHIC PROCEDURE

A = 2% acetonitrile in water; B = 0.05 *M* potassium dihydrogen phosphate buffer with 2% acetonitrile, pH 3.35; C = 0.25 *M* potassium dihydrogen phosphate buffer with 0.50 *M* potassium chloride and 2% acetonitrile, pH 5.25.

Time (min)	Volume per cent		
	A	B	C
0	80	20	0
10	60	40	0
20	44	44	12
25	40	40	20
50	25	25	50
75	10	10	80
85	7.5	7.5	85
90	7.5	7.5	85

elution the solutions were deaerated with helium. The flow-rate was kept constant at 1.3 ml/min.

Lymphoid cell fractionation

Lymphoid cells from children with non-B-non-T-cell acute lymphoblastic leukemia (non-B-non-T ALL) before treatment, were fractionated from bone marrow aspirates or from peripheral blood. Lymphocytes from normal persons were isolated from the peripheral blood. Bone marrow aspirates (1.0—2.0 ml) were collected in heparin and purified on a one-step Ficoll—Isopaque gradient according to the description of Bøyum [14]. Peripheral blood lymphocytes were fractionated in the same way. Cells floating at the interface were collected in 1 ml of Tris-buffered minimum essential medium supplemented with 5% foetal calf serum.

Nucleotide extraction

Nucleotides were extracted in the cold by addition of perchloric acid (PCA). A cell suspension ($10—20 \cdot 10^6$ cells) was centrifuged at 900 *g* for 3 min at 4°C. One hundred microlitres of 0.4 *M* ice-cold PCA were added to the cell pellet; the suspension was vortexed and kept on ice for 15 min. Proteins were precipitated by centrifugation at 2200 *g* for 5 min at 4°C. The supernatant was collected and adjusted to a pH between 6.0 and 6.5 with an ice-cold mixture of 4 *M* potassium hydroxide and 1 *M* dipotassium hydrogen phosphate (approximately 30 µl). The neutralized supernatant was kept on ice for another 15 min and the precipitated potassium perchlorate was removed by centrifugation. The extract was kept at —20°C until injection.

RESULTS AND DISCUSSION

Chromatographic conditions

The retention behaviour of a standard mixture of 5'-mono-, 5'-di- and

5′-triphosphates, cyclic AMP, NAD^+, $NADP^+$ and UDPG is shown in Fig. 1. A multi-step gradient was essential for optimal separation of the 5′-monophosphates, cyclic AMP and NAD^+, and of $NADP^+$ and the 5′-diphosphates. In one-step linear gradients poor resolutions were obtained either between the monophosphates or between $NADP^+$, CDP and UDP, or between ADP and IDP.

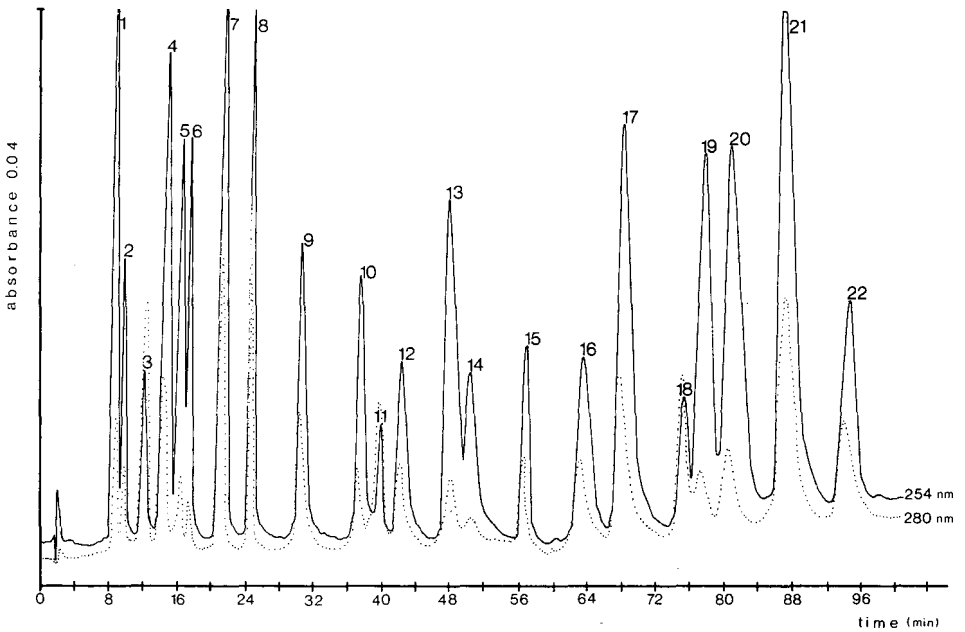

Fig. 1. Elution profile of a test mixture of cyclic AMP, NAD^+, $NADP^+$, UDPG and 5′-mono-, 5′-di- and 5′-triphosphates, as detected at 254 nm and 280 nm (separation conditions are described in Experimental). Retention times in minutes are as follows: (1) cyclic AMP, 8.9; (2) NAD^+, 10.0; (3) CMP, 12.0; (4) UMP, 15.1; (5) AMP, 16.6; (6) IMP, 17.6; (7) GMP, 21.4; (8) UDPG, 24.8; (9) XMP, 30.8; (10) $NADP^+$, 37.4; (11) CDP, 39.2; (12) UDP, 42.0; (13) ADP, 47.7; (14) IDP, 50.2; (15) GDP, 56.8; (16) XDP, 63.4; (17) UTP, 68.0; (18) CTP, 75.1; (19) ITP, 77.7; (20) ATP, 80.8; (21) GTP, 86.9; (22) XTP, 94.4.

Between successive runs the column was equilibrated for 30 min under initial conditions (Table I). In this way the retention times were reproducible. Before and after each series of runs, a standard mixture was analysed. Calibration curves were made by means of standard solutions of different concentrations. Integrated peak areas were calculated from UV scan patterns (254 nm). In addition, peak identification was facilitated using the E_{280}/E_{254} ratios. In Figs. 1—3 the scan patterns at both wavelengths (254 nm and 280 nm) are given. NAD^+, $NADP^+$, UDPG, cyclic AMP and the monophosphates could be quantitated with a lower detection limit of 10 pmol per injection. The diphosphates and triphosphates could be quantitated with a lower detection limit of 20 pmol and 40 pmol, respectively. The linearity of the calibration curves was considered to be sufficient, correlation coefficients being at least 0.997 in a concentration range between detection limit and 5 nmol per in-

jection. The relative standard deviation of peak areas for twenty successive injections of 1 nmol was 0.5%.

Nucleotide recovery after extraction

In order to check the stability of the nucleotides under experimental conditions, standard solutions of AMP, ADP, ATP, CTP, GTP and UTP were extracted with PCA (see Experimental). Thus, 10 μl of a 0.3 m*M* nucleotide standard solution was mixed at 0°C with 100 μl of 0.4 *M* PCA, neutralized with cold KOH—K_2HPO_4 solution and centrifuged.

In the supernatant, CTP and GTP appeared to be the most labile nucleotides with maximal losses of 8% and 5%, respectively. AMP, ADP, ATP and UTP had recoveries greater than 97%.

Analysis of nucleotide pools in human cells

Typical scans of PCA extracts from normal peripheral blood lymphocytes and lymphoblasts of children with non-B-non-T-cell ALL are shown in Figs. 2 and 3, respectively.

Results of studies with 25 patients and 21 controls are given in Table II. The leukemic blasts from patients with ALL before treatment have significantly higher ATP and UTP concentrations than normal lymphocytes. The ATP/ADP, CTP/CDP, GTP/GDP and UTP/UDP ratios (Table III) were higher than those reported by others [13, 15]. Furthermore, the energy charges, (TP + ½DP)/(TP + DP + MP), of the adenosine, guanosine, cytidine and uridine nucleotides were all higher than 0.90 (Table III). The high ratios and the

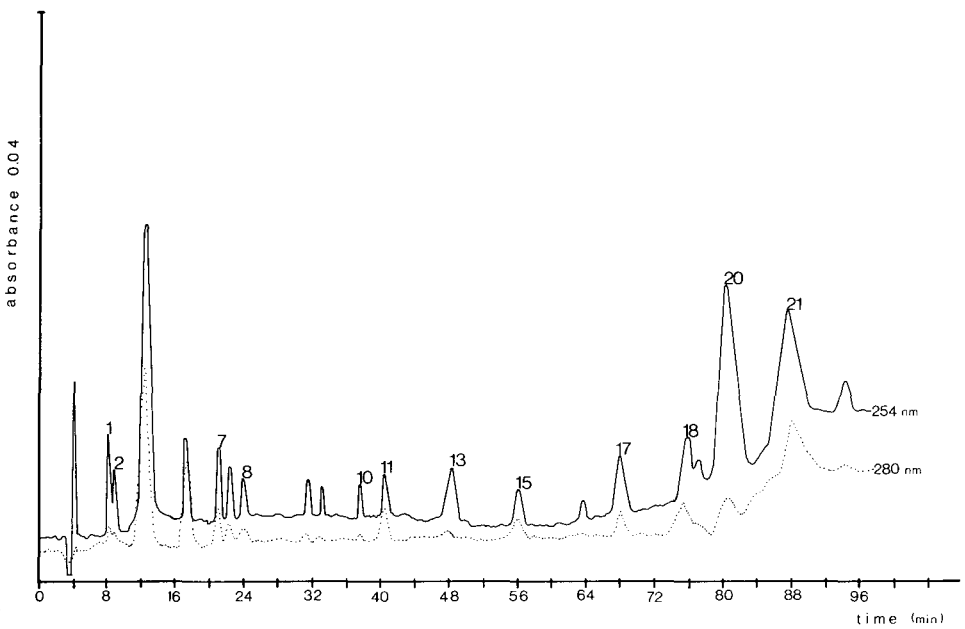

Fig. 2. HPLC profile of nucleotides in a cell extract from normal peripheral blood lymphocytes (for extraction procedure, see Experimental). The peaks can be identified by referring to the legend for Fig. 1.

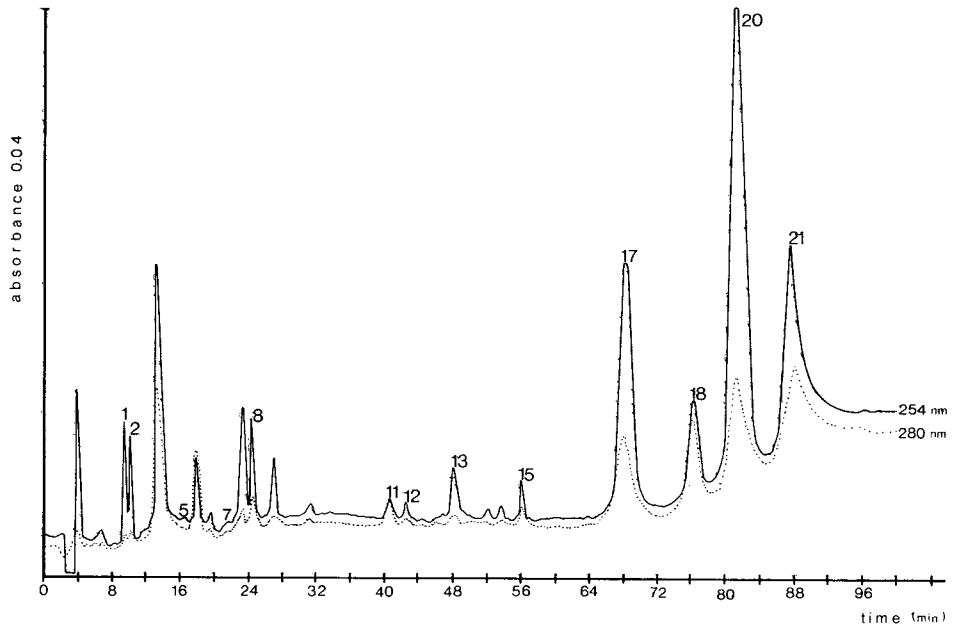

Fig. 3. HPLC profile of nucleotides in a cell extract from lymphoblasts from a patient with non-B-non-T ALL (for extraction procedure, see Experimental). The peaks can be identified by referring to the legend for Fig. 1.

TABLE II

NUCLEOTIDE CONCENTRATIONS IN BONE MARROW (BM) AND PERIPHERAL BLOOD (PB) LYMPHOBLASTS FROM PATIENTS WITH NON-B-NON-T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA AND IN NORMAL PERIPHERAL BLOOD LYMPHOCYTES

n = number of individuals.

Nucleotide	Concentration (pmol per 10^6 cells; mean ± S.D.)		
	Normal lymphocytes	Leukemic lymphoblasts	
	PB (n = 21)	BM (n = 25)	PB (n = 12)
AMP	25 ± 11	34 ± 11	28 ± 15
ADP	53 ± 40	66 ± 22	62 ± 38
ATP	500 ± 140	1005 ± 205	912 ± 210
GMP	7 ± 5	15 ± 10	12 ± 6
GDP	22 ± 10	33 ± 14	21 ± 10
GTP	128 ± 47	211 ± 43	213 ± 55
CDP	12 ± 12	21 ± 18	21 ± 21
CTP	73 ± 44	138 ± 64	108 ± 40
UDP	14 ± 12	23 ± 11	18 ± 7
UTP	102 ± 31	295 ± 107	347 ± 119

TABLE III

NUCLEOTIDE TRIPHOSPHATE/NUCLEOTIDE DIPHOSPHATE RATIOS (TP/DP) AND ENERGY CHARGES, (TP + ½DP)/(TP + DP + MP), CALCULATED FROM MEASURED ADENOSINE, GUANOSINE, CYTIDINE AND URIDINE NUCLEOTIDE CONCENTRATIONS IN NORMAL LYMPHOCYTES AND IN LYMPHOBLASTS FROM PATIENTS WITH NON-B-NON-T ACUTE LYMPHOBLASTIC LEUKEMIA

n = number of individuals.

Nucleotides	Lymphocytes (n = 21)		Lymphoblasts (n = 25)	
	TP/DP (mean ± S.D.)	Energy charge (mean ± S.D.)	TP/DP (mean ± S.D.)	Energy charge (mean ± S.D.)
Adenosine nucleotides	13 ± 7	0.90 ± 0.04	18 ± 8	0.93 ± 0.01
Guanosine nucleotides	6 ± 3	0.91 ± 0.03	9 ± 5	0.92 ± 0.05
Cytidine nucleotides	9 ± 7	0.91 ± 0.03	11 ± 8	0.92 ± 0.03
Uridine nucleotides	11 ± 6	0.93 ± 0.04	17 ± 11	0.95 ± 0.03

high energy charges are supplementary indications that the extraction procedure and the chromatographic method presented here are reliable enough to measure the nucleotide concentrations in lymphoid cells. In conclusion, a more refined separation of a great number of purine and pyrimidine nucleotides can be achieved using our multi-gradient system.

Further studies are now being performed in our laboratory to make an inventory of the nucleotide levels in different immunologically characterized lymphoid cells and to study the effect of cytostatics on these levels.

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CHROMBIO. 1061

DETERMINATION OF OPHIDINE IN HUMAN URINE

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(First received May 21st, 1981; revised manuscript received July 10th, 1981)

SUMMARY

Ophidine (β-alanyl-3-methylhistidine) was first detected in the urine of two patients and later in two members of the laboratory staff loaded with whale meat, by column chromatography, high-voltage paper electrophoresis and two-dimensional paper chromatography.

The ophidine peak was detected between homocarnosine and dimethylarginine using a lithium buffer gradient in column chromatography. In paper chromatography the ophidine spot was detected at a position close to anserine and homocarnosine. The ophidine in the urine from the patients was of dietary origin since it was absent in the urine a few weeks later.

INTRODUCTION

One or two of the dipeptides carnosine (β-alanyl-histidine), anserine (β-alanyl-1-methylhistidine) or ophidine (β-alanyl-3-methylhistidine) (Fig. 1) occur in the muscle tissue of most vertebrates so far examined. In muscles of whales and snakes the main imidazole dipeptide is ophidine [1] (Table I).

The physiology of these dipeptides is unknown. Their possible role in glycolysis, muscle contraction, or as buffers associated with muscles that maintain their energy-rich phosphate ester supplies by anaerobic means has been suggested [1].

Urine samples from two brothers with mental retardation were examined for inborn errors of metabolism. By column chromatography using a lithium buffer a large unknown peak was found about 30 min after 3-methylhistidine. The urine was then examined using high-voltage paper electrophoresis and two-dimensional paper chromatography. The urine was concentrated and the unknown ninhydrin-positive peak was collected, hydrolyzed and identified as ophidine.

Two normal adults were then loaded with whale meat, and when their urines were examined they both contained large amounts of ophidine.

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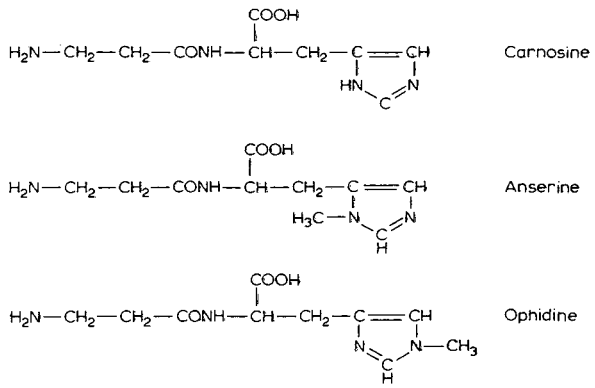


Fig. 1. Structural formulae of three imidazole dipeptides of β-alanine.

TABLE I

AMOUNTS OF CARNOSINE, ANSERINE AND OPHIDINE FOUND IN VERTEBRATE MUSCLE BY VARIOUS WORKERS*

Class	Common name	Muscle	Dipeptide** conc. (μmol/g tissue)		
			Car	Ans	Oph
Mammalia	Ox	Thigh	6.6	1.0	
	Horse	Longissimus dorsi	19.3		
	Cat	Gastrocnemius	6.6	8.3	
	Rabbit	Gastrocnemius	3.5	20.9	
	Fine whale, *Balaenoptera physalus*	L. dorsi	4.3		45.0
	Blue whale, *B. musculus*	L. dorsi	0.4		44.9
	Sei whale, *B. borealis*	Dorsal			37.5
	Bottle-nosed dolphin (newborn)		2.8		3.0
	Dolphin (adult)		5.8		20.2
	Sperm whale	L. dorsi	2.9	7.9	
Aves	Chicken	Pectoral	12.3	40.9	
	Pigeon	Pectoral	0.9	4.6	
Reptilia	Boa constrictor		1.8		0.8
	Cotton-mouthed moccasin		2.2		2.9
	King cobra				5.0
	Sea snake				23.3
	Black-banded sea snake				33.1
	Crocodile		1.4		
Osteichthyes	Atlantic salmon		17.7		

*Modified from Crush [1].
**Car = carnosine, Ans = anserine, Oph = ophidine.

MATERIALS AND METHODS

Urines were obtained from two brothers with mental retardation and from two normal persons. The normal persons were loaded with meat from the whale *Balaenoptera acutorostrata*, which lives along the west coast of Norway. The urines were collected over 4 h after the meal.

Sample preparation

For the determination of the urinary amino acid, the urine was deproteinized by adding 5 mg of sulfosalicylic acid per ml of urine. Before collecting the "ophidine fraction" the urine was first extracted with three volumes of ethyl acetate (pH 1.5—2) and the aqueous layer was concentrated by a rotavapor at 40°C. The concentrated urine was then filtered on an ultrafilter (molecular weight cut-off, > 10,000) and the filtrate was evaporated to dryness on a rotavapor at 40°C. Before analyzing, the dried residue was suspended in water corresponding to ten times the concentration of the urine.

Hydrolysis

The concentrated fraction (100 μl) was hydrolyzed in 6 *N* HCl for 20 h at 100°C. After evaporation to dryness, the hydrolyzates were dissolved in 100 μl of distilled water.

Two-dimensional paper chromatography

Whatman paper No. 1, 20 × 20 cm, was used in Shandon equipment for ascending chromatography at 23°C. In the first dimension the solvent was pyridine—water—acetone—ammonia (45 : 30 : 20 : 5, v/v). In the second dimension the solvent was isopropanol—formic acid—water (75 : 12.5 : 12.5, v/v).

Spray reagents

Ninhydrin (3 g) was dissolved in collidine (50 ml) and made up to 1000 ml with isopropanol.

High-voltage paper electrophoresis

A Pherograph original (Nach Wieland Pfleiderer DBCM. No. 1713858, Frankfurt, G.F.R.) was employed with Macherey Nagel paper (No. 214, 35 × 40 cm). The buffer (0.05 *M* $NaB_4O_7 \cdot 10H_2O$) was adjusted to pH 9.25 with 0.1 *N* NaOH and the electrophoresis was run for 4 h. The paper was then dried at 50°C and a second run carried out with isopropanol—acetic acid—water (8 : 1 : 1, v/v) for ascending paper chromatography.

Liquid column chromatography

The amino acids were analyzed using a Technicon amino acid analyzer. The analyzer was equipped with a glass column (140 × 0.6 cm I.D.) packed with Chromobeads Type B resin. The buffer solution for the gradient elution contained lithium according to the method of Perry et al. [2]. The column was operated at 35°C until the glutamine was eluted, whereupon the temperature was raised to 70°C. The Technicon integrator—calculator was used to

quantitate all of the amino acids. Norleucine was used as the internal standard. The concentration of the ninhydrin-positive substances is expressed as μmol/mmol of creatinine in the urine.

RESULTS

An unknown ninhydrin-positive peak was detected in the urine from the two brothers about 30 min after the 3MeHis peak on the amino acid analyzer (Fig. 2). The retention time was 1134 min (see Table II). When two members of the laboratory staff were loaded with whale meat, a peak with the same retention time was obtained (Fig. 3).

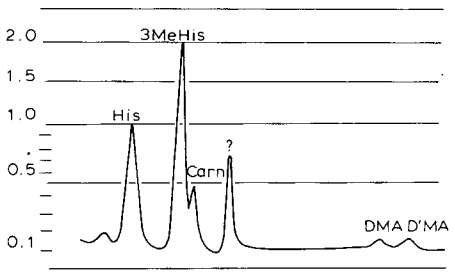


Fig. 2. The chromatogram of ninhydrin-positive histidine compounds from one of the patients using the Technicon amino acid analyzer. The unknown peak was eluted about 30 min after 3MeHis. DMA = Guanidino-N,N-dimethylarginine; D'MA = guanidino-N,N'-dimethylarginine.

TABLE II

THE RETENTION TIMES OF HISTIDINE AND ITS DERIVATIVES, USING THE TECHNICON AMINO ANALYZER WITH LITHIUM GRADIENT

Histidine compounds	Retention time (min)
Histidine	1064
1-Methyl-histidine	1085
Anserine	1098
3-Methyl-histidine	1102
Carnosine	1111
Homocarnosine	1114
Ophidine	1134

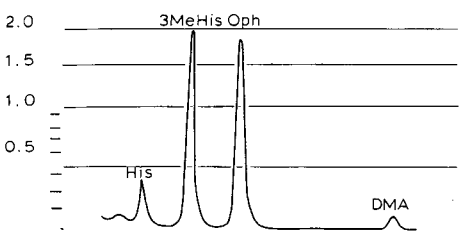
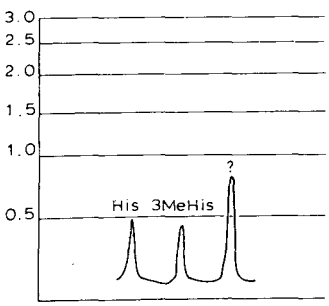


Fig. 3. The chromatogram shows the histidine and histidine derivatives from one of the two normal persons loaded with whale meat. The ophidine peak was eluted about 30 min after 3MeHis.

After extraction with ethyl acetate and ten-fold concentration of the urine samples from the brothers and the normal persons, the unknown ninhydrin-positive peak was collected on a sample collector. Fig. 4 shows the concentrated fraction from one of the brothers with added His and 3MeHis. In Fig. 5 the hydrolyzed products from the same fraction are shown together with added His. The hydrolyzed fraction contained only βAla and 3MeHis, which proves that the unknown peak is ophidine.

The positions of the histidine and the histidine derivatives occurring in human urine upon two-dimensional paper chromatography are shown in Fig. 6.



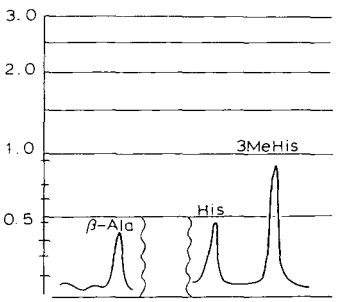


Fig. 4. Concentrated fraction of the unknown substance from the urine of one of the patients. 3MeHis and His are added to the fraction as reference substances.

Fig. 5. Hydrolysis products of the fraction shown in Fig. 3. His was added as a reference.

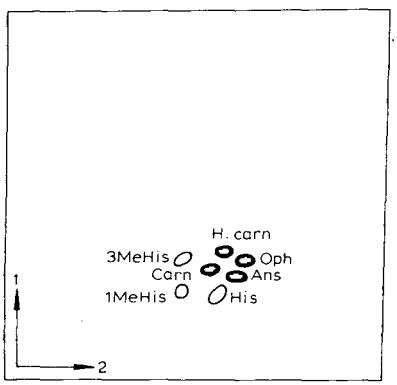
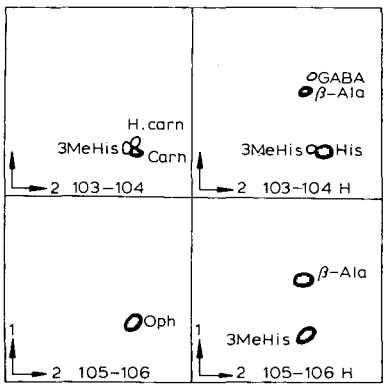


Fig. 6. Separation of standard solutions of histidine and histidine derivatives by two-dimensional paper chromatography.

When the concentrated fractions of the urines from the two brothers and two normal persons were collected, both the fractions and their hydrolysis products were separated by two-dimensional paper chromatography. Fig. 7 shows the unknown ninhydrin-positive fraction (105—106) from one of the brothers' urine, and the fraction (103—104) before it. The same fractions (103—104 H and 105—106 H) after hydrolysis prove that fraction 103—104 contains both homocarnosine (γ-aminobutyryl-histidine) and carnosine, while fraction 105—106 contains only ophidine.

In Fig. 8 the paper chromatogram of urine from one of the two persons loaded with whale meat is shown. No homocarnosine was present, but otherwise the hydrolyzed carnosine and ophidine fractions (103—104 H and 105—106 H) show the same picture as the hydrolyzed fractions from the patient (Fig. 7).



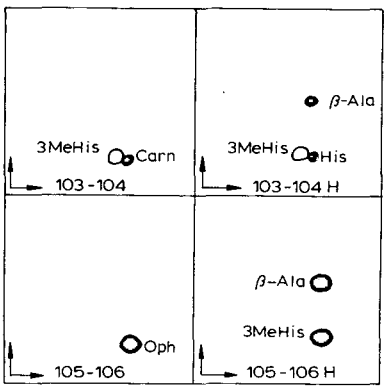


Fig. 7. Paper chromatography of two concentrated fractions collected after column chromatography of the urine from one of the two patients. The two chromatograms to the right are of the hydrolysis products. H = hydrolyzed.

Fig. 8. Paper chromatography of the two concentrated fractions collected from a normal person loaded with whale meat. H = hydrolyzed.

To verify further that the patients really excreted ophidine (containing 3MeHis) and not anserine (containing 1MeHis), the hydrolyzed fraction of the unknown peak was separated by high-voltage paper electrophoresis. In this run the histidine and methylhistidines are clearly separated. In fraction 105—106 H from the patient only 3MeHis was detected (as shown in Fig. 9), which proves the presence of ophidine.

Urine samples received from the two brothers six weeks later did not contain ophidine.

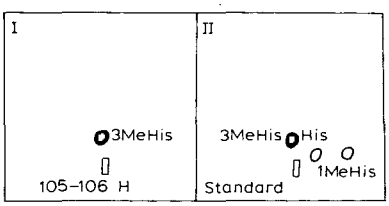


Fig. 9. Separation of histidine and histidine derivatives using high-voltage electrophoresis as the first run and paper chromatography as the second run. (I) hydrolyzed fraction from the concentrated urine from one of the patients. (II) standard solution of His, 1MeHis and 3MeHis. ▯ represents the spot of application. H = hydrolyzed.

DISCUSSION

Quantitative determination of amino acids in physiological fluids using

an automatic amino analyzer is one of the routine procedures for detecting inborn errors of metabolism. This analyzer was used to quantitate the amino acids in the urine from two brothers with mental retardation. They were excreting an excessive amount of an unknown compound and when their urines were hydrolyzed a large amount of βAla and 3MeHis appeared. The unknown peak was collected from the amino acid analyzer and examined before and after hydrolysis by paper chromatography and high-voltage electrophoresis and identified as the dipeptide ophidine.

There is evidence that the diet can affect the dipeptide content of tissues. It has been found that the carnosine content of white muscle of the cat could be decreased by fasting the animal or by preventing use of the muscles. On the other hand, carnosine could be increased by liberal feeding of meat [3]. In salmon on a histidine-deficient diet, the histidine and carnosine in the skeletal muscle were depleted, but the anserine concentration was maintained [4].

Anserine is normally excreted by man after eating rabbit or chicken meat which are rich in anserine (see Table I).

Ophidine has been described in earlier papers [1] as a peptide in the muscle of whales and snakes, together with carnosine (see Table I). When two normal persons were loaded with whale meat they both excreted large amounts of ophidine, just as the two brothers did.

Urine samples from the two brothers collected six weeks after the first sample did not contain any ophidine. This fact indicates that the dipeptide ophidine in the urine from these two brothers was of dietary origin.

ACKNOWLEDGEMENT

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DETERMINATION OF NEOPTERINE IN HUMAN URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Since the analysis of urinary neopterine is important in diagnosing malignancy, a method has been developed for its rapid and sensitive separation and quantitation using high-performance liquid chromatography (HPLC) on reversed phase. Eluted neopterine is monitored by fluorescence at an excitation wavelength of 353 nm and measured at 438 nm. Separation was optimized by elution with 15 mmol/l potassium phosphate (pH 6.4) at a flow-rate of 0.8 ml/min.

Urinary neopterine was related to creatinine with the aim of reducing variations due to fluctuating urinary concentrations. The proposed method has good performance characteristics and is not influenced by the presence of reduced neopterine in urine.

Using this HPLC method, urinary neopterine related to creatinine was determined for 148 healthy male adults (mean neopterine/creatinine 113 μmol/mol), 146 healthy female adults (mean neopterine/creatinine 140 μmol/mol) and 60 healthy children (mean neopterine/creatinine 163 μmol/mol). The neopterine levels for four healthy individuals were measured daily over a period of one month.

INTRODUCTION

Determination of pteridines in biological fluids is considered to be important, since there are reports showing a correlation between high pteridine excretion rates and increased growth or proliferation. Previous investigations conducted in our laboratory [1,2] have revealed a connection between urinary excretion of a fluorescent compound and malignant growth. These preliminary observations were backed up by experiments on mice with Ehrlich ascites tumor, which showed a significant increase of a urinary fluorescent metabolite in response to the tumor [3]. We identified this fluorescent metabolite as 6-hydroxylumazine originating during analysis from non-fluorescent 7,8-

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dihydro-6-hydroxylumazine by autoxidation [4] and the metabolite from human urine as neopterine [5]. Recently, it was shown that in patients with haematological neoplasmias the urinary neopterine levels correlated well to clinical features and to tumor staging [6]. Moreover, the pteridine excretion is generally altered when there is cell proliferation or growth [7—11].

The reports demonstrate that a need exists for a simple and rapid method of measurement of pteridines in biological fluids, particularly of urinary neopterine. Published methods for determining pteridines by ion-exchange separation [12,13] have proved to be tedious and time-consuming. This paper, therefore, proposes a simple and rapid technique for a specific and sensitive measurement of urinary neopterine by high-performance liquid chromatography (HPLC) on reversed phase. The method is well-suited for automation and routine clinical laboratory use together with a quantification of creatinine and uric acid levels [14,15].

This report also describes an application of the method to quantify neopterine excreted in the urine of healthy subjects as a function of sex and age.

EXPERIMENTAL

Chemicals

Acetonitrile (chromatography grade), dipotassium hydrogen phosphate and potassium dihydrogen phosphate (analytical grade) were purchased from Merck (Darmstadt, G.F.R.). Neopterine was obtained from Fluka (Basle, Switzerland).

Apparatus

Chromatography was performed on a Varian Model LC 5000 instrument (Varian Assoc., Palo Alto, CA, U.S.A.) equipped with an automatic sample injection system (Auto Sampler 8000, Varian) in conjunction with a 10-μl injection valve (Valco Instruments, Houston, TX, U.S.A.). The effluent was monitored with a spectrofluorometer Model SFM 23 (Kontron, Eching/Munich, G.F.R.) and with a Model PM 2 DLC variable-wavelength UV absorption detector (Zeiss, Oberkochen, G.F.R.). Peak areas were measured with a Model CDS 111 chromatography data system (Varian).

Column

Ready-to-use columns (stainless steel, 300 × 3.9 mm I.D.), packed with 10-μm Bondapak C_{18}, were purchased from Waters Assoc., Milford, MA, U.S.A.

Liquid chromatography

Chromatographic elution is performed with an aqueous 15 mmol/l potassium phosphate buffer (pH 6.4, column temperature 30°C). The buffer is degassed under vacuum just prior to use. During a period corresponding to the first 7 min, a flow-rate of 0.8 ml/min is suitable for separation of urinary pteridines. During the ensuing period, from 7—8 min, a linear flow gradient between 0.8 and 1.4 ml/min and a linear elution gradient between 0 and 1.218 mol/l acetonitrile can be used to cleanse the column. In the next period, from 8—12.5 min, the flow and composition remain constant. From 12.5—13.5 min the starting

conditions are restored. The effluent is monitored by setting the fluorescence detector for excitation at 353 nm and emission at 438 nm. The absorption detector is set at 235 nm. Chromatograms are quantified by means of a chromatography data system employing the external standard method.

Collection of specimens

The first morning urine was collected from 148 healthy male and 146 female volunteers and from 60 healthy children. Samples were either analysed immediately, or stored at −20°C in the dark. When the urine samples were kept in a frozen state we added 100 mg of disodium ethylenediamine tetraacetate to 10 ml of urine.

To protect neopterine from photooxidation, all operations were conducted in dim light.

Sample preparation

To avoid column contamination we prepurified the urinary samples through commercially available Sep-Pak C_{18} cartridges (Waters Assoc.); 1.0-ml aliquots of urine were pressed through the cartridge using a 10-ml syringe with Luer end fitting. The high-polarity components such as neopterine, creatinine and uric acid were eluted with 9 ml of water. The 1.0-ml and the 9-ml elution were mixed and a 10-µl aliquot corresponding to 1 µl of urine is injected on to the column.

Sediment formation

To take the possibilities of error due to urinary sediments into account, the following sediments were precipitated in the presence of neopterine and creatinine contained either in standard solutions or urinary sample: calcium phosphate, calcium oxalate, calcium carbonate, sodium urate and uric acid [16]. After centrifugation the precipitates as well as the supernatants were tested for neopterine and creatinine. Urine, or standard solutions containing 5.92 µmol/l neopterine, were irradiated with light of a wavelength of either 254 nm or 366 nm, using a Model Uvis mercury lamp (Desaga, Heidelberg, G.F.R.). The distance from the lamp was 12 cm. Assays for the oxidation of neopterine (5.92 µmol/l) of 7,8-dihydroneopterine (14.9 µmol/l) and of 5,6,7,8-tetrahydroneopterine (10.89 µmol/l) were carried out by stirring with manganese dioxide and hydrogen chloride, by digesting with hydrogen peroxide, or by blowing in atmospheric oxygen.

RESULTS

Development of separation

The experimental conditions were optimized to permit quantitation of polar components such as neopterine and creatinine. The mobile phase was varied by altering the concentration and pH of aqueous potassium phosphate buffer and by adding acetonitrile, methanol or tetrahydrofuran. The best separation was obtained using the buffer alone. Although a slightly superior resolution resulted with lower phosphate concentrations, we chose 15 mmol/l phosphate. Thus better reproducibility of retention times was achieved due to higher

buffering capacity. Separation decreased in the presence of various concentrations of acetonitrile, methanol or tetrahydrofuran. It was found that retention times were mostly sensitive to changes in pH. A pH optimum for the separation of neopterine, creatinine and uric acid was reached at a value of 6.4. At this pH, superior resolution resulted, especially in the first part of the chromatogram.

A flow-rate of 0.8 ml/min was found to be suitable for obtaining high resolution in the interesting part of the chromatogram. After elution of neopterine, a flow and concentration program was instituted by adding acetonitrile. This permitted rapid purification of the column, thus reducing analysis time. Fig. 1 shows the separation of a urine sample chromatographed under the described conditions.

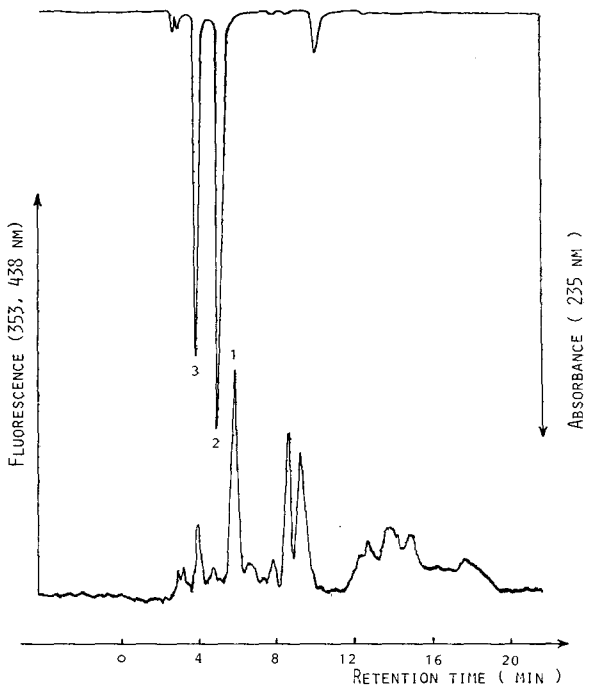

Fig. 1. Chromatogram of neopterine (1.5 nmol), creatinine (13 μmol) and uric acid (2.0 μmol) in a 1-ml urine sample. Upper chromatogram was obtained with the UV detector (0.64 a.u.f.s. at 235 nm). Lower chromatogram was obtained with the fluorescence detector (353 nm excitation and 438 nm measuring). Separation of urine was on a 10-μm Bondapak C_{18} column (300 × 3.9 mm I.D.). Solvent, 15 mmol/l potassium phosphate buffer (pH 6.4); flow-rate, 0.8 ml/min; column temperature, 30°C. Peaks: 1 = neopterine, 2 = creatinine, 3 = uric acid.

Elution through Sep-Pak

Urinary components more non-polar than xanthine were removed by sample preparation with Sep-Pak C_{18} cartridges. By this prepurification we avoided column contamination by solid or non-polar urinary components. Further, the dilution obtained has the advantage of remaining in the linear range for creatinine estimation. Although the precision of absolute neopterine and

creatinine values was diminished slightly by this sample prepurification, the neopterine/creatinine ratio is much less affected and has an acceptable level of precision. Using standard solutions, 99.7% creatinine and 99.7% neopterine were eluted with 9 ml of water. In this sample preparation the neopterine/creatinine ratio did not appear to be affected.

Relation of urinary neopterine to creatinine

Because of physiologically variable concentrations of urine, it is useful to relate urinary neopterine to creatinine. Table I shows that the neopterine/creatinine ratio of three typical individuals remains relatively constant during a 24-h period. On the other hand, the neopterine and creatinine levels show distinct variations. Use of such a neopterine/creatinine relation decreases the physiological variation in the urinary concentration as well as eliminating possible errors due to sample preparation or sample injection.

Urinary sediments

Urinary sediments composed of calcium phosphate, calcium oxalate or calcium carbonate did not contain neopterine or creatinine. Here, all the neopterine and creatinine were recovered in the supernatant. In sediments of uric acid or sodium urate using standard solutions or urine, the precipitate contained neopterine and creatinine. In the supernatant the neopterine levels were 16% lower and the creatinine levels 10% lower than the starting values. From this result it is to be concluded that, when the urine samples are kept frozen, sediment formation has to be prevented, for example through the addition of 100 mg of disodium ethylenediamine tetraacetate to 10 ml of urine.

Stability of neopterine and of its reduced forms

Neopterine is not oxidized by air within 7 h at pH 6.4 and pH 1.0 in standard solution or urine, respectively. Since neopterine is found in human urine in reduced as well as in oxidized form, the measurement of both is possibly of interest. In assaying the oxidation of 7,8-dihydroneopterine and 5,6,7,8-tetrahydroneopterine, best results were obtained using manganese dioxide and hydrogen chloride for 15 min. In standard solutions, 7,8-dihydroneopterine produced only 60—70% neopterine. Using a urine sample or tetrahydroneopterine the yield varied even more. Thus, we found that the oxidation of reduced neopterines by these procedures appeared not to give consistent and comparable results.

Upon irradiation at 254 nm and 366 nm, neopterine photooxidizes markedly within 5 min, showing the importance of always working in a dim light.

Performance characteristics

Linearity and sensitivity. Excellent linearity was noted in the relationship of the peak area of standard solutions to neopterine concentration, which ranged from 200 nmol/l to at least 40 μmol/l. The following regression equation was obtained: $y = 1.006\,x - 2.94$; $r = 0.9999$.

Sensitivity and selectivity were increased by measuring fluorescence at an excitation wavelength of 353 nm and an emission wavelength of 438 nm. The

BLE I

VELS OF URINARY CREATININE, NEOPTERINE AND NEOPTERINE IN RELATION TO CREATININE OF THI
VIDUALS DETERMINED DURING A 24-h PERIOD

	Individual I (37 years, female)			Individual II (26 years, male)			Individual III (51 years, female)		
	Creatinine	Neopterine	Neopterine/ creatinine	Creatinine	Neopterine	Neopterine/ creatinine	Creatinine	Neopterine	Neopteri creatinir
	7	7	7	6	6	6	6	6	6
	10.4 mmol/l	1.53 μmol/l	151 μmol/mol	11.5 mmol/l	1.05 μmol/l	92 μmol/mol	19.0 mmol/l	2.06 μmol/l	109 μmol/m
.D.	5.0 mmol/l	0.70 μmol/l	26 μmol/mol	2.4 mmol/l	0.22 μmol/l	14 μmol/mol	5.4 mmol/l	0.58 μmol/l	16 μmol/m
/. (%)	48.1	45.8	17.2	20.9	21.0	15.2	28.4	28.2	14.7

sensitivity at a peak-to-noise ratio of 5 : 1 was determined to be 120 nmol/l of undiluted sample, or 120 fmol per injection.

Precision and accuracy. Within-run precision was obtained by assaying 25 aliquots of three urine samples containing different concentrations of neopterine and creatinine. In the same way, day-to-day precision was observed for 15 days, using three urine samples. Table II shows the reproducibility within-day and between-day for neopterine in relation to creatinine. Recovery experiments with six spiked urine samples yielded a mean value of 102.2% for neopterine and of 99.3% for neopterine in relation to creatinine.

TABLE II

PRECISION OF URINARY NEOPTERINE IN RELATION TO CREATININE DETERMINED BY HPLC ON REVERSED PHASE

	Sample No.					
	1	2	3	4	5	6
	Within-run			Day-to-day		
n	20	24	24	15	15	15
$\bar{x}$ (μmol/mol)	95	214	244	183	260	229
± S.D. (μmol/mol)	6.2	8.1	9.3	13.6	7.3	16.3
C.V. (%)	6.5	3.8	3.8	7.4	2.8	7.1

Specificity and interferences. Urine contains only a few components having similar polarity or similar fluorescence characteristics; most are pteridines. The following pteridines did not interfere because of their different elution times: 6-pterincarboxylate, monapterine, biopterine, xanthopterine, isoxanthopterine, pterine and 6-hydroxymethylpterine. 7,8-Dihydroneopterine and 5,6,7,8-tetrahydroneopterine eluted with the same retention time as neopterine. Because of the very low fluorescence of 7,8-dihydroneopterine at the fluorescence wavelengths used and because tetrahydroneopterine did not fluoresce, any positive bias in measuring neopterine remains negligible.

A comparison of fluorescence spectra for standard and eluted urine samples provides further information on possible interferences. The two fluorescence maxima were identical for both samples (353 and 438 nm). Additionally, the fluorescence maxima of 7,8-dihydroneopterine (362 and 408 nm) were detected in urine, indicating that this compound is eluted together with neopterine. This result corroborates the specificity of the urinary neopterine estimation.

Practicability. Analysis of one urine sample, including preparation and column regeneration, takes 20 min.

Application

The technique was applied to the study of neopterine related to creatinine in healthy subjects as a function of sex and age. Table III shows the results for 148 male and 146 female individuals with mean age of 40.3 and 42.2 years, respectively. The mean urinary neopterine level related to creatinine is lower in males than in females. The mean values vary only a little between different age groups.

TABLE III

URINARY NEOPTERINE IN RELATION TO CREATININE AS A FUNCTION OF SEX AND AGE

Sex	Age (years)	Number of individuals	Neopterine/creatinine (μmol/mol)		C.V. (%)
			$\bar{x}$	± S.D.	
Male	3—18	35	168	64.8	38.6
	19—25	39	118	29.9	23.7
	26—35	25	97	32.6	33.6
	36—45	28	113	29.5	26.1
	46—55	23	103	36.2	35.2
	56—65	18	115	44.2	38.4
	66—80	15	120	39.8	33.2
	19—80	148	113	35.3	31.2
Female	3—16	25	155	62.5	40.3
	17—25	38	128	34.4	25.9
	26—35	21	124	31.3	25.2
	36—45	26	142	40.2	28.3
	46—55	21	147	29.9	20.3
	56—65	20	156	34.4	22.1
	66—85	20	146	37.1	25.4
	17—85	146	140	35.7	25.5
Children					
Male	3—18	35 } 60	163	65.1	39.9
Female	3—16	25 }			

In the children's group notably higher mean urinary neopterine values were observed than in the adult group. Similarly, the mean value is slightly higher in females above the age of 35 years. Within a particular age group the level of neopterine related to creatinine shows a very low standard deviation and coefficient of variation, indicating a relatively constant excretion of neopterine. In addition, the urinary neopterine levels were controlled daily in four normal individuals over a one-month period. Table IV shows that the level of neopterine related to creatinine varies within the same range as is found to be typical for people of the same sex and age group.

TABLE IV

NEOPTERINE IN RELATION TO CREATININE DETERMINED OVER A PERIOD OF ONE MONTH FOR FOUR INDIVIDUALS USING THE FIRST MORNING URINE

	Individual			
	1	2	3	4
Sex	Female	Female	Female	Male
Age (years)	36	37	26	26
n	31	31	31	28
$\bar{x}$ (μmol/mol)	154	111	115	105
± S.D. (μmol/mol)	43	30	36	30
C.V. (%)	27.9	32.5	31.9	28.6

DISCUSSION

Analytical considerations

The method described permits very rapid and sensitive measurement of urinary neopterine, creatinine and uric acid in a single chromatographic run. Quantification of these urine components inclusive of sample prepurification can be accomplished in 20 min. The technique is suitable for routine clinical laboratory use.

Application to healthy individuals

The higher mean neopterine value for children indicates fast growth behaviour. Interpretation of the slightly increased mean neopterine values of females over 35 years of age is the subject of further investigations.

Quantitation of neopterine present in oxidized or reduced form

Since it is well known that neopterine originates through biosynthesis from guanosine triphosphate via reduced forms [17], estimation of neopterine in its reduced forms should also be of interest. Measurement is restricted to urinary neopterine in the oxidized form, because oxidation of reduced forms is not yet possible with the desired precision and accuracy. Reduced forms of neopterine do not fluoresce or only slightly. Thus the neopterine present in oxidized form alone can be determined with a high level of sensitivity. Further, under dim light, neopterine as well as its reduced forms remain sufficiently stable for hours when exposed to atmospheric oxygen. This method, therefore, gives good precision and is not subject to distortion through the appearance of reduced forms of neopterine or through the disappearance of the latter as the result of oxidation. The fact that measurement of urinary neopterine without determination of reduced forms showed slight biological variations among healthy individuals, as well as good correlations to diagnosis in cases of malignancy, indicates that this method of estimation can be useful in the diagnosis and monitoring treatment of neoplastic diseases.

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ASSAY OF BROMHEXINE IN HUMAN PLASMA BY CAPILLARY GAS—LIQUID CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION AND SELECTED ION MONITORING

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SUMMARY

A specific, sensitive method for the determination of bromhexine in human plasma is described. It comprises a selective extraction procedure and a specific determination with capillary gas—liquid chromatography and nitrogen-selective flame ionization detection. The detection limit of the assay is about 0.5 ng/ml. The specificity of the assay was checked by gas chromatography—mass spectrometry. The method is applied to the pharmacokinetics of bromhexine in humans.

INTRODUCTION

Bromhexine (Bisolvon®, Thomae, Biberach, G.F.R.), N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)-amine, is widely used as a mucolytic agent in human and veterinary medicine [1—3].

Pharmacokinetic and metabolic investigations of ^{14}C-labelled bromhexine in man and in animals have shown that bromhexine undergoes extensive and rapid metabolism, yielding mainly dealkylated and hydroxylated metabolites and conjugates [4—7].

Successful therapy in man with bromhexine can be achieved using very low doses (8—15 mg). This low dosage, the rapid biotransformation, and the large distribution volume resulting from the lipophilic character of the drug [8] are the reasons for the very low plasma levels (nanogram range). Until now, only a few methods have been published concerning the quantitative determination of bromhexine in biological material. Eichler and Kreuzer [9] have used gas—liquid chromatography (GLC) with electron-capture (EC) detection for analysing bromhexine residues in animal tissues and in milk. Plasma levels have

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been determined by Matsumara et al. [10] using GLC with nitrogen-selective flame ionization detection (N-FID). De Leenheer and Vandecasteele-Thienpont [11] have reported on the GLC behaviour of bromhexine and derivatization methods, as well as on the determination of plasma levels in man [12] by EC-GLC.

The published methods are not satisfactory for several reasons. Although multiple extraction steps are performed, no [9] or no appropriate [10] internal standard has been used, and neither accuracy nor precision have been evaluated. On the other hand, in methods with good precision [12], no studies have been performed on the specificity. Based on our experience, the use of packed columns is not sufficient for the separation of the parent compound from its many similar basic metabolites; the stationary phases used were SE-30 [9, 12], OV-101 [10], mixed phase SE-30 and DEGS [9]. This was found also by Eichler and Kreuzer [9], and they therefore used a thin-layer chromatography clean-up step. In addition, the sensitivity of all the published methods is too low for detailed pharmacokinetic studies, with the exception of a recent gas chromatographic—mass spectrometric (GC—MS) assay using derivatisation and packed columns [13]. We have therefore developed a method for the determination of bromhexine in human plasma with appropriate precision, specificity and sensitivity.

EXPERIMENTAL

Reagents and chemicals

Bromhexine [Bisolvon®, N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)-amine (Fig. 1, I), the internal standard [N-cyclopentyl-N-methyl-(2-amino-3,5-dibromobenzyl)-amine] (Fig. 1, II), and the metabolites, named according to Schraven et al. [4], were of analytical grade. The most important metabolites are: M II = N-cyclohexyl-N-(2-amino-3,5-dibromobenzyl)-amine; M V = N-(*trans*-4-hydroxy-cyclohexyl)-N-methyl-(2-amino-3,5-dibromobenzyl)-amine; M VIII = N-(*trans*-4-hydroxy-cyclohexyl)-N-(2-amino-3,5-dibromobenzyl)-amine; and M XI = 6,8-dibromo-3-(*trans*-4-hydroxy-cyclohexyl)-1,2,3,4-tetrahydro-quinazoline.

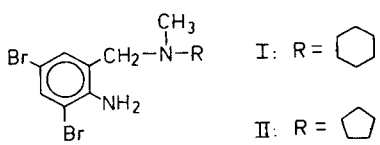

Fig. 1. Structural formulae of bromhexine (I) and the internal standard (II).

^{14}C-Labelled bromhexine (specific activity = 23.4 μCi/mg = 8.65 · 10^5 dps per mg) was synthesized in the isotope laboratory of Dr. Karl Thomae GmbH. It was labelled in the benzylic position.

n-Hexane (Nanograde quality, No. 4159) was from Mallinckrodt (Wesel, G.F.R.). All other reagents were purchased from E. Merck (Darmstadt, G.F.R.): p.a. quality, sulfuric acid (No. 731), sodium hydroxide (No. 6498) and 1 *M* KH_2PO_4 solution (No. 4881) were used. The buffer solutions for the partition studies were made by mixing 1 *M* KH_2PO_4 with 1 *M* H_3PO_4 (No. 552)

(pH range 1—4) and 1 *M* KH_2PO_4 with 1 *M* Na_2HPO_4 (No. 6589) (pH range 4.5—7). The perchlorate solutions were made by mixing 1 *M* $NaClO_4$ (No. 6564) and 1 *M* $HClO_4$ (No. 519).

The internal standard (Fig. 1, II) was used in a concentration of 10 ng/ml in hexane.

Apparatus

The gas chromatograph used was a Hewlett-Packard 5840 equipped with a nitrogen—phosphorus-sensitive-detector, Model No. 18848A, and an autosampler No. 7672.

The column was a 25 m × 0.3 mm I.D. WCOT capillary column with OV-17 as the stationary phase (Perkin-Elmer, Überlingen, G.F.R.).

Operating conditions were injector temperature 240°C, detector temperature 260°C. After injection in the splitless mode, the split was opened 1 min later and the temperature program was started. The column temperature was programmed from 60°C to 140°C at 10°C/min and then to 230°C at 5°C/min. Gas flow-rates were helium (carrier) 2 ml/min, hydrogen 3 ml/min, air 50 ml/min, helium (auxiliary gas) 35 ml/min.

The gas chromatograph—mass spectrometer was a Finnigan 3300 with 6015 data system. The gas chromatograph was equipped with the same column as the GC—N-FID system. The operating conditions in this case were injector temperature 260°C. After injection in the splitless mode, the split was opened 1 min later and the temperature program was started. The column was programmed from 60°C to 230°C at 20°C/min. The gas flow-rate was about 2 ml/min. The column was directly connected to the transfer line.

The mass spectra of bromhexine and the internal standard are depicted in Fig. 2. The following ions were monitored: m/z = 293 [M^+ — cyclohexyl (C_6H_{11}) = 83 mass units] and m/z = 264 [M^+ — N-methyl-cyclohexyl ($C_7H_{14}N$) = 112 mass units).

The helium was always purified by an Oxisorb-Kit purchased from Messer-Griesheim (Frankfurt, G.F.R.) to improve the service life of the column. The evaporation of the organic extracts was carried out with a Vortex-Evaporator (Searle-Buchler, Fort Lee, NJ, U.S.A.).

Analytical procedures

Extraction. The blood was sampled using heparinized syringes (Monovette®, Sarstedt, Nümbrecht, G.F.R.), centrifuged and the plasma kept at −30°C in glass tubes. It was thawed at room temperature and 1-ml portions were pipetted into 25-ml centrifuge tubes. For plasma levels lower than 5 ng/ml, 2 ml of plasma were used, and for levels above 40 ng/ml 0.5-ml samples were used. Then 2 ml of buffer and 10 ml of internal standard solution were added to the plasma. The pH attained was about 5.2. After extraction and phase separation the aqueous layer was discarded and the organic layer was extracted with 1.0 ml of 2 *N* sulfuric acid. After phase separation the aqueous phase was washed once with 5 ml of hexane. Then the aqueous phase was adjusted to pH 12.5 with 0.5 ml of 5 *N* sodium hydroxide. The aqueous phase was extracted twice with 5 ml of hexane.

The hexane was removed in silanized tubes by the evaporator. The residue

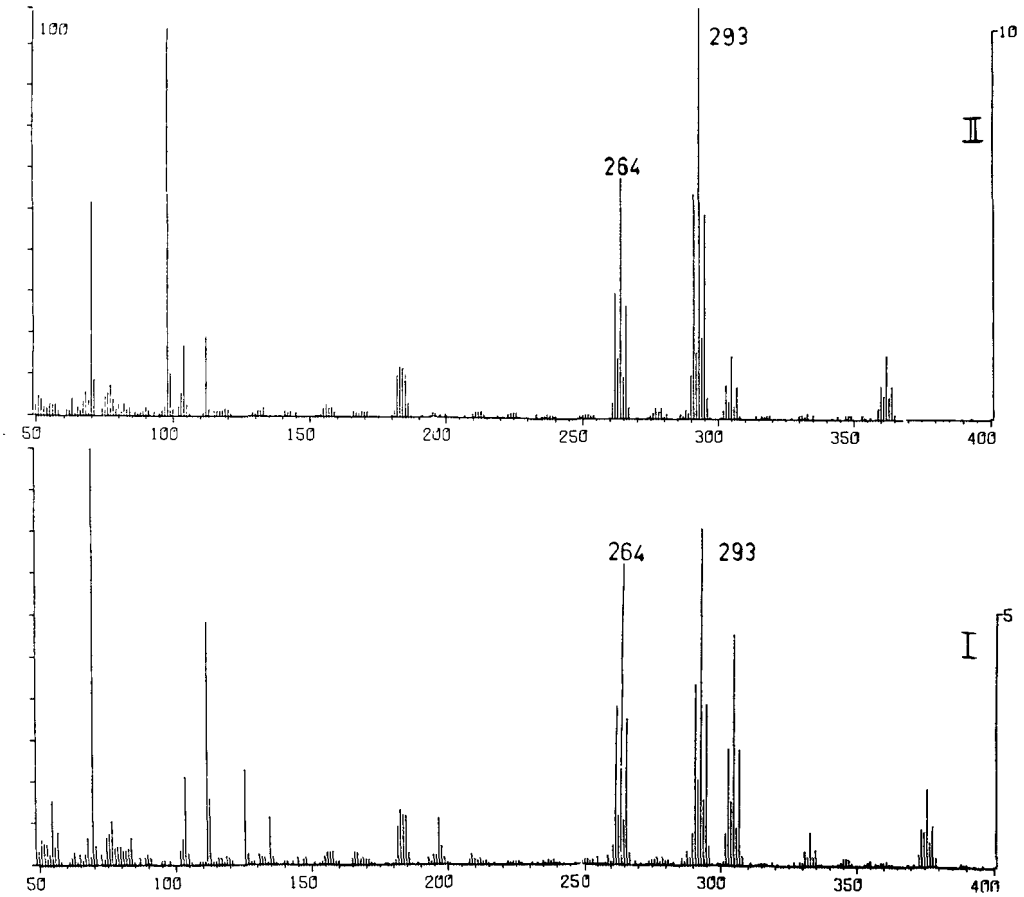

Fig. 2. Electron-impact (70 eV) mass spectra of bromhexine (I) and internal standard (II).

was then dissolved in 25 μl of hexane and transferred into autosampler vials; 2 μl were injected into the chromatograph. For GC—MS, the volume was reduced by concentration to about 5 μl and 2 μl were injected.

All extraction steps were performed with a shaking machine (120 strokes per min) for 15 min. Phase separation was always performed by freezing out the aqueous phase at −20°C and decanting the organic layer.

Preparation of the calibration curve. The calibration curve was constructed by preparing an aqueous stock solution containing 400 ng/ml bromhexine hydrochloride. From this aqueous solution 2.5 ml were added to 47.5 ml of human plasma. After incubation (2 h, 37°C), this plasma (20 ng/ml) was diluted with non-spiked plasma to obtain a concentration range of 1—20 ng/ml. An equilibrium of bromhexine binding with plasma protein was thus established, as the equilibration time for protein binding is in the range of seconds [14]. The standard samples were analysed by the same procedure as described above.

Partition studies. For the partition studies, about 10 μg of the compounds were dissolved in the organic solvents (5 ml) and the extraction with aqueous phase (5 ml) was performed by shaking for 15 min with a shaking machine.

After centrifugation, aliquots of both phases were measured. The concentrations were determined photometrically or by liquid scintillation counting if ^{14}C-labelled compounds were used.

Recovery studies. For the recovery studies, 100 ng and 10 ng of ^{14}C-labelled bromhexine were used and extracted as described under Extraction, but without the addition of internal standard.

Human studies

Five healthy volunteers participated in the study: R.R., 23 years, female, 58 kg; H.D.S., 30 years, male, 72 kg; J.B., 25 years, male, 68 kg; J.E., 26 years, male, 75 kg; R.K., 24 years, female, 60 kg.

During the test no other drugs were taken. The formulation used was Lot No. 6203. An amount of 15 mg (7.5 ml) was administered intravenously by a continuous 45-sec infusion. Two hours later, the volunteers received a standard breakfast (tea and toast with ham). Four hours later a normal dinner was served.

RESULTS AND DISCUSSION

Extraction procedure

The experiments presented in Table I show that hexane is a very favourable extraction medium. The result at pH 5.2 is particularly interesting. We attribute this behaviour to the highly lipophilic character of the compound. A similar effect was observed for methaqualone [15].

TABLE I

PARTITION RATIOS (C_{org}/C_{aq}) OF BROMHEXINE BETWEEN DIFFERENT SOLVENTS AND DIFFERENT pH VALUES OF THE AQUEOUS PHASE

Aqueous phase	Organic solvent		
	Hexane	Diethyl ether	Toluene
NaOH, 0.1 *N*	99	>100	100
Phosphate buffer, pH 7.2	>100	>100	100
Phosphate buffer, pH 5.2	23	95	83
H_2SO_4, 0.2 *N*	<0.01	<0.01	0.04

In Fig. 3 the pH dependence of the partition behaviour between hexane and phosphate buffer, hexane and perchlorate solution, and chloroform and perchlorate is plotted. Bromhexine is a weak base, $pK_{a1} = 8.5$ (benzylamino group) $pK_{a2} = -0.3$ (anilino group), and a very lipophilic compound. The calculated log P for octanol—water, according to Hansch and Leo [16] is 6.2. From the results of Fig. 3, the true partition coefficient, P_t, was calculated from measurements of the apparent partition coefficient, P_a, the pK_a and the pH according to $\log P_t = \log P_a + pK_{a1} - pH$. The $\log P_t$ calculated in this way is about 6.4. The relatively low values of P_a in Fig. 3 at a pH above 6 is due to the great influence of small amounts of ^{14}C-labelled degradation products having a lower partition coefficient than bromhexine. These results mean that only the system

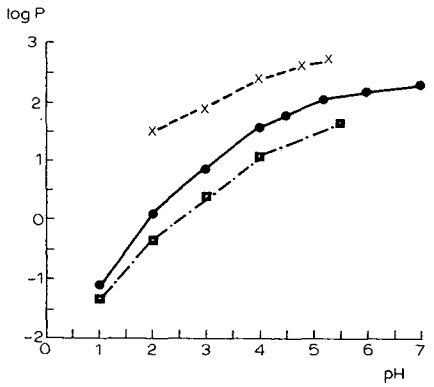

Fig. 3. Log P values of bromhexine between chloroform and perchlorate (×), hexane and phosphate (•), and hexane and perchlorate (▪).

TABLE II

PARTITION RATIOS (C_{org}/C_{aq}) OF BROMHEXINE METABOLITES BETWEEN THE ORGANIC PHASES AND PHOSPHATE BUFFER AT pH 5.2

Substance	Hexane	Toluene
Bromhexine	23	83
M II	23	100
M V	0.43	16.6
M VIII	0.26	0.61

chloroform—perchlorate reveals ion-pair mechanisms.

The use of silanized glass is strongly recommended (see under Assay parameters — recovery) to minimize losses caused by adsorption. This is of special importance since we used hexane without adding alcohol. Silanization was done according to the vapor phase procedure of Fenimore et al. [17].

Chromatographic properties of bromhexine and metabolites

The chromatographic properties of bromhexine and its derivatives have been described by Eichler and Kreuzer [9] and De Leenheer and Vandecasteele-Thienpont [11]. They demonstrated that bromhexine can be satisfactorily separated without derivatisation. These observations are confirmed by our chromatograms. We conclude that the chromatographic behaviour of free bromhexine is due to the fact that both amino groups are shielded sterically and that the anilino group has a weak basic character (pK_a of anilino-hydrochloride is −0.3). As the N-FID sensitivity is not decisively increased by derivatisation steps, we used underivatized bromhexine.

The retention times of bromhexine, the internal standard and the metabolites are summarized in Table III. The figures demonstrate that a sufficient separation between bromhexine (I) and M II is achieved only on the OV-17 phase.

TABLE III

RETENTION TIMES OF BROMHEXINE (I), INTERNAL STANDARD (II) AND METABOLITES ON OV-101 AND OV-17

The length of the column was 25 m in each case.

Compound	Retention time (min)		
	OV-101 (70—220°C, 20°C/min)	OV-17 (isothermal, 230°C)	OV-17 (actual temperature programme)
I	9.95	2.55	20.63
II	9.28	2.21	19.12
M II	9.93	2.89	21.15
M V	12.83	6.79*	—
M VIII	13.08	6.96*	—
M XI	15.35	7.71*	—

*M V, M VIII, M XI had no appropriate behaviour on this column and only showed peaks at very high concentrations.

Chromatographic system

For ultra trace analysis in the lower parts per 10^9 range, every means to gain sensitivity must be used. Capillary GLC reduces peak width to a few seconds. This means that for nearly all types of GLC detectors the detection limit with capillary columns is lower than with packed columns. The theoretical background has been published by Yang and Cram [18] and Franzen [19]. The gain in sensitivity is about four times using an N-FID and MS, but about 80 times in the case of an EC detector [18].

A second consideration leads to the use of capillary columns. In plasma samples there is always interfering material of various origins: endogenous compounds, nutritional components and smoking (flavours, caffeine, nicotine and metabolites thereof, etc.) and drug metabolites. The lower the blood levels of the drug, the greater the probability that there are interfering endogenous compounds. A chromatographic system with extreme separation power, however, reduces the probability of interference on just probability considerations. Furthermore, capillary columns favour the application of internal standards which are structurally very similar to the drug. These facts have been recently summarised [20].

The sample injection process is a critical step in quantitative high-resolution GC analysis. For trace analysis [21] the split mode cannot be used. The direct sampling and the falling-needle device are not yet usable for automatic sampling. Only splitless injection can be performed with an autosampler and this therefore appears to be the method of choice for day and night operation.

Detection

The detector used for drug monitoring must be sensitive, selective, robust and compatible with capillary columns. The nitrogen detector used meets all these requirements. Its high sensitivity (25 pg of pure substance) reduces the plasma volumes needed for analysis. The detection limit of the GC—MS systems was about 100 pg; the detection limit of the EC detector was not established.

The robust performance of a detector reduces the servicing time. The FID and N-FID are particularly insensitive to depositions since all substances eluting from the column are combusted by an oxygen-rich flame. This is the major advantage of this kind of detector as compared to a mass spectrometer or EC detector. Additionally, the small dead volume of the N-FID does not spoil the resolving power of capillary columns [18].

Assay parameters

The analytical method described corresponds to the IFCC recommendations [22] concerning sensitivity, specificity, accuracy and precision.

Fig. 4 shows the chromatograms of extracts from plasma compared with the plasma blanks, which are very low (< 0.2 ng/ml at the detection limit of the system) and nearly constant for all volunteers. The detection limit of the assay is about 0.5 ng/ml and for the pure substance about 25 pg. The detection limit is determined by the volume of blood available (2 ml) and the fact that only one-tenth (2 μl from 20 μl) of the extract can be injected. The back-extraction with sulfuric acid in necessary for two reasons. Firstly, we gain selectivity because interfering non-basic compounds are not extracted. Without back-extraction we have a detection limit of about 2 ng/ml. Secondly, this procedure is necessary to improve the life-time of the column, because otherwise it would

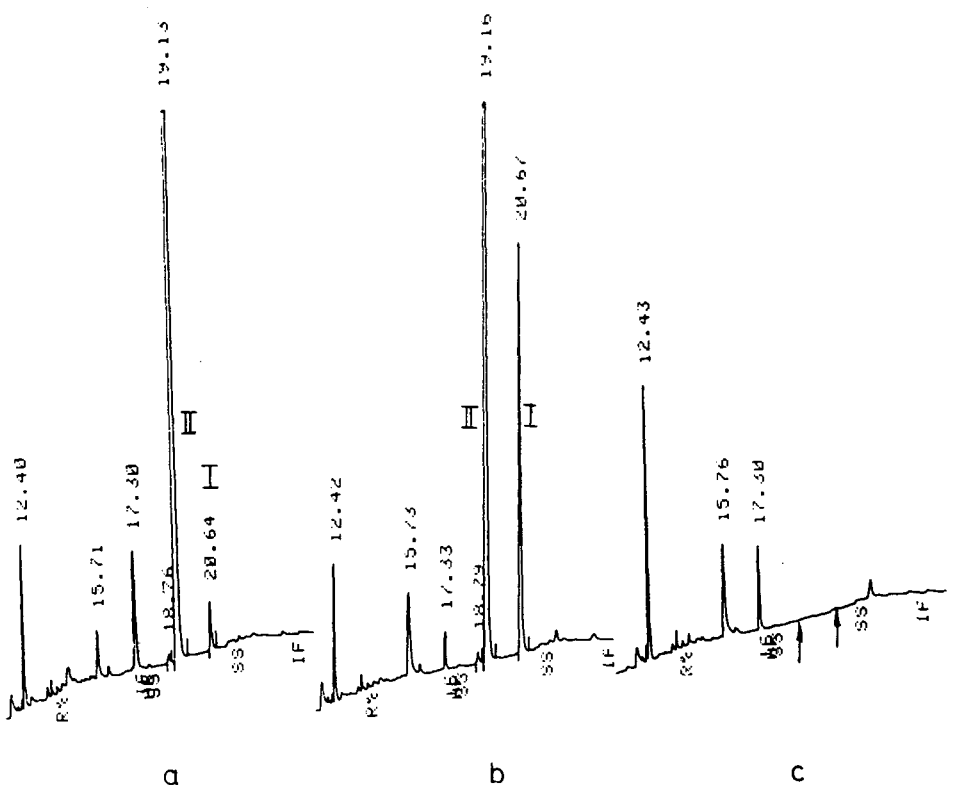

Fig. 4. Chromatograms of human plasma showing bromhexine at a level of (a) 1.5 ng/ml, (b) 18.7 ng/ml, (c) blank. I = bromhexine, II = internal standard.

become polluted with about 200 μg of triglycerides and 100 μg of cholesterol after each injection. Up to now the life-time of the column is about 900 injections with the back-extraction procedure.

The calibration curve is linear in the range 1—20 ng/ml bromhexine. It can be described by the equation $y = 1.84 \cdot 10^{-2} x - 5.5 \cdot 10^{-3}$. The coefficient of correlation is $r = 0.9978$.

The repeatability was studied by preparing plasma samples containing different amounts of bromhexine and analysing them on different days. The results are shown in Table IV.

TABLE IV

REPRODUCIBILITY OF BROMHEXINE DETERMINATION ON ONE DAY (A) AND BETWEEN DAYS (B)

	Bromhexine added (ng/ml)	Bromhexine found (ng/ml)	No. of determinations	S.D. (%)
A	2.0	2.03	4	4.2
	7.5	7.68	6	4.6
	15.0	15.16	5	3.5
B	10.0	9.66	7	5.7

Using liquid scintillation counting, the recovery of the ^{14}C-labelled bromhexine was 83.4 ± 5.9% (n = 4) at a concentration of 10 ng/ml and 85.1 ± 0.9% (n = 4) at 100 ng/ml. This experiment was performed without internal standard to check losses due to adsorption. A recovery of 96.5 ± 1.0% was found for 100 ng and 101.7 ± 5.0% for 10 ng of bromhexine when we measured all partition phases. This satisfactory result is due to the use of silanized glass tubes.

The specificity of the method with respect to known metabolites is summarized in Table III, with respect to the blanks shown in Fig. 4. We further analysed bromhexine by GC—MS. In Fig. 5 the multiple ion detection traces of a plasma extract containing 2.9 ng/ml bromhexine show the sensitivity of the GC—MS assay.

Fig. 6 shows the regression line of the bromhexine levels from a pharmacokinetic study as determined by GC—N-FID and simultaneously by GC—MS. The regression line was calculated by the sum of least squares. The regression coefficient is 0.996 with a slope of 0.99 (both calculated from non-logarithmic data) and is therefore compatible with the assumption that there are no systematic deviations due to unknown metabolites.

The GC—N-FID method is useful for serial analyses. As the extraction steps are performed in centrifuge tubes and the phase separation is done by freezing out, 30 samples can be analysed per day by one technician. The advantages of phase separation by centrifuging and freezing out [23] have been emphasized recently [24]. Using an autosampler, the time needed for one run (35 min) is of minor importance since day and night operation is possible.

Fig. 7 shows the mean plasma level of five human volunteers after administration of 15 mg of bromhexine by a continuous 45-sec infusion. The values measured are fitted by a three-compartment model with two side-compart-

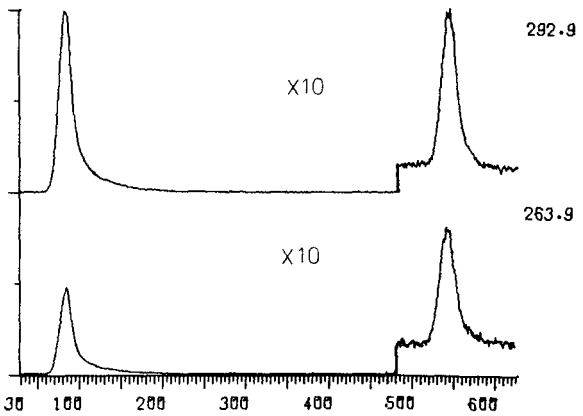

Fig. 5. Multiple ion detection traces of a plasma extract containing 2.9 ng/ml bromhexine, the actual retention times being 14.05 min and 16.32 min.

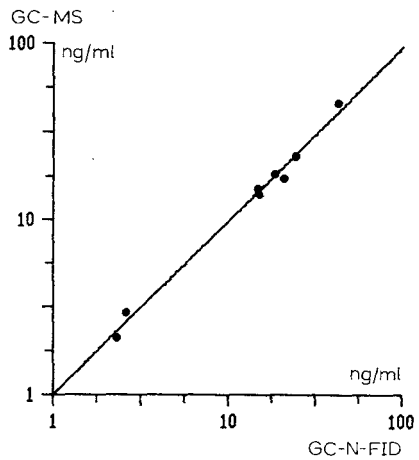

Fig. 6. Regression line of the bromhexine levels from a pharmacokinetic study determined by GC—N-FID and GC—MS.

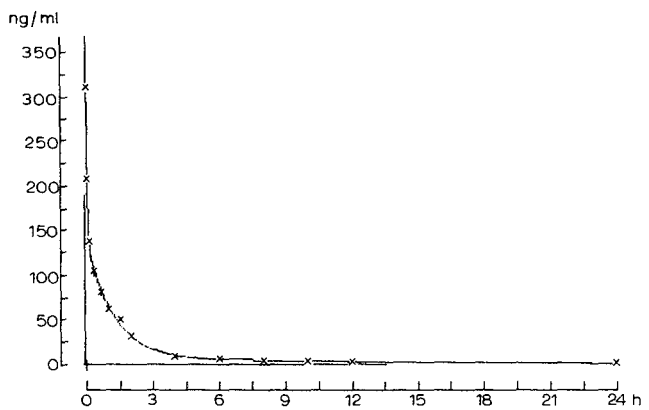

Fig. 7. Plasma levels of bromhexine in human subjects (n = 5) following intravenous administration of 15 mg by an infusion lasting 45 sec. (X), Experimental values; (—), computer-fitted curve.

ments using our Thomae-Topfit program [25]. In accordance with older studies [6] in which only total radioactivity was measured, we find that bromhexine is rapidly metabolised. The total clearance [26] is about 1.1 l/min. The terminal elimination half-life is about 15 h and is determined by the back-diffusion of bromhexine from the tissues.

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CHROMBIO. 1043

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF 6-THIOGUANINE APPLICABLE TO PHARMACOLOGIC STUDIES IN HUMANS

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SUMMARY

6-Thioguanine (6TG) and its metabolites were analyzed in human plasma with a reversed-phase high-performance liquid chromatographic method. 6TG and related compounds were extracted from plasma with an equal volume of 2 *N* perchloric acid at a 50—100% recovery efficiency. The neutralized extracts were chromatographed on a μBondapak C_{18} column by two separate isocratic conditions. 6TG, 6-thiouric acid, 6-thioxanthine, 6-thioguanosine, and 6-methylthiouric acid were analyzed with 0.01 *M* sodium acetate, pH 3.5—10% methanol as the mobile phase and 340 nm for detection. 6-Methylthioguanine and three unknown metabolites were separated with acetate—25% methanol and 310 nm detection. One of the unknowns was identified as 6-methylthioguanosine. External standard calibration was used for quantitation. The 6TG detection limit was 0.8 nmol/ml in plasma.

INTRODUCTION

6-Thioguanine (6TG) has been used as a cancer chemotherapeutic agent for several decades. The analytical techniques developed over the years for the pharmacokinetic study of this drug, however, are lacking for a number of reasons. An early method that separated metabolites on Dowex columns followed by spectrometric quantitation [1], is time consuming. The use of ^{35}S-labelled 6TG for detection and paper chromatography for separation of metabolites [2] involves the problems inherent in administration of radio-labelled compounds to humans. A spectrofluorometric assay following oxidation with hydrogen perioxide [3] suffers from both a lack of specificity for metabolites and poor sensitivity. Another method entailing oxidation to a fluorescent product with alkaline permanganate [4] followed by anion-exchange chromatography [5] does not allow detection of 6-thiouric acid (6TU),

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6-thioxanthine (6TX), or 6-methyl metabolites. An anion-exchange high-performance liquid chromatographic (HPLC) method [6,7] although suited for the analysis of one of the 6TG metabolites, 6-thioguanosine monophosphate, requires a long time to analyze each sample. A recent report has suggested that intermittent high dose 6TG may have utility in the treatment of colorectal carcinoma [8]. The initiation of this protocol prompted us to investigate a reversed-phase HPLC system for the routine and sensitive assay of 6TG and related compounds in patient plasma.

EXPERIMENTAL

Materials

6-Thioguanine (NSC-752) was obtained from the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). 6TX, 6-thioguanosine (6TGS) and guanosine monophosphate were obtained from Sigma (St. Louis, MO, U.S.A.). 6TU, 6-methylthioguanine (6MeTG), 6-methylthiouric acid (6MeTU), and 6-methylthioxanthine (6MeTX) were kindly provided by Dr. Gertrude Elion of Burroughs-Wellcome (Research Triangle Park, NC, U.S.A.). Acetate buffer (0.01 *M*) was made from glacial acetic acid (Mallinckrodt, St. Louis, MO, U.S.A.) and de-ionized water (Millipore, New Bedford, MA, U.S.A.), and the pH was adjusted with 5 *M* sodium hydroxide. This buffer was filtered (Millipore HA 0.45 μm) and degassed before the addition of degassed methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) to make the mobile phase. The pH 2.5 mobile phase was adjusted with 1 *N* hydrochloric acid. Oxidation to fluorescent species was carried out by the method of Finkel [4] using potassium permanganate (Baker reagent) and an SPF-125 spectrofluorometer (Aminco, Silver Spring, MD, U.S.A.). 6TU, 6TX and 6MeTG could not be oxidized to fluorescent products by this method.

HPLC

A Spectra-Physics Model 3500B (Santa Clara, CA, U.S.A.) liquid chromatograph is used for solvent delivery. Samples are injected through a Valco 6-port rotary valve fitted with a 100-μl sample loop onto a 30 cm × 3.9 mm μBondapak C_{18} column (particle size 10 μm, Waters Assoc., Milford, MA, U.S.A.). 6TG and metabolites are detected with an SP 770 variable-wavelength UV detector (Spectra-Physics) at a sensitivity of 0.04 a.u.f.s. Peaks are traced on a Hewlett-Packard 7130A strip chart recorder at 0.25 in./min using a 10 mV setting, and integrated with a Spectra-Physics Autolab System I Computing Integrator. Optimization of conditions was carried out with acetate buffer as the A solvent and methanol—acetate (1:1, v/v) as the B solvent. Two separate conditions are used for the detection of metabolites in clinical specimens. 6TG, 6TU, 6TX, 6TGS, 6MeTU and 6MeTX are eluted with a mobile phase of 0.01 *M* sodium acetate, pH 3.5—10% methanol (v/v) and detected at 340 nm. 6MeTG is eluted with 0.01 *M* sodium acetate, pH 3.5—25% methanol (v/v) and detected at 310 nm. Flow-rates are 2.0 ml/min and produce column pressures of 11.1 MPa and 13.8 MPa respectively. The column is flushed daily with methanol—water (1:1, v/v).

Extractions

Triplicate 1.0-ml plasma samples containing known concentrations of 6TG or metabolite standards were precipitated with 1.0 ml of cold 2 *N* perchloric acid (PCA), and centrifuged at 48,000 *g* at 4°C for 20 min. Aliquots (1.0 ml) of the resulting supernatants were removed, adjusted with 0.150 ml 4 *N* potassium hydroxide to pH 10—12, and stored at 4°C for two days to precipitate all of the perchlorate salts [9]. The solutions were adjusted to pH 2—3 with 0.150 ml of 1 *N* hydrochloric acid (to avoid dissolution of the silica support of the column) and centrifuged at 700 *g* at room temperature for 10 min. Appropriate aliquots were injected onto the column.

An alternative extraction was attempted using trichloroacetic acid (TCA). Triplicate 1.0-ml plasma samples containing 6TG or metabolites were precipitated with 1.0 ml 14% TCA (w/v). Following centrifugation at 48,000 *g* for 20 min, 1.0 ml of the supernatant was removed and extracted three times with 1.0 ml diethyl ether. Nitrogen was blown on the aqueous layer for 1.0 min and aliquots injected onto the chromatograph.

A patient (R.E.) who received 1800 mg (1000 mg/m^2) of 6TG as a 60-min intravenous infusion had blood collected into heparinized tubes. The blood samples were centrifuged (700 *g*, 10 min), and the plasma supernatant stored at −20°C until extracted in triplicate with perchloric acid as above.

Quantitation

The areas of 6TG peaks in plasma extracts were compared to a calibration line of peak area versus nmol injected. The calibration line was generated daily by injections from a stock solution of 80 μM 6TG. For extraction efficiency experiments, separate calibration lines were made from stock solutions of each metabolite. These calibration lines were used to calculate relative response factors for the various metabolites compared to 6TG. Subsequently, quantitation of metabolite peaks in patient plasma entailed multiplying the metabolite peak area by the appropriate response factor and comparing this value to a 6TG calibration line. Unknown metabolites are assigned a response factor of 1.0. Quantitation of metabolites detected at 310 nm is carried out in a similar manner with a 6MeTG calibration line.

Analysis of unknowns

Pooled patient urine was adjusted to pH 10 with 10 *N* sodium hydroxide and then extracted three times with equal volumes of ethyl acetate. The ethyl acetate was removed in vacuo and the residue redissolved in 0.01 *M* acetate buffer, pH 3.5. Repeated injections were made onto the analytical column using a mobile phase of 0.01 *M* acetate, pH 3.5—20% methanol which gave baseline resolution of unknowns 1, 2, and 3. After rechromatography, the collected fractions were then alkalinized, extracted into ethyl acetate and dried. The residues were subjected to direct probe electron impact analysis on a VG Micromass 30F (Altrincham, Great Britain) mass spectrometer. The source settings were: 70 eV, 180°C, 4 kV, and 170 μA. UV spectra were obtained on a Cary 118 UV spectrometer (Varian, Palo Alto, CA, U.S.A.). Acidic hydrolysis of unknown 3 was done in 0.1 *N* hydrochloric acid for 10 min at 100°C and analyzed directly by HPLC.

RESULTS AND DISCUSSION

A preliminary separation of 6TG and metabolites was accomplished by a modification (0.01 *M* KH_2PO_4, pH 5.5—10% methanol, isocratic elution) of the method of Hartwick and Brown [10] for nucleosides and bases. However, after less than 100 samples had been injected using this mobile phase for elution, the characteristics of the column suddenly changed as evidenced by a shift in retention times and severe tailing of the 6TG peak. Upon changing the mobile phase to 0.01 *M* sodium acetate, pH 3.4—10% methanol (v/v), the 6TG peak was restored to a sharp and symmetrical shape.

These results prompted a study of the effect of the mobile phase pH and methanol concentration on the resolution of 6TG and related metabolites. Of the compounds studied (6TU, 6TG, 6TX, 6TGS, and 6MeTG), only 6MeTG showed a sensitivity to the pH (Fig. 1). As the pH was raised (with methanol concentration constant at 10%), 6MeTG was eluted at a larger k'. Therefore, when the 6-thio group is methylated, the partition coefficients of these compounds exhibit a sharp dependency on mobile phase pH.

The methanol concentration was varied at pH 3.5 to affect retention. As the polarity of the mobile phase increased with the decreasing methanol concentrations, 6TG and metabolites had longer retention times. An interesting observation is that 6TGS was eluted before 6TX at 15% methanol, but, at lower methanol concentrations it was eluted after 6TX.

Based on these data and the fact that 6MeTG has a λ_{max} at 310 nm and very little absorption at 340 nm, two operating conditions (Fig. 2) were chosen for the study of 6TG pharmacology in patients: (1) elution with 0.01 *M* acetate, pH 3.5—10% methanol and detection at 340 nm for analysis of 6TU, 6TG, 6TX, 6TGS; and (2) elution with 0.01 *M* acetate, pH 3.5—25% methanol and detection at 310 nm for analysis of 6MeTG. Once these conditions were chosen, the mobile phase was premixed and the gradient mixer on the instrument was not used. The premixed mobile phase, however, eluted 6TX and 6TGS together and the k' values for 6TU and 6TG were slightly lowered

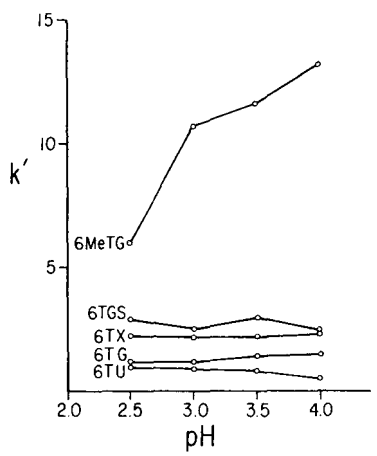

Fig. 1. Capacity factors versus pH of mobile phase. The mobile phase in each case was 0.01 *M* acetate—10% methanol (v/v). 6MeTG was detected at 310 nm.

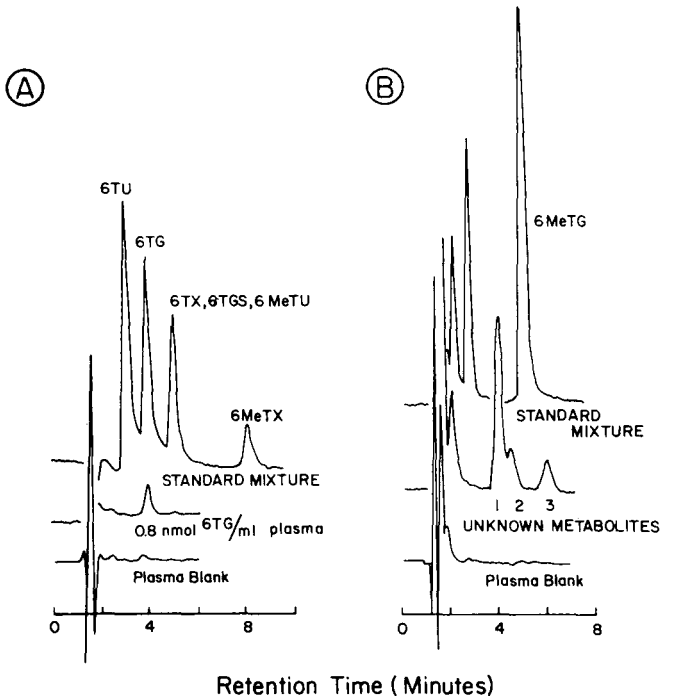

Fig. 2. Chromatograms of 6TG and metabolites in the two isocratic systems used. (A) Elution with 0.01 *M* acetate, pH 3.5—10% methanol and detection at 340 nm. The traces show a plasma blank, the detection limits and a mixture of 6TG and metabolites used as standards. (B) Elution with 0.01 *M* acetate, pH 3.5—25% methanol and detection at 310 nm. The traces show a plasma blank, a typical patient's plasma extract containing the three unknown metabolites, and a mixture of metabolite standards.

TABLE I

COMPOUNDS CHARACTERIZED BY REVERSED-PHASE HPLC

Compound	k'*	Response factor
6TU**	0.57 ± 0.01	1.30
6TG	1.34 ± 0.08	1.00
6TX	1.94 ± 0.08	1.60
6TGS	1.94 ± 0.06	1.78
6MeTU	1.93 ± 0.02	–
6MeTX	3.73 ± 0.05	–
Metabolite 1***	1.72 ± 0.09	1.00
Metabolite 2	2.09 ± 0.14	1.00
6MeTG	2.68 ± 0.02	1.00
Metabolite 3	3.27 ± 0.09	1.00

*Average ± standard deviation of at least four injections using premixed mobile phase.

**Compounds were chromatographed with 0.01 *M* acetate, pH 3.5—10% methanol and detected at 340 nm. Response factors are relative to 6TG.

***Compounds were chromatographed with 0.01 *M* acetate, pH 3.5—25% methanol and detected at 310 nm. Response factors, relative to 6MeTG, are assumed.

(Table I). This observation indicates the probable inaccuracy of the gradient module of the instrument in mixing the methanol concentration and accounts for the differences in k' values between Table I and Figs. 1 and 2. When 6MeTU was obtained and chromatographed, its k' was found to be identical with that of 6TX and 6TGS. Since metabolites seen at this retention time, if present at all, are minor compounds in plasma the resolution of 6TX, 6TGS and 6 MeTU became a moot point, and the premixed 0.01 *M* acetate—10% methanol buffer was kept as the mobile phase in the interest of analysis time.

The extraction efficiencies of 6TG and metabolites with PCA, within the concentration range studied (Table II), gave reasonably consistent values with low standard deviations. Extractions of 6TG in the range of 0.4—3.2 nmol/ml (not shown) gave efficiencies of 38—60% with a lower detection limit of 0.8 nmol/ml (3:1 signal-to-noise ratio). The efficiencies and standard deviations obtained in the concentration range of clinical interest (1—100 nmol/ml) gave confidence in the reproducibility of the assay. An extraction using TCA was also attempted. Although the extraction efficiency was high for 6TG at 32 nmol/ml, the extraction efficiency dropped to low levels for 6TX and 6TU

TABLE II

EXTRACTION EFFICIENCIES OF 6TG AND RELATED COMPOUNDS

Recovery (%), average of 3 extractions ± standard deviation using PCA.

Compound	Concentration (nmol/ml)				
	4	8	16	24	32
6TG	42.5 ± 16	67.0 ± 3.2	82.0 ± 5.2	101.0 ± 8.6	100.3 ± 0.6 (99.7 ± 12.5)★
6TX	76.4 ± 9.8	92.9 ± 10.8	87.4 ± 15.3	101.4 ± 13.3	95.3 ± 5.1 (20 ± 3.5)
6TU	51.9 ± 5.4	48.6 ± 5.3	58.6 ± 1.3	56.4 ± 9.4	69.2 ± 5.7 (32 ± 1.7)
6TGS	63.8 ± 10.4	62.6 ± 2.2	58.2 ± 4.6	58.7 ± 1.6	59.0 ± 2.1
6MeTG	53.0 ± 3.3	78.6 ± 8.1	88.3 ± 4.3	85.2 ± 2.8	95.7 ± 9.2
6TG + guanine★★	75.7 ± 3.2	70.9 ± 0.9	84.0 ± 8.5	82.2 ± 1.6	71.1 ± 2.1

★Using TCA extraction.
★★1.0 μmol/ml.

(Table II) and this extraction was not investigated further. The use of an internal standard would have been preferred, however, the search for a compound with similar chemical properties and appreciable absorbance near 340 nm was fruitless. All available thiopurines are suspected or proven metabolites except mercaptopurine which coeluted with 6TG.

The pattern of 6TG extraction efficiency indicated that a fixed amount rather than a fixed percent of 6TG was lost in each extract. The extraction study was repeated in the presence of a large excess of guanine (1.0 μmol/ml) in the hope that guanine would saturate any plasma or glassware binding sites for 6TG. The only noticeable improvement in extraction efficiency was at 4 nmol/ml of 6TG where the recovery was increased to 76%. Therefore, guanine was not used as a carrier molecule in subsequent extractions.

The standard plots generated for each metabolite during the extraction efficiency studies were used to compute response factors (Table I) of the compounds relative to 6TG. In all cases the correlation coefficients of the calibration plots were greater than 0.996. These response factors were used to quantify known metabolites with a 6TG calibration line. Response factors were not

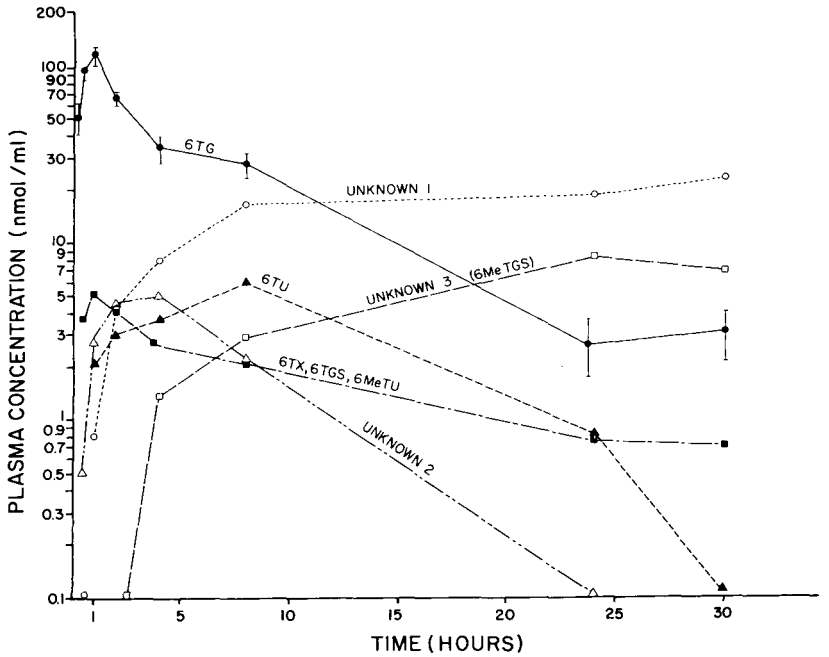

Fig. 3. Plasma concentrations of 6TG and metabolites in patient (L.W.) receiving 1800 mg 6TG as a 60-min infusion. 6TG (•——•), 6TU (▲ - - - ▲), 6TX, 6TGS, 6MeTU (▪—— — ——▪), metabolite 1 (○ - - - ○), metabolite 2 (△—- - △), metabolite 3 (□ - - - □). Points represent the mean ± standard deviation of triplicate extractions.

computed for 6MeTX or 6MeTU since there were impurities present in these standards and the concentrations of stock solutions were unknown.

Analysis of extractions from patient plasma revealed, besides large amounts of 6TG, a surprising number of metabolites (Fig. 3). 6TU and 6MeTG (not seen in this patient), were detected as previously reported [2] and identified in extracts by virtue of their retention times. Plasma concentrations of 6TU were detected by 1.0 h and peaked at 8 h. The group of metabolites (6TX, 6TGS, 6MeTU) which are eluted at k' 1.94 peaked at 1 h where they reached a total concentration of 0.045 of the 6TG concentration and declined thereafter. When analyzing the extracts at 310 nm, three peaks, designated metabolites 1, 2, and 3 were seen besides 6MeTG (Fig. 2). The retention time of these peaks did not correspond to any of our known standards. By 24 h, metabolite 1 had become the principal metabolite detected in plasma.

Sufficient quantities of unknown metabolites for structural analysis were obtained by extracting alkalinized pooled patient urine with ethyl acetate. This extraction had an efficiency of greater than 90% for all three unknowns. Attempts at isolating purified unknown metabolites were made by repeated injections onto the analytical HPLC column. Rechromatography of collected fractions, however, revealed that unknowns 1 and 2 degraded rapidly to unknown 3. UV analysis of unknown 3 gave a spectrum similar to 6MeTG (λ_{max} = 310 nm, 1.0 *N* sodium hydroxide; λ_{max} = 328 nm, 1.0 *N* hydrochloric acid). Mild acid treatment caused hydrolysis to 6MeTG as assayed by HPLC. The mass spectrum of unknown 3 (Fig. 4) exhibited the classic fragmentation [11]

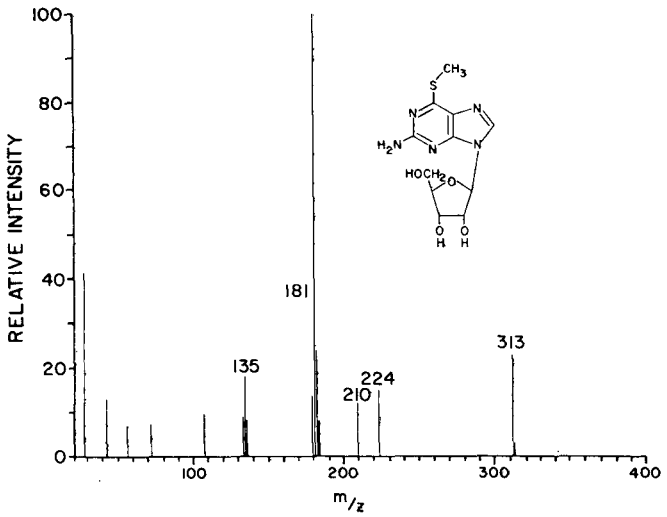

Fig. 4. Mass spectrum of unknown 3. The sample was analyzed by direct probe insertion at 180°C. The metabolite was deduced to be 6-methylthioguanosine.

of a purine riboside [*m/e* 313 $(M)^+$, 224 $(M-89)^+$, 210 $(B+30)^+$, and 181 $(B+H)^+$]. The identity of unknown 3 was concluded to be 6-methylthioguanosine (6 MeTGS). The extractability of unknowns 1 and 2 from base into ethyl acetate, their absorbance at 310 nm, and their high retention on the column suggest that they are 6-thiomethylated metabolites. These characteristics also rule out the possibility of phosphorylated riboside derivatives. All attempts at isolating unknown metabolites 1 and 2 have proven futile and their identity remains undefined.

The finding of 6MeTGS in human plasma is particularly relevant to the recent finding of Lee and Sartorelli [12] that sarcoma 180 cells in vitro actively metabolize 6TG to 6TGS by purine nucleoside phosphorylase. A similar pathway operating in humans, either preceeding or following methylation of the thio functionality, may account for the high concentrations of 6MeTGS we found. Another possibility may be that 6MeTGS is produced from dephosphorylation of 6MeTGS monophosphate during the extraction procedure. To investigate this possibility we extracted guanosine monophosphate (since neither 6MeTGS monophosphate nor 6TGS monophosphate were available) at 20 nmol/ml from plasma and water with PCA as described in Experimental. The extraction efficiency was 97.3 ± 5.5% from water, but dropped to 68.2 ± 2.7% from plasma. The missing monophosphate was totally accounted for by conversion to guanosine. Thus degradation of 6MeTGS monophosphate, if present, may account for some of the 6MeTGS seen in patient plasma; however, considering the high concentrations of 6MeTGS found, a degradative formation would not account for all of the 6MeTGS present.

In either case, the results may question the rationale of using high dose 6TG regimens. High doses of 6TG may merely be shunted into 6TGS and/or 6MeTGS with no correspondingly higher level of the cytostatic 6-thioguanosine monophosphate attained.

In summary we have delineated an isocratic reversed-phase HPLC method for

the routine analysis of 6TG and metabolites in human plasma. We have observed three previously undescribed metabolites in humans and we have definitively identified one of them as 6-methylthioguanosine.

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CHROMBIO. 1047

HIGH-PERFORMANCE LIQUID COLUMN AND THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF HUMAN SERUM GLIBENCLAMIDE AT THERAPEUTIC LEVELS

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SUMMARY

For glibenclamide bioavailability studies in serum, high-performance liquid column and thin-layer chromatographic methods were introduced. Both methods are specific, accurate and sensitive with detection limits of at least 5 ng of glibenclamide per ml of serum. Detection is performed in the ultraviolet at wavelengths of 200 nm for liquid chromatography or 300 nm for thin-layer chromatography.

Serum levels determined by either method correlated well with those determined by an already existing radioimmunoassay. Some pharmacokinetic data were computed using a one-compartment open model.

INTRODUCTION

Glibenclamide (HB 419)* (Fig. 1) is a highly potent antidiabetic agent. For pharmacokinetic purposes, especially for bioavailability studies of various preparations, analytical methods are required. They have to be practicable, specific, accurate and sensitive, allowing assaying at the low ng/ml of serum level.

Previously published methods employed radioimmunoassay [1—3], gas chromatography [4], polarography [5], colorimetry [6], fluorimetry [6, 7], and, more recently, high-performance liquid chromatography (HPLC) [8, 9]. Polarography, colorimetry and fluorimetry were not specific. For radioimmunoassay cross-reactions with sulfonylureas and their metabolites are encountered. Gas chromatography required a derivatization step. Furthermore,

*Trademarks: Daonil® and Euglucon® of Hoechst AG (Frankfurt, G.F.R.) and Boehringer Mannheim GmbH (G.F.R.), respectively.

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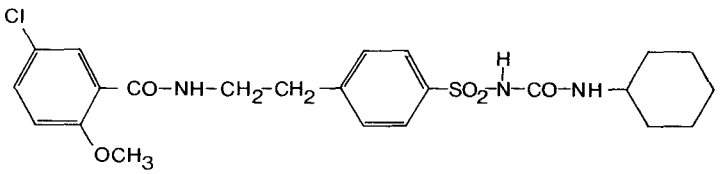


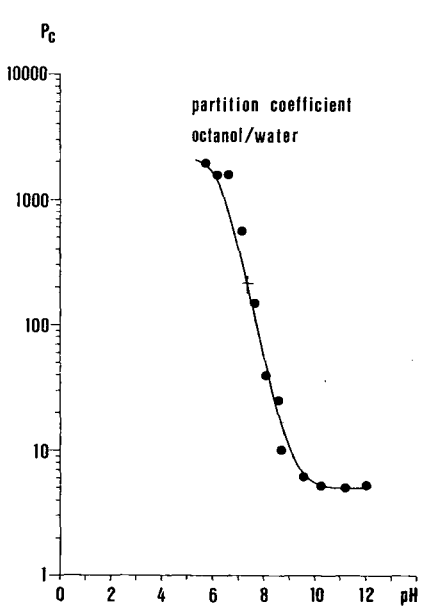
Fig. 1. Structure of glibenclamide.

the sensitivity of HPLC was not sufficient to follow a single oral treatment with a 2.5-mg tablet (cf. Fig. 10).

We therefore developed highly sensitive assays based on HPLC and thin-layer chromatography (TLC).

PHYSICOCHEMICAL PROPERTIES OF GLIBENCLAMIDE

Knowledge of the physicochemical properties of a compound is essential for obtaining optimal conditions for its extraction from serum and for choosing the most suitable conditions of measurement. The UV spectrum, solubility and partition of glibenclamide in ether/buffer have been published by Hajdú et al. [6]. Furthermore, we studied partition in octanol/Britton-Robinson buffer in the pH range 5—12. From these data, partition coefficients for the free acid and for the salt of ca. 2300 and 5.0 ± 0.3, respectively, were calculated (Fig. 2). The pK_a = 6.15 was determined using an extrapolation procedure [10] (Fig. 3) following tritrations in various methanolic solutions. This value equals the pK_a = 6.4 ± 0.2 resulting from the partition study.



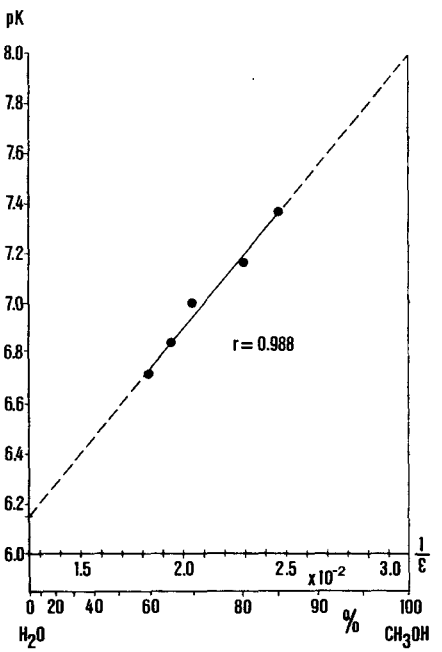


Fig. 2. Partition coefficient (octanol/water) of glibenclamide.

Fig. 3. Determination of pK_a following titration in methanolic solutions and extrapolation of pK_a (ϵ) to pK_a in aqueous solution.

EXPERIMENTAL

HPLC analysis

Reagents. The reagents used were 0.1 *N* hydrochloric acid AR, 5 *N* hydrochloric acid AR, 0.1 *N* sodium hydroxide AR, 1/15 *M* phosphate buffer solution (pH 7), diethyl ether AR, acetonitrile (HPLC grade S, Rathburn Chemicals, Walkerburn, Great Britain), the 4-methylcyclohexyl analogue of glibenclamide [N-(4-(β-5-chloro-2-methoxybenzamido-ethyl)benzenesulfonyl)-N′-(4-methylcyclohexyl)-urea] as internal standard (20 μg/ml in methanol). The mobile phase was acetonitrile—1/15 *M* phosphate buffer pH 7 (2:5, v/v).

Apparatus. The chromatograph consisted of a Waters M6000A pump with a Rheodyne 7100 injection port (100-μl sample loop) and a Schoeffel SF 770 spectrophotometer, equipped with a 10 cm × 4.6 mm I.D. column and a 3 cm × 4.6 mm I.D. pre-column filled with Spherisorb-ODS 5 μm (Brownlee MPLC system).

For sample clean-up a Vortex mixer, glass-stoppered tubes (ca. 12 ml capacity) and conical glass-stoppered tubes (ca. 8 ml capacity) were used.

Procedure. In a glass-stoppered tube, 1 ml of serum was treated with 0.2 μg of internal standard (10 μl) and 0.1 ml of 1 *N* hydrochloric acid. The serum was extracted for 20 sec with 5 ml of diethyl ether on a Vortex mixer; the phases were then separated by centrifugation (5 min) and 4.5 ml of the organic phase were transferred into a conical tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase and 75 μl were injected into the chromatograph. The compound was detected at 200 nm (Fig. 4). At a flow-rate of 2 ml/min the retention times (t_R) found and k' values calculated were t_R = 4.0 min (k' = 7.9) for glibenclamide, and t_R = 7.6 min (k' = 15.9) for the internal standard (cf. Fig. 5). Quantitation was based on the peak height ratio of glibenclamide/internal standard.

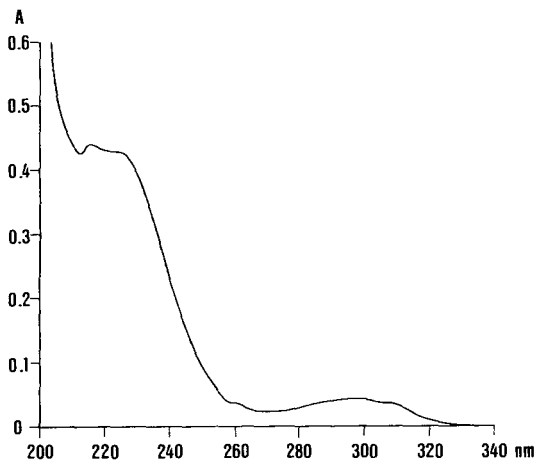


Fig. 4. UV spectrum of glibenclamide in buffered aqueous solution, 10 μg/ml of buffer (pH 6).

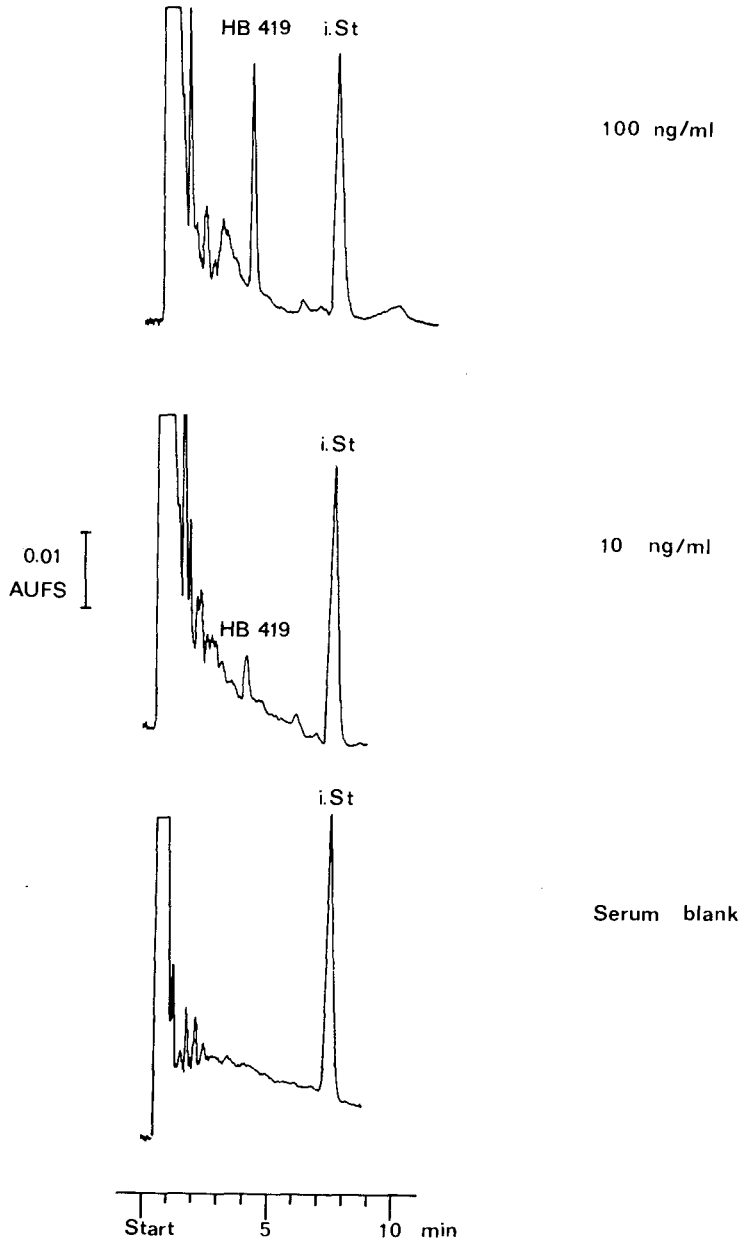


Fig. 5. Determination of glibenclamide (HB 419) in serum by HPLC, 10 and 100 ng/ml serum, compared with a serum blank.

TLC analysis

Reagents. The reagents used were buffer pH 4.62 (No. 36050, Riedel-de Haën, Seelze-Hannover, G.F.R.), diethyl ether AR, chloroform AR freshly distilled, methanol AR. The solvent system was chloroform—methanol—concentrated ammonia (15:3:0.2).

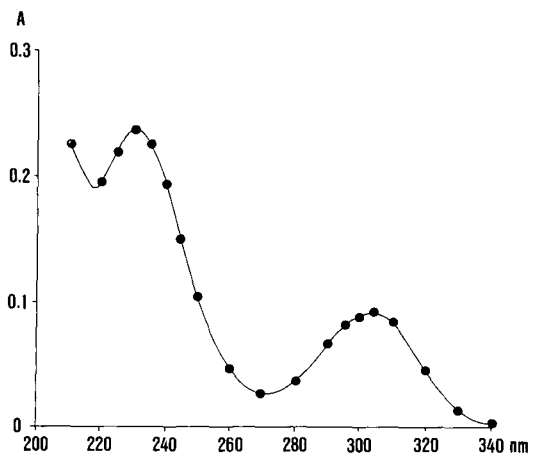
Equipment. A Zeiss KM3 chromatogram spectrophotometer with micro-optics and Servogor® 210 (Metrawatt) recorder were used. Separation was performed on F_{254} HPTLC plates (No. 5642, E. Merck, Darmstadt, G.F.R.) in a Camag twin-trough HPTLC chamber 20 cm × 10 cm (No. 25254).

For sample clean-up and spotting, a Vortex mixer, a centrifuge, glass-stoppered tubes (ca. 8 ml), conical glass-stoppered tubes (ca. 8 ml) and a Desaga Autospotter were used.

Procedure. In a glass-stoppered tube, 0.5 ml of serum was treated with 0.5 ml of buffer (pH 4.62). The serum was extracted with 5 ml of diethyl ether for 30 sec on a Vortex mixer, the phases were separated by centrifugation (5 min), and 4 ml of the organic phase were transferred into a conical tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 µl of chloroform. Using the Desaga Autospotter*, 75 µl were transferred on to the HPTLC plate as a series of consecutive droplets of ca. 100 nl volume each. Since each of these drops evaporated before the next one fell, narrow spots were obtained suitable for a high-performance TLC procedure.

The twin-trough HPTLC developing chamber contained 10 ml of the solvent in one compartment. The plate was developed over a distance of 6 cm in the dark and without previous saturation. The R_F value of glibenclamide was 0.5.

Measurements of the plates were carried out in the reflectance mode in the direction of the solvent flow with an effective slit (micro-optics) of 4.5 mm × 0.15 mm at a wavelength of 300 nm** (Fig. 6), scanning speed 50 mm/min and paper speed 240 mm/min. Peak areas of glibenclamide were evaluated and quantified by means of a calibration graph based on parallel analysis of known serum standards on the same plate.



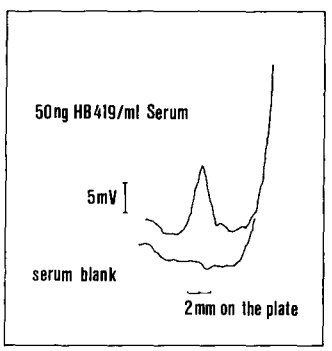


Fig. 6. In situ UV spectrum of glibenclamide on an HPTLC plate, 0.5 µg per spot.

Fig. 7. Determination of glibenclamide (HB 419) in serum by HPTLC, 50 ng/ml of serum, compared with a serum blank.

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

**Peak heights at 230 nm are considerably greater. However, as we experienced, interference from biological matter then induces higher variations of the results.

RESULTS

For HPLC and TLC the compound was admixed independently to blank serum in, respectively, seven and eight concentrations covering the range 2—500 ng/ml serum. Each sample was split into six portions, so that six equal series were formed. Each series was then analyzed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria

In correspondence with the recommendations of IUPAC [11] and IFCC [12], quality criteria of an analytical method are precision, accuracy, sensitivity and specificity. The corresponding parameters are derived from the analytical results given in Table I.

TABLE I

GLIBENCLAMIDE DETERMINATION BY HPLC AND TLC, RECOVERY AND ASSAY PRECISION

n = 6 determinations.

Added (ng/ml)	Found (ng/ml)	
	HPLC	TLC
500	501 ± 21	498 ± 13
250	247 ± 13	—
200	—	199 ± 5.2
100	98 ± 9.2	100 ± 3.3
50	49 ± 4.0	51 ± 2.5
25	23 ± 2.3	—
20	—	24 ± 2.3
10	11 ± 1.1	10 ± 1.8
5	6 ± 3.1	4 ± 1.0
2	—	2 ± 1.6
Blank	0	0
Precision*	± (3.9% ± 2.5 ng/ml)	± (2.3% ± 1.3 ng/ml)
Accuracy**	−0.7 ± 1.7 ng/ml	+0.1 ± 1.8 ng/ml

*See text under Precision.
**See text under Accuracy.

Precision. The standard deviations (S.D.) of the results obtained from each sample were considered to be a measure of precision. Experience has shown that precision decreases with decreasing concentration. Considering this effect, we expressed the standard deviation of the method as a function of concentration: glibenclamide by HPLC, S.D. = 2.5 ng/ml +3.9% of concentration in ng/ml; glibenclamide by TLC, S.D. = 1.3 ng/ml +2.3% of concentration in ng/ml.

Accuracy. Accuracy was depicted by the deviation (bias) at the mean value of the results from the theoretical value. In the case of glibenclamide, average

accuracy was < 1 ng/ml for both analytical methods. Regression coefficients were better than 0.999.

Sensitivity. The limit of detection is considered to be a measure of sensitivity. Whereas the limit of detection in a purely qualitative chromatographic separation is defined by the signal-to-noise ratio, it is a function of the precision of a method in the case of a quantitative procedure. As the precision decreases with decreasing values, a threshold value is obtained whose standard deviation is so large that statistically significant differentiation from zero is no longer possible. This value is defined as detection limit (DL)

$$DL = t_{n,95\%} \cdot \sigma_{c \to 0}$$

which for $n = 6$ becomes

$$DL = 2.02 \cdot \sigma_{c \to 0}$$

Thus, for glibenclamide the limit of detection calculated from the precision of the method for values approximating zero was 5 ng/ml in the case of HPLC compared to 3 ng/ml in the case of TLC.

Specificity. Specificity can only be defined verbally: so far, neither metabolites nor other drugs interfering in the determination of glibenclamide are known. However, it should be pointed out that this statement can not be definitive and should be reconsidered whenever applying this method to other species or in the case of new, concurrent medication.

Comparison with radioimmunoassay

Accuracy as described above refers only to spiked samples. Accuracy in true samples, i.e. from human or animal trials, has to be proved by comparison with results obtained from identical samples using an independent method. Both the TLC and HPLC methods were therefore compared independently with the radioimmmunoassay method, which up to now served as the standard procedure in our company [13]. The results correlated well (cf. Figs. 8 and 9).

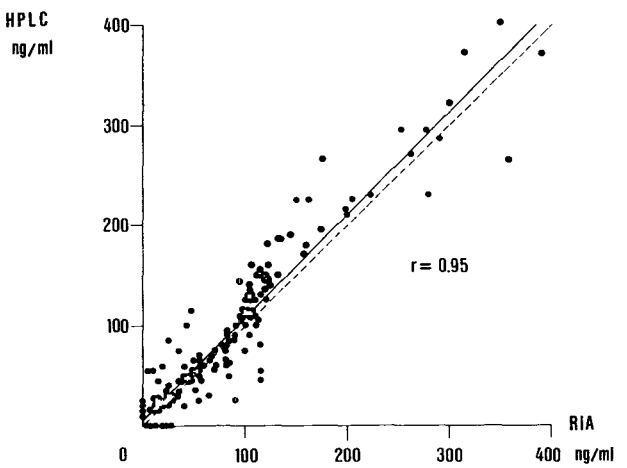


Fig. 8. Parallel analyses of 174 human serum samples by radioimmunoassay (RIA) and HPLC. HPLC: $\overline{c}$ = 88 ng/ml, S.D. = 23 ng/ml ≈ 27% of $\overline{c}$. RIA: $\overline{c}$ = 80 ng/ml, S.D. = 25 ng/ml ≈ 31% of $\overline{c}$. C_{HPLC} = (1.03 ± 0.03 · C_{RIA} + (5 ± 3) ng/ml. C_{RIA} = (0.88 ± 0.02) · C_{HPLC} + (3 ± 3) ng/ml.

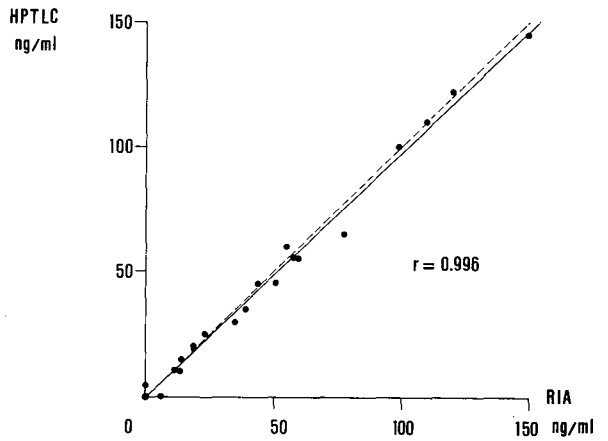


Fig. 9. Parallel analyses of 27 human serum samples by radioimmunoassay (RIA) and HPTLC. HPTLC: $\overline{c}$ = 36 ng/ml, S.D. = 3.7 ng/ml ≈ 10% of $\overline{c}$. RIA: $\overline{c}$ = 37 ng/ml, S.D. = 3.7 ng/ml ≈ 10% of $\overline{c}$. C_{HPTLC} = (0.98 ± 0.02) · C_{RIA} + (−0.6 ± 0.9) ng/ml. C_{RIA} = (1.02 ± 0.02) · C_{HPTLC} + (0.8 ± 1.0) ng/ml.

Pharmacokinetics

Glibenclamide was administered orally in doses of 2.5 mg to three healthy male volunteers (volunteer I, age 32 years, height 1.84 m, weight 89 kg; II, age 36 years, height 1.92 m, weight 69 kg; III, age 50 years, height 1.92 m, weight 80 kg)*. Serum levels were determined and the pharmacokinetic profiles were calculated based on the Bateman function. This is demonstrated by the serum kinetics obtained from the three volunteers in Fig. 10.

After a lag time of 1.8 ± 0.7 h, glibenclamide was absorbed with a half-life of 0.6 ± 0.2 h. Maximum serum levels of 123 ± 9 ng/ml serum were observed 3.2 ± 0.9 h post administration. The drug was eliminated with a half-life of 1.6 ± 0.3 h.

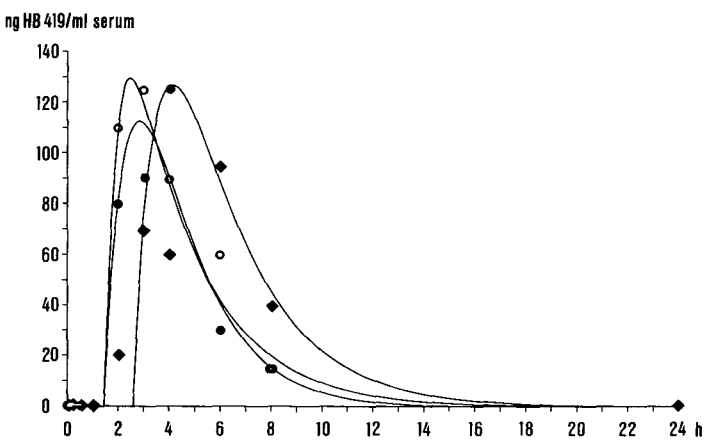


Fig. 10. Serum pharmacokinetics after oral application of 2.5 mg glibenclamide (HB 419) to volunteers I (•), II (♦) and III (○).

*These studies were performed by Drs. W. Rupp and M.J. Badian, Hoechst AG.

DISCUSSION

Both analytical methods, HPLC and TLC, were shown to be practicable and sensitive enough for routine determinations of glibenclamide in human serum. Therefore either of these methods may be employed whenever the use of the radioimmunoassay is not possible. Performance differences between both assays presented here are marginal.

Because of its flexibility, TLC should be preferred when small numbers of samples are to be analyzed. For larger series we prefer HPLC for which automated equipment has become more easily available. Since, meanwhile, TLC scanning also may be automated [14], the choice of the "appropriate" method should be based in any instance on a laboratory's equipment as well as experience.

ACKNOWLEDGEMENTS

We would like to thank Mr. Gottfried Fischer and Mr. Lutz Hack for technical assistance.

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SIMULTANEOUS DETERMINATION OF CHLORPROMAZINE AND LEVOMEPROMAZINE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ELECTROCHEMICAL DETECTION

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(First received April 22nd, 1981; revised manuscript received July 21st, 1981)

SUMMARY

A rapid, selective and sensitive method for the simultaneous determination of chlorpromazine and levomepromazine in human plasma and urine has been developed using high-performance liquid chromatography with electrochemical detection.

The unchanged drugs and internal standard extracted from plasma and urine were separated by reversed-phase high-performance liquid chromatography. The influence of acetonitrile concentration and of the pH of the mobile phase were investigated. The detection limits were 100 pg for chlorpromazine and for levomepromazine. In comparison with three other detection systems this was found to be the most sensitive method.

This method was successfully applied to the simultaneous determination of chlorpromazine and levomepromazine in human plasma and urine for pharmacokinetic studies.

INTRODUCTION

Antipsychotic drugs have been widely used in the treatment of psychosis.

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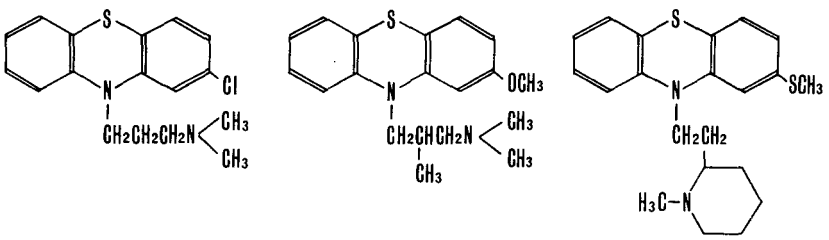
Also, it is well known that several drugs are simultaneously used in psychiatric practice. However, there is no convincing evidence for their efficacy [1]. To study the clinical utility of the polypharmacy from the aspect of pharmacokinetic evaluation we have developed a sensitive and selective method by reversed-phase high-performance liquid chromatography (HPLC) using electrochemical detection for the simultaneous determination of chlorpromazine (CPZ) and levomepromazine (LPZ) (Fig. 1) in biological fluids. Detection of the drugs is based on their electro-oxidizable properties.



(I) (II) (III)

Fig. 1. Structures of chlorpromazine (I), levomepromazine (II) and thioridazine (III).

Gas—liquid chromatography (GLC) is a satisfactory and practical means for the determination of phenothiazines in biological materials. Using electron-capture detection (ECD) [2—5], CPZ and its metabolites in plasma can be determined in nanogram quantities, whereas phenothiazines (e.g. LPZ) having no halogen can not be detected. By HPLC using UV [6—11] or fluorescence [12] detection, difficulties in the determination of phenothiazines by GLC based on high polarity, low volatility and thermal instability could be eliminated. HPLC using electrochemical detection (voltammetric detection, VMD) [13, 14] has made it possible to determine the low levels of electroactive CPZ and LPZ in subnanogram quantities.

EXPERIMENTAL

Reagents

All reagents were of analytical grade. Chlorpromazine hydrochloride was kindly supplied by Yoshitomi Pharmaceutical Co. (Osaka, Japan), levomepromazine hydrochloride by Shionogi Pharmaceutical Co. (Osaka, Japan) and thioridazine (TRD) by Sandoz (Basle, Switzerland).

Stock solution

Ten milligrams each of CPZ, LPZ and TRD (internal standard) were weighed into respective 10-ml volumetric flasks and dissolved in methanol to the concentration of 1 mg/ml. Under refrigeration these solutions were stable for several weeks.

Materials

Blood and urine samples for recovery experiments were collected from several volunteers. Samples from patients who had received both CPZ and LPZ

for at least two weeks were analyzed in clinical studies. Urine samples were collected for 24 h under shaded light.

Apparatus

Analyses were performed on a ALC/GPC 204 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Waters Model U6K injector and a Yanaco electrochemical detector Model VMD-101 (Yanagimoto Manufacturing Co., Kyoto, Japan). The column (150 mm × 4 mm I.D.) was stainless steel and packed with Nucleosil C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) of 5 μm particle size. The detector consisted of a low-volume thin-layer cell housing a three-electrode detection system, i.e. a glassy carbon working electrode, a stainless tube auxiliary electrode and an Ag/AgCl reference electrode (Fig. 2), and an electrochemical control unit. The detector output was connected to a linear potentiometric recorder.

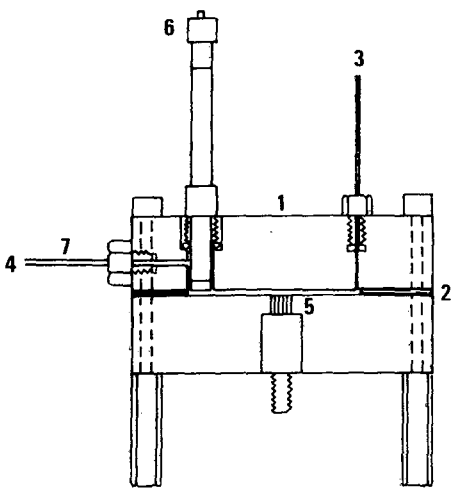


Fig. 2. Cross-section of the low-volume thin-layer cell for HPLC. 1 = cell body (polychlorotrifluoroethylene); 2 = spacer (polytetrafluoroethylene film, 50 μm); 3 = solute inlet; 4 = solute outlet; 5 = glassy carbon working electrode; 6 = Ag/AgCl reference electrode; 7 = stainless tube auxiliary electrode.

In GLC analyses a Yanagimoto GC-1800 gas chromatograph equipped with a flame ionization detector (FID) (Yanagimoto Manufactoring Co.) was used. The glass column (2.0 m × 2 mm I.D.) was packed with 4% PEG 20M on Chromosorb W AW HMDS (80—100 mesh).

Chromatographic conditions

Conditions for HPLC. The mobile phase consisted of pyridine—tetrahydrofuran—acetonitrile—0.1 *M* acetate buffer (pH 3.5) (0.1:1.0:68.9:30.0, v/v) containing 20 mmol/l of $NaClO_4$. The solvent mixture was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) and degassed before use under negative pressure. The flow-rate was adjusted to 0.7 ml/min, generating a pressure of 1200—1300 p.s.i. (ca. 8.2—9.0 MPa).

Conditions for GLC. The operating conditions were as follows: oven tem-

perature 246°C; detector temperature 290°C; inlet temperature 290°C; carrier gas (nitrogen) flow-rate 25 ml/min; hydrogen flow-rate 20 ml/min; air flow-rate 750 ml/ min.

All operations in both HPLC and GLC were carried out at room temperature.

Extraction procedures

Plasma. To 1 ml of plasma were added 4 ml of distilled water, 1 ml of internal standard solution (TRD, 160 ng/ml) and 0.8 ml of 1 *N* sodium hydroxide. The mixture was extracted three times with 15 ml of *n*-heptane containing 1.5% isoamyl alcohol by shaking for 10 min. After centrifugation at 700 *g*, the combined organic layer was evaporated to dryness. The residue was dissolved in 10 ml of 0.05 *N* hydrochloric acid, and 20 ml of diethyl ether were added subsequently. After shaking for 3 min, the ether was removed by aspiration. The acidic aqueous layer was alkalinized with the addition of 1 ml of 5 *N* sodium hydroxide and extracted again with 10 ml of *n*-heptane—isoamyl alcohol mixture. After shaking and centrifugation, the organic layer was evaporated to dryness. The residue was dissolved in 1 ml of acetonitrile and 50 μl were injected into the chromatograph.

Urine. To 20 ml of urine were added 3 ml of 1 *N* hydrochloric acid and 1 ml of internal standard solution (TRD, 1 μg/ml). The procedure is summarized as follows. Washing with diethyl ether, alkalinization of the acidic aqueous layer and extraction with *n*-heptane—isoamyl alcohol mixture are successively carried out as described above. Finally, the residue is dissolved in 1 ml of acetonitrile and 10 μl are injected into the chromatograph.

Calculation

The calculations of the concentrations of CPZ and LPZ in plasma and urine were always made by the internal standard method using the peak-height ratio technique.

RESULTS AND DISCUSSION

Chromatographic conditions

In order to achieve the best chromatographic conditons, the capacity factor (k') was measured as a function of acetonitrile content and pH of the mobile phase. When the acetonitrile concentration exceeded 80%, the resolution became poorer and the retention time decreased. By lowering the acetonitrile content the resolution was improved but the retention time was more than 15 min (Fig. 3). As the pH was increased above 4 the k' and the retention time gradually increased. At a pH of less than 3.5 excellent resolutions were attained in a short time (Fig. 4). In order to achieve good resolution it is necessary that the acetonitrile content and the pH of the mobile phase are fixed at 68% and 3.5, respectively.

The addition of pyridine to the mobile phase caused the oxidation potential to shift to a more negative potential [15]. Sodium perchlorate was added as a supporting electrolyte to improve conductivity. TRD was chosen as an internal standard because it is a structural analogue to CPZ and was not co-administered to patients in these experiments.

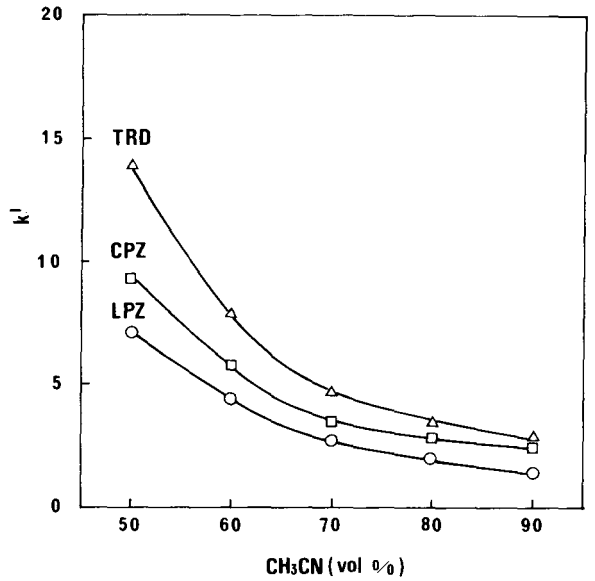


Fig. 3. Effect of the solvent acetonitrile content at pH 3.5 on capacity factors (k') of CPZ (□), LPZ (○) and internal standard (△). A 5-μl volume of the standard mixture (5 ng of CPZ and LPZ, and 7 ng of internal standard) was injected. Other conditions are described in the text.

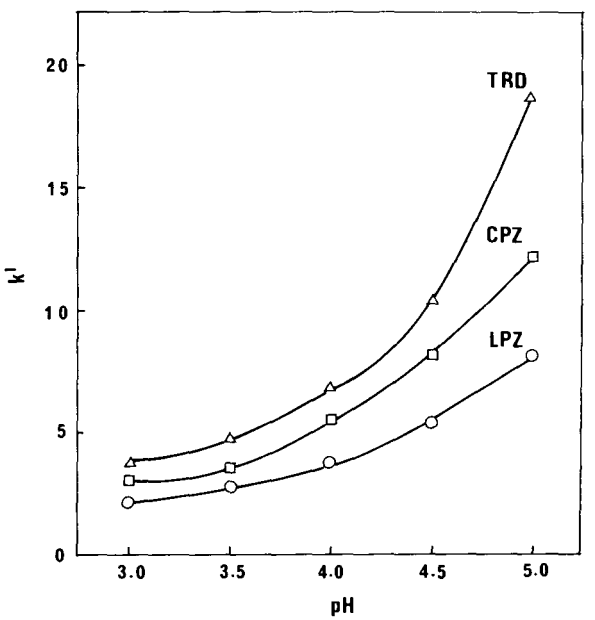


Fig. 4. Effect of the solvent pH on capacity factors (k') of CPZ (□), LPZ (○) and internal standard (△). Other conditions are described in Fig. 3.

Under the chromatographic conditions described above, the retention times of CPZ, LPZ and internal standard were 8.0, 7.0 and 9.5 min, respectively. Fig. 5 shows a typical chromatogram of a standard mixture. No interfering peaks in

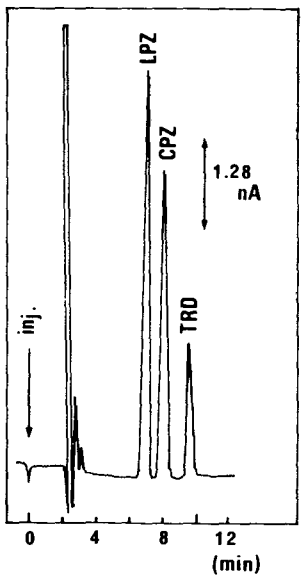


Fig. 5. Chromatogram of standard mixture. A 5-μl volume of the standard mixture (5 ng of CPZ and LPZ, and 7 ng of internal standard) was injected. Chromatographic conditions are described in the text.

the region of the standards have been observed from the blank plasma and urine samples analyzed.

Detection and sensitivity

Electrochemical detection is extremely selective because the sensitivity for each compound can be changed by altering the potential applied on the working electrode. We obtained three hydrodynamic voltammograms from the chromatographic peak currents of the respective drugs at each potential (Fig. 6). The limiting currents reached a plateau at 0.85, 0.80 and 0.75 V for CPZ,

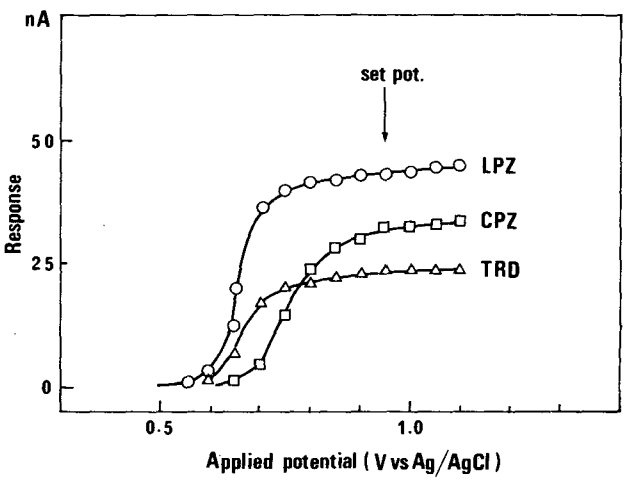


Fig. 6. Current—potential curves for CPZ, LPZ and internal standard. A 32-ng amount of each compound was injected. Chromatographic conditions are described in the text.

LPZ and TRD, respectively. When the applied potential was set at more than 0.85 V against the Ag/AgCl electrode, each drug could be quite sensitively and selectively oxidized in the VMD cell. In these experiments the potential was fixed at 0.95 V vs. Ag/AgCl.

The sensitivity of the electrochemical detector gradually declined in the continuous analysis. But it was restored by cleaning the electrode surface with a soft cloth loaded with hexane once a month, and by setting the voltammetric detector at —0.5 V for a few minutes before the analysis.

Table I shows comparable results obtained using four detection systems, minimum detectable amounts for CPZ and LPZ and the detection limits in plasma and urine. VMD and UV detection revealed a more sensitive response to the drugs than FID-GLC. ECD-GLC could sensitively respond to CPZ, but not to LPZ. With FID-GLC these drugs were simultaneously detected with poorer sensitivity. For simultaneous determination VMD is found to be the most sensitive method.

TABLE I

DETECTION LIMIT OF CPZ AND LPZ IN FOUR DETECTION SYSTEMS

The final residue from the extraction procedure for GLC was dissolved in 0.3 ml of acetone and 5 μl were injected. Other conditions were as described in the text.

Method	Detection method	Minimum detectable amount (ng)		Detection limit (ng/ml)			
				Plasma		Urine	
		CPZ	LPZ	CPZ	LPZ	CPZ	LPZ
HPLC	UV	1	1	20	20	5	5
	VMD	0.1	0.1	2	2	0.5	0.5
GLC	FID	10	10	600	600	30	30
	ECD*	0.2	—	12	—	0.6	—

*Values cited from ref. 16.

The precision and linearity of this method were determined by injecting 10- or 50-μl aliquots of the standard mixture, in which the concentration of CPZ or LPZ was between 0.002 and 6.4 μg/ml, and that of TRD 0.16 or 1.0 μg/ml. Linear regression analyses were carried out by plotting the ratios of the peak height of CPZ or LPZ to that of internal standard as a function of the ratios of the weight of each drug to that of the standard. Both calibration graphs gave excellent linearity with a correlation coefficient (r) of 0.999 (n = 8). The linear regression equations calculated by the least-squares method were $y = 1.335x + 0.048$ for CPZ and $y = 1.952x - 0.028$ for LPZ when the concentration of TRD was 1.0 μg/ml. The reproducibility of the method was studied by the repeated injection (n = 10) of 1 or 10 ng of each drug. The standard deviations of peak currents were 1.8% and 1.6% for CPZ and LPZ, respectively.

Recovery

The recovery and reproducibility of the extraction were determined by spiking blank plasma and urine samples with known amounts of CPZ, LPZ and internal standard (plasma 20, 50 and 100 ng/ml; urine 0.1 and 1.0 μg per 20 ml) and extracting as described under procedures. The average recovery from plasma samples was 89.8 ± 1.38% (S.D.) for CPZ, 93.2 ± 2.07% for LPZ and 82.1 ± 2.34% for TRD; from urine the average recoveries were 84.0 ± 3.00% (S.D.) for CPZ, 98.0 ± 3.20% for LPZ and 80.5 ± 1.35% for TRD. The coefficients of variation in plasma samples were calculated to be 1.54%, 2.22%, and 2.85% for CPZ, LPZ and TRD, respectively, while those in urine samples were 3.62%, 3.27% and 1.61% for CPZ, LPZ and TRD, respectively.

Clinical studies

The HPLC—VMD system was applied to the analysis of plasma and urine samples from patients receiving daily 200-mg doses of CPZ and LPZ. Fig. 7a shows typical chromatograms of extracts of blank plasma and of plasma of patients receiving the drugs. Fig. 7b also shows the chromatograms of extracts from urine samples. Fig. 8 shows two types of patterns (I and II) of CPZ and LPZ levels in the plasma of patients who received the drugs on a divided-dose schedule. Urinary excretion of the drugs in the patients for 24 h is given in Table II.

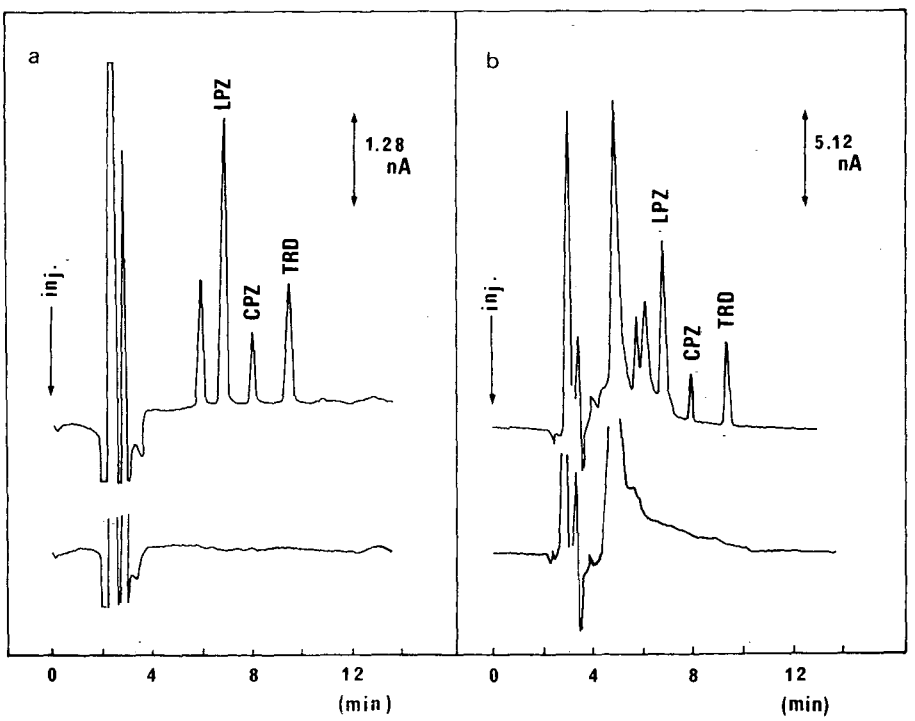


Fig. 7. (a) Chromatograms of extracts of blank plasma and of plasma of patients receiving daily 200 mg of CPZ and 200 mg of LPZ. (b) Chromatograms of extracts of blank urine and of urine of patients receiving the drugs as in part (a). These were determined at a different VMD sensitivity from the measurements in (a).

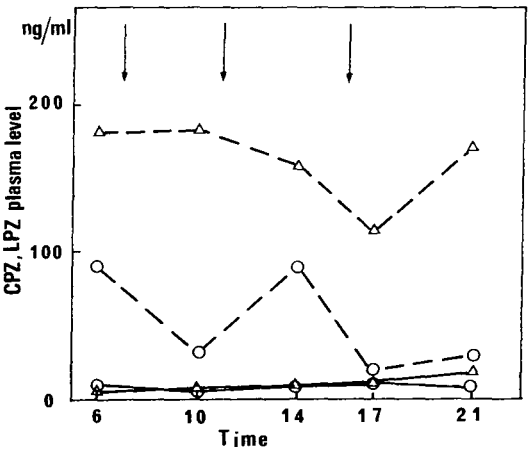


Fig. 8. The patterns (type I, — — —; and type II, ——) of CPZ (○) and LPZ (△) levels in plasma of patients receiving the drugs on a divided-dose schedule. Arrows show the times the patients received the drugs (200 mg per day of each drug).

TABLE II

URINARY EXCRETION OF CPZ AND LPZ IN PATIENTS

Patient	Drug administration			Urine volume for 24 h (ml)	Urinary excretion (mg/day)	
	Dose (mg/day)		Duration (months)			
	CPZ	LPZ			CPZ	LPZ
A	200	350	3	1210	1.343	0.762
B	200	100	17	1090	0.458	0.425
C	200	250	1	1100	0.319	0.112
D	200	150	20	1500	1.395	0.315
E	300	150	3	1650	1.419	0.611

Our preliminary results of CPZ and LPZ levels in plasma and urine show as follows. In type I pattern the drugs maintain relatively high concentrations in plasma all day, and in type II lower concentrations. LPZ is always at a higher concentration than CPZ in both types. More CPZ than LPZ tends to be excreted in the urine. These results might suggest that CPZ would inhibit the metabolism of LPZ or LPZ would activate that of CPZ in patients. Our subsequent paper will present a further detailed report about the interaction between phenothiazines in psychotic patients.

The system has proved to be useful for pharmacokinetic studies in humans.

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DETERMINATION OF (*R*)- AND (*S*)-ALPRENOLOL AND (*R*)- AND (*S*)-METOPROLOL AS THEIR DIASTEREOMERIC DERIVATIVES IN HUMAN PLASMA BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol in human plasma. Separation of the enantiomers was accomplished after preparation of diastereomeric derivatives with symmetrical anhydrides of *tert.*-butoxycarbonyl-L-leucine followed by treatment with trifluoroacetic acid at 0°C to remove the *tert.*-butoxycarbonyl group. The separations of the diastereomeric derivatives were performed using a reversed-phase system with μBondapak C_{18} as support and phosphate buffer pH 3.0 with the addition of acetonitrile as the mobile phase. High stability of the chromatographic system was achieved.

The reproducibilities in the determination of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol in human plasma were 9.4 and 9.8% (relative standard deviation) for alprenolol and metoprolol, respectively, at drug levels of 0.5 ng/ml.

In two subjects who received single oral doses of alprenolol (100-mg tablet) and metoprolol (50-mg tablet) the plasma levels of the (*R*)-isomers were lower than for the (*S*)-isomers.

INTRODUCTION

Most commercially available adrenergic beta-receptor blocking drugs are racemic mixtures. The (*S*)-forms are considered to cause most of the pharmacological effects [1].

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Recently we developed a high-performance liquid chromatographic (HPLC) technique enabling separation and quantitation of (*R*)- and (*S*)-propranolol as their diastereomeric derivatives with N-trifluoroacetyl-L-proline or L-leucine [2, 3], and we found that the two isomers had different plasma disposition kinetics after giving the racemate to man [2]. Similar results were reported thereafter [4]. Differences in the kinetics of optical isomers may be important clinically and in studies on concentration—effect relationships. It is of general interest to make possible such studies also with other beta-receptor blocking drugs. Therefore we have developed a HPLC method for the separation and quantitation of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol in plasma after administration of clinical doses of the racemic drugs to man.

This paper presents studies of the formation rates of the diasteromeric L-leucine derivatives of the two drugs, using the technique described previously [3], as well as the chromatographic behaviour of the diastereomeric derivatives. Plasma concentrations of the (*R*)- and (*S*)-isomers of the two drugs in two healthy volunteers given single doses, 100 mg of alprenolol and 50 mg of metoprolol, are also presented.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was built up from an Altex Model 100 solvent delivery system and a Waters Model U6K injector. A Schoeffel FS 970 L.C. fluorimeter was used as the detector. The detector was operated with an excitation wavelength of 193 nm (for metoprolol determinations) or 198 nm (for alprenolol determinations) without cut-off emission filter. The mass spectrometer was an LKB 2091 equipped with a digital PDP 11/05 computer system and operated in the electron-impact mode at 70 eV. Infrared (IR) spectra were recorded with a Perkin-Elmer 457 spectrophotometer. pH was measured with an Orion Research Model 801 A/ digital pH meter equipped with an Ingold combined electrode Type 401.

Chemicals

Racemic alprenolol hydrochloride, (*R*)- and (*S*)-alprenolol tartrate and racemic metoprolol tartrate were kindly supplied by AB Hässle, Mölndal, Sweden. N-*tert.*-Butoxycarbonyl-L-leucine (Boc-L-Leu) and N-*tert.*-butoxycarbonyl-L-alanine (Boc-L-Ala) were obtained from Sigma (St. Louis, MO, U.S.A.). N,N-Dicyclohexylcarbodiimide and phosgene were obtained from Fluka (Buchs, Switzerland). The support, μBondapak C_{18} (10 μm), was obtained from E. Merck (Darmstadt, G.F.R.). The acetonitrile was of grade S quality and purchased from Rathburn Chemicals (Walkerburn, Great Britain). Triethylamine "zur Synthese" and trifluoroacetic acid (TFA) "für die Spektroskopie" were obtained from E. Merck. The triethylamine was treated overnight with sodium hydroxide pellets to remove water. The sodium hydroxide pellets were removed by filtration and naphthylisocyanate (2%, v/v) was added to the triethylamine before distillation to remove primary and secondary amines [5]. All other chemicals used were of analytical or equivalent grade and were used without further purification.

Chromatographic technique

Chromatographic analyses were performed at room temperature. The mobile phases were prepared from phosphoric acid (final phosphate concentration 0.1 *M*), appropriate amounts of sodium hydroxide (pH was adjusted to 3.0) acetonitrile and water. The mobile phase was degassed in an ultrasonic bath before use. The volume of the mobile phase in the column, V_M, was measured either by injection of water or phosphate buffer pH 7.5.

The asymmetry factor was calculated by drawing a perpendicular to the baseline from the vertex formed by the two peak tangent lines. The back part of the peak baseline divided by the front part gives the asymmetry factor.

Column preparation

The column (100 × 3.2 mm) was made of 316 stainless steel with a polished inner surface, equipped with modified Swagelok connections and Altex stainless-steel frits (2 μm). The column was packed by a modification of the balanced density slurry technique described by Majors [6]. The support was suspended in 10 ml of chloroform and poured into the packing column which was filled by hexane. Acetone was used as driving liquid in the Haskel pump which was operated at 36.2 MPa. After packing the column was washed by pumping 50 ml of acetonitrile through the column followed by 100 ml of acetonitrile—water (50:50). The column was then equilibrated with 150 ml of the mobile phase.

Synthesis of the reagents

Symmetrical anhydrides of Boc-L-Ala and Boc-L-Leu were prepared in two different ways: either by use of phosgene [7] or N,N-dicyclohexylcarbodiimide [8].

Phosgene method. A solution of 1 mmole of the Boc-amino acid and 1.1 mmole of triethylamine in 5.0 ml of methylene chloride was chilled in a dry ice—ethanol bath to −40°C. One half mmole of phosgene (a 20% phosgene solution in toluene) was added and after 5 min the reaction mixture was analysed by IR spectroscopy. The symmetrical anhydrides have three characteristic absorption bands at 1830, 1750 and 1720 cm^{-1} [7]. The reagents were stored at −20°C until they were used.

N,N-Dicyclohexylcarbodiimide method. To a solution of 1 mmole of the Boc-amino acid in 3.0 ml of methylene chloride was added 0.5 mmole of N,N-dicyclohexylcarbodiimide (2.0 ml of a 0.25 *M* N,N-dicyclohexylcarbodiimide solution in methylene chloride) and allowed to react for 1 h at 0°C. The reaction mixture was filtered to remove the urea formed during the reaction and finally analyzed by IR spectroscopy as above.

Evaluation of reaction conditions

A reaction scheme for the derivatization process is given in Fig. 1.

Reaction of alprenolol and metoprolol with the leucine reagent. (1) Racemic alprenolol hydrochloride and metoprolol tartrate corresponding to 97 and 104 ng as base, respectively, were added to 1.0 ml of 1 *M* carbonate buffer pH 9.9. Six millilitres of water-saturated diethyl ether were added and the tubes were agitated for 15 min. After centrifugation at 4200 *g* for 5 min, 5.0 ml of the

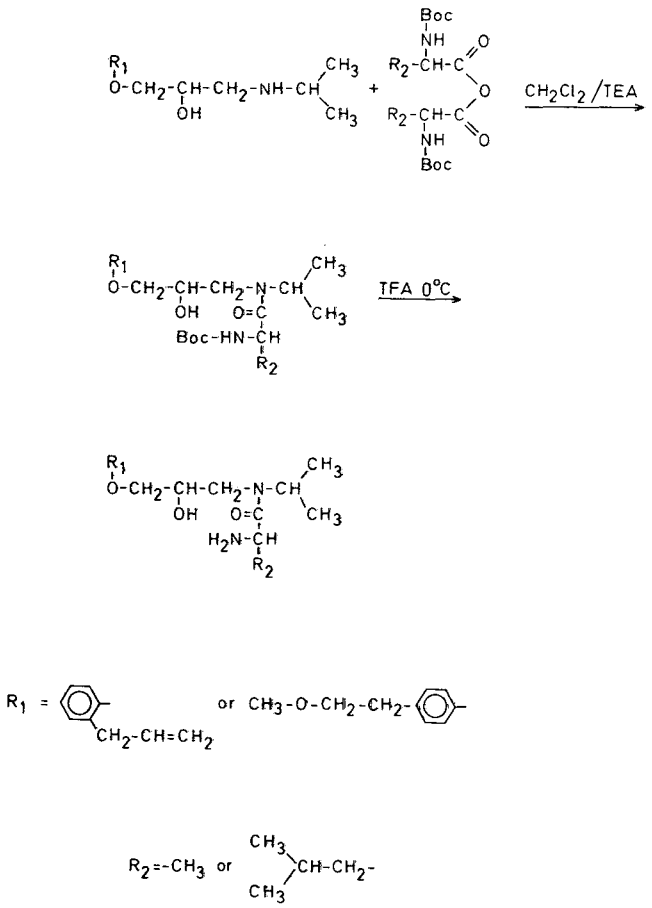


Fig. 1. Reaction of alprenolol and metoprolol with symmetrical anhydrides of Boc-amino acids. TEA = triethylamine.

ether phases were evaporated at 35°C in a stream of dry nitrogen.

(2) The residues were dissolved in 250 μl of a solution of triethylamine in methylene chloride (for alprenolol 89 μmoles of triethylamine and for metoprolol 35.6 μmoles of triethylamine, see below) and 100 μl of the reagent (Boc-L-Leu) were added. After suitable intervals samples were taken out in duplicate and evaporated under nitrogen at 35°C. Two millilitres of 0.1 *M* sodium hydroxide were added to the tubes and the tubes were agitated for 10 min to hydrolyse excess reagent. Six millilitres of ether were added and the solutions were extracted with ether for 15 min. After centrifugation 5.0 ml of the ether phases were evaporated with dry nitrogen at 35°C.

(3) The residues were treated for 10 min with 250 μl of TFA in an ice-bath (see below). The reaction was stopped by addition of 2.0 ml of 2 *M* sodium hydroxide. The solutions were extracted with 6.0 ml of ether and 5.0 ml of the ether phases were collected and extracted with 100 μl (for metoprolol) or 150 μl (for alprenolol) of 0.1 *M* phosphoric acid.

(4) An aliquot of 94 μl (for metoprolol) or 145 μl (for alprenolol) of the phosphoric acid extracts was injected on to the column. The alprenolol-L-Leu derivatives were separated using a mobile phase consisting of 35% acetonitrile in phosphate buffer pH 3.0. The separation of the metoprolol-L-Leu derivatives was performed using 30% acetonitrile in phosphate buffer pH 3.0.

Dependence of the yield on the triethylamine concentration. The dependence of the yield of the alprenolol-Boc-L-Leu and the metoprolol-Boc-L-Leu derivatives on the triethylamine concentration were evaluated by reaction of alprenolol and metoprolol according to the procedure given above. The amount of triethylamine in the reaction mixture was varied between 1.78 and 124.6 μmoles. The samples were allowed to react for 30 min with the Leu reagent.

Removal of the Boc group from the alprenolol-Boc-L-Leu and metoprolol-Boc-L-Leu derivatives. One millilitre of a solution containing 6.3 μg of racemic metoprolol tartrate per ml or 4.5 μg of racemic alprenolol hydrochloride per ml was extracted and treated as described in (1) and (2) above with the exception that 20 ml of ether were used for the extraction of the derivatives (2).

One-millilitre portions of the ether phase were taken out and evaporated in a stream of dry nitrogen at 35°C. The tubes were cooled in an ice-bath and 250 μl of TFA were added. After suitable intervals the reaction was stopped in duplicate tubes and treated as in (3) above. Aliquots of the phosphoric acid extracts were injected on to the column. The mobile phases described in (4) above were used.

Standard curves and analytical procedure for quantitation of (R)- and (S)-metoprolol and (R)- and (S)-alprenolol in human plasma

Standard curves were prepared by adding known amounts of racemic alprenolol hydrochloride (corresponding to 1—97 ng as base) and metoprolol tartrate (corresponding to 1—104 ng as base) to 1.0 ml of 1 *M* carbonate buffer pH 9.9 and 1.0 ml of drug-free plasma (for standard samples). Standard and original plasma samples (1.0 ml) were treated according to the procedure given under *Reaction of alprenolol and metoprolol with the leucine reagent.* Samples containing alprenolol were reacted for 30 min, while a 15-min reaction time was used for the samples containing metoprolol.

Standard curves were constructed by plotting the peak height versus the sample concentration.

Identification of the derivatives

Racemic metoprolol tartrate and alprenolol hydrochloride corresponding to about 50 μg as base of the two substances were extracted and derivatised as described above. The pH of the phosphoric acid extracts (3) was adjusted to 11 with sodium hydroxide and the derivatives were extracted to ether. An aliquot of each ether phase was collected and evaporated in a stream of dry nitrogen at 35°C. The residues were dissolved in 50 μl of methylene chloride and an aliquot was injected directly into the ion source of the mass spectrometer which was operated with an electron energy of 70 eV.

RESULTS AND DISCUSSION

Extraction procedures

Alprenolol and metoprolol are secondary amines with a pK_a of 9.63 ± 0.02 and 9.68 ± 0.02, respectively [9]. Alprenolol is quantitatively extracted by ether from 1 *M* carbonate buffer pH 9.9 with a phase-volume ratio (V_{org}/V_{aq}) of 3.0. Under the same conditions 98.5% of metoprolol is extracted by ether. These extraction studies were performed with alprenolol hydrochloride and metoprolol tartrate concentrations of 96.1 and 139.7 μg/ml, respectively.

After the removal of the Boc protection group, the L-Leu derivatives of alprenolol and metoprolol were extracted by ether from the 2 *M* sodium hydroxide solution. The degree of extraction of these derivatives was determined by quantitation of the diastereomeric derivatives in the two phases using the liquid chromatographic systems described under Experimental (4). It was found that the two diastereomeric derivatives of alprenolol were quantitatively extracted to the organic phase, while 94.7% of the L-Leu derivative of (*S*)-metoprolol and 98.7% of the (*R*)-metoprolol derivative was extracted into ether under the same conditions. This study was performed using alprenolol- and metoprolol-L-Leu concentrations of 4.9 and 5.0 μg/ml, respectively.

The L-Leu derivatives of alprenolol and metoprolol were extracted by 0.1 *M* phosphoric acid in the last extraction step. The degree of extraction of the derivatives by the phosphoric acid was determined by quantitation of the derivatives in the two phases chromatographically. With a phase-volume ratio (V_{org}/V_{aq}) of 50, the two metoprolol derivatives were found to be quantitatively extracted into the aqueous phase. The degree of extraction of the more lipophilic alprenolol derivatives was 91.7 and 85.6% for the (*S*)- and (*R*)-alprenolol derivatives, respectively, with a phase-volume ratio (V_{org}/V_{aq}) of 33.3. The same sample concentrations as for the above extraction study were used. All extraction yields are the mean values of two determinations.

Synthesis of the reagent

The symmetrical anhydride of Boc-leucine was prepared in two different ways, either by reaction of the triethylammonium salt of the Boc-amino acid with phosgene or by reaction of Boc-leucine with N,N-dicyclohexylcarbodiimide. It was, however, sometimes observed that by-products are formed when the phosgene method is used. Fig. 2A demonstrates an IR spectrum of a contaminated symmetrical anhydride of Boc-leucine. The peaks at 1685 and 1785 cm^{-1} are probably the result of an excess of phosgene in the reaction mixture [7].

The second method, in which N,N-dicyclohexylcarbodiimide was used for the synthesis of the Leu reagent, is easier to reproduce and the peaks at 1685 and 1785 cm^{-1} in the IR spectrum have never been observed using this method. An IR spectrum of a symmetrical anhydride of leucine of good quality is demonstrated in Fig. 2B.

Preparation of the Leu reagent by the latter method has the disadvantage that it is slower than the phosgene method.

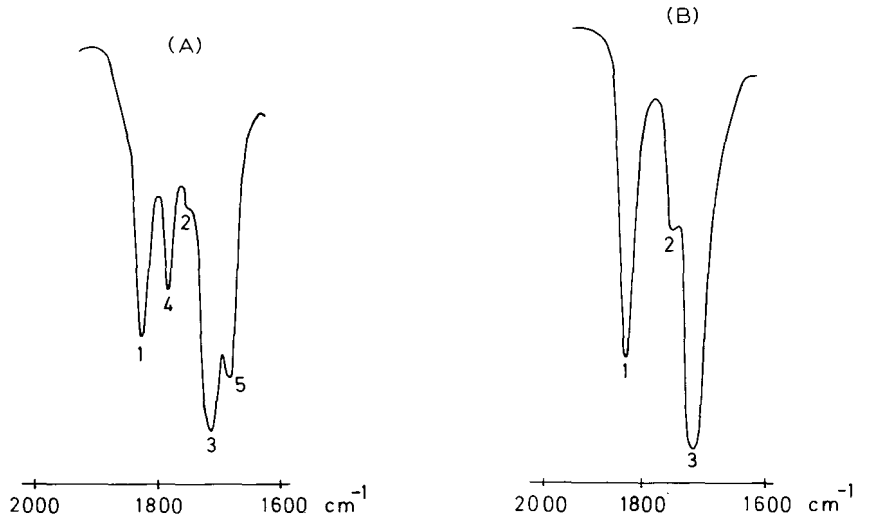


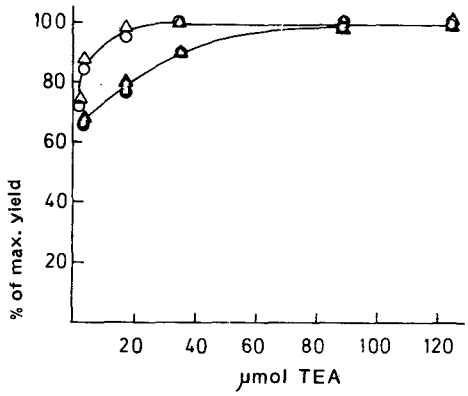
Fig. 2. IR spectra of symmetrical anhydrides of Boc-L-leucine. (A) Contaminated reagent prepared by the phosgene method. (B) Reagent of good quality prepared by the N,N-dicyclohexylcarbodiimide method. Peaks 1 (1830 cm^{-1}) and 2 (1760 cm^{-1}) arise from the carbonyl group of the symmetrical anhydride; peak 3 (1720 cm^{-1}) arises from the carbonyl group of the Boc protection group. Peaks 4 (1790 cm^{-1}) and 5 (1690 cm^{-1}) are by-products [7].

Derivatization procedure

Diastereomeric derivatives of alprenolol and metoprolol were prepared by reaction with a symmetrical anhydride of leucine, and by use of triethylamine as the catalyzing agent. This derivatization technique has been described in a previous paper for preparation of diastereomeric derivatives of propranolol [3].

It was observed that the content of triethylamine in the reaction mixture influences the yield of the diastereomeric derivatives of alprenolol and metoprolol. This study was performed by reacting the two substances with the Leu reagent for 30 min and by varying the triethylamine content in the reaction mixture between 1.78 and 124.6 μmoles. The result of this study is demonstrated in Fig. 3, and it was observed that the maximum catalyzing effect was obtained with 36 and 90 μmoles of triethylamine, corresponding to a molar excess of triethylamine of 95,000 and 222,000 times for metoprolol and alprenolol, respectively.

The relationship between the yield of the Boc-L-Leu derivatives of alprenolol and metoprolol and the reaction time was evaluated as described under Experimental. Fig. 4 demonstrates the result of this study; maximum yield of the metoprolol derivatives was obtained after 15 min whereas a 30-min reaction time was required for alprenolol. No significant level of underivatized substance was detected in the reaction mixture after the reaction with the Leu reagent for the stated times. This was assessed by reaction of alprenolol and metoprolol according to the procedure given under Experimental. After the reaction with the Leu reagent and extraction with ether (paragraph 2 of the procedure), an aliquot of the ether phase was extracted with 0.1 *M* phosphoric acid, which was analyzed chromatographically.



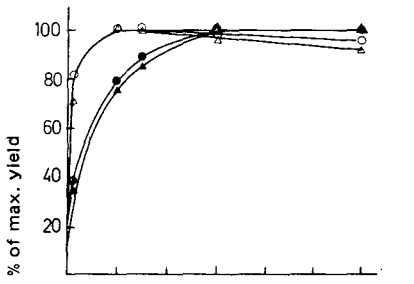


Fig. 3. Influence of triethylamine concentration on the yield of alprenolol- and metoprolol-Boc-L-Leu. (○), (*S*)-Metoprolol-Boc-L-Leu; (△), (*R*)-metoprolol-Boc-L-Leu; (●), (*S*)-alprenolol-Boc-L-Leu; (▲), (*R*)-alprenolol-Boc-L-Leu.

Fig. 4. Formation rates of the alprenolol- and metoprolol-Boc-L-Leu derivatives. Symbols as in Fig. 3.

Deprotection of the Boc-L-Leu derivatives of alprenolol and metoprolol

The last step in the derivatization procedure is deprotection of the primary amino group of the Boc-L-Leu derivatives of alprenolol and metoprolol. The Boc group is removed by reaction with TFA in an ice-bath at 0°C as described previously [3]. Maximum yield of the L-Leu derivatives of the two substances is obtained after 10 min, and no indications of non-completion of this reaction were found. In accordance with previous findings for propranolol, the Boc-L-Leu derivatives of the (*R*)-isomers of alprenolol and metoprolol are more slowly hydrolyzed than the corresponding (*S*)-isomer derivatives [3]. This effect is demonstrated in Fig. 5 where the peak area ratios (*S*/*R*) for the diastereomeric pairs are plotted against the reaction time. From Fig. 5 it can be seen that

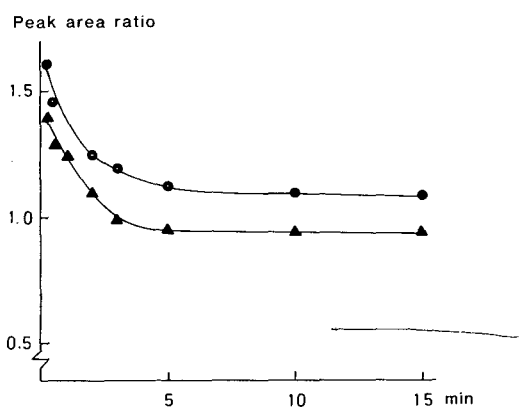


Fig. 5. *S*/*R* peak area ratios after different times of hydrolysis by TFA. (●), Alprenolol-L-Leu; (▲), metoprolol-L-Leu.

initially the S/R peak area ratios rapidly decrease, but after 5 min the curves level off and reach constant values at about 1.1 and 0.94 for the alprenolol and the metoprolol derivatives, respectively. The discrepancy from 1.0 of the S/R peak area ratios is probably caused by the extraction procedure used in the isolation step. It was shown under *Extraction procedures* that the two diastereomeric alprenolol derivatives are not extracted to the same extent in the last extraction step (into 0.1 *M* phosphoric acid) and a peak area ratio (S/R) of 1.07 is created by this extraction. It was also observed that the metoprolol-L-Leu derivatives are not extracted to the same degree into ether from a 2 *M* sodium hydroxide solution (see *Extraction procedures*). This extraction step creates a ratio (S/R) of 0.96 which is in good agreement with the 0.94 found in Fig. 5.

Racemization test

To confirm that the discrepancies from 1.0 of the S/R peak area ratios are not caused by racemization during the derivatization procedure, (S)- and (R)-alprenolol were reacted separately according to the procedure given under Experimental. Fig. 6A and B show the result of this study. Only one peak in each chromatogram was detected, corresponding to the L-Leu derivatives of (S)- and (R)-alprenolol, respectively. This is in accordance with previous findings for propranolol derivatized with symmetrical anhydrides of Boc-amino acids [3].

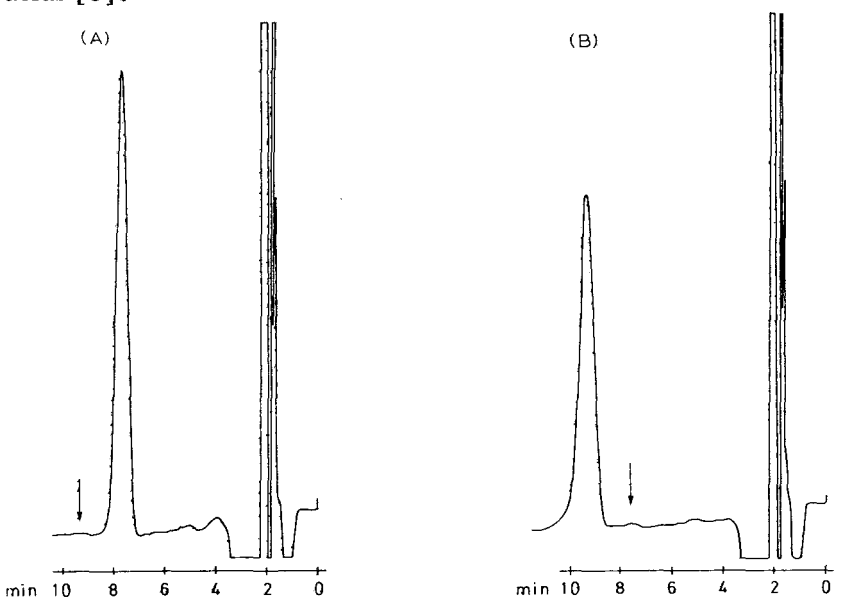


Fig. 6. Racemization test. (A) (S)-Alprenolol (48.5 ng as base) derivatized according to the procedure given under Experimental. Arrow indicates the retention time for the (R)-alprenolol-L-Leu derivative. (B) (R)-Alprenolol (48.1 ng as base) derivatized as above. Arrow indicates the retention time for the (S)-alprenolol-L-Leu derivative. Column: 100 × 3.2 mm packed with μBondapak C_{18} (10 μm). Mobile phase: 35% acetonitrile in phosphate buffer (pH 3.0); flow-rate, 0.5 ml/min.

Identification of the derivatives

The identity of the derivatives was confirmed by mass spectrometry. The fragmentation pattern coincided with that for the L-Leu derivatives of propranolol and the base peaks were in accordance with the propranolol-L-Leu derivatives m/e = 86 [3]. Molecular ions with low intensity were also present in the spectra at m/e 362 and 380 for the alprenolol and the metoprolol derivatives, respectively. Peaks at m/e = 229 were also obtained for the two derivatives, which arise from cleavage of the ether linkage.

Chromatographic studies

The diastereomeric derivatives of alprenolol and metoprolol were separated using μBondapak C_{18} as support and acetonitrile in phosphate buffer pH 3.0 as the mobile phase. In a previous study the L-Leu derivatives of propranolol were separated using LiChrosorb RP-18 as support and a mobile phase consisting of phosphate buffer (pH 3.0)—acetonitrile, with addition of the tertiary amine N,N-dimethyloctylamine [3]. Addition of the tertiary amine to the mobile phase was a prerequisite for obtaining good chromatographic performance of the propranolol derivatives. It was observed, however, that by use of μBondapak C_{18} as support the tertiary amine could be excluded from the mobile phase without loss of column efficiency or peak symmetry, which also has been observed previously during chromatography of secondary and tertiary amines [10].

The capacity factor of the L-Leu derivatives of alprenolol and metoprolol can be regulated over a wide range by varying the content of acetonitrile in the mobile phase; this is demonstrated in Fig. 7. The selectivity increases with de-

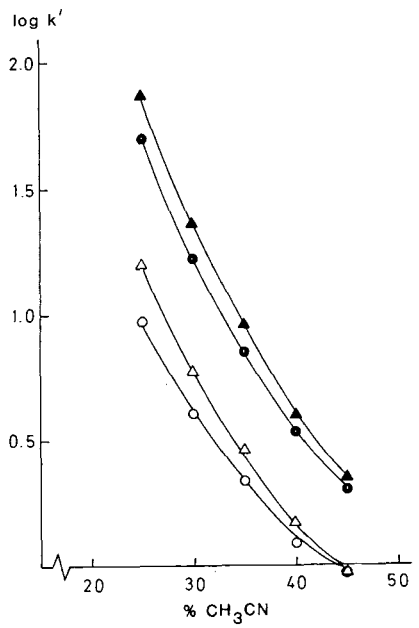


Fig. 7. Regulation of the capacity factor (k') by the acetonitrile concentration. Column and flow-rate: as in Fig. 6. Mobile phase: phosphate buffer (phosphate concentration 0.1 M) pH 3.0 with different concentrations of acetonitrile. Symbols as in Fig. 3.

creasing concentration of acetonitrile, and the separation factor, α, ranges between 1.0—1.67 and 1.10—1.49 for the metoprolol and the alprenolol derivatives, respectively. Baseline separation of the diastereomeric alprenolol and metoprolol derivatives was obtained using mobile phases containing 35 and 30% (v/v) acetonitrile in phosphate buffer (pH 3.0), respectively. Typical chromatographic data for the derivatives using these mobile phases are given in Table I. The separation efficiency shows a strong dependence of the capacity factor and a drastic increase of H (the height of a theoretical plate) was observed with capacity factors below 5. The alanine derivatives of the two drugs were also prepared and tested but the separation factors obtained were lower compared to the L-Leu derivatives. It was also observed that no resolution at all was obtained by chromatography of the Boc-L-Leu derivatives of alprenolol and metoprolol. This is in accordance with a previous observation during chromatography of the Boc-L-Leu derivatives of 3,4-dihydroxyphenylalanine [11]. The high selectivity obtained between the two diastereomeric pairs by the coupling of L-Leu to the two substances contributes to the high sensitivity obtained with the described method. Large separation factors allow the use of a short column, which gives low interstitial volumes and an increase in the maximum concentration in the eluted peak [12].

TABLE I

CHROMATOGRAPHIC DATA

Support: μBondapak C_{18} (10 μm) in a 100 × 3.2 mm column. Mobile phase: phosphate buffer (pH 3.0) containing 30% (for metoprolol derivatives) or 35% (v/v) acetonitrile (for alprenolol derivatives); flow-rate, 0.5 ml/min.

Compound	k'	α	H (μm)	Asymmetry factor
(*S*)-Alprenolol-L-Leu	7.16	1.27	66	1.07
(*R*)-Alprenolol-L-Leu	9.11		78	0.84
(*S*)-Metoprolol-L-Leu	4.03	1.45	87	1.11
(*R*)-Metoprolol-L-Leu	5.86		120	0.96

Fluorimetric detection

Fluorimetric detection was used for quantitation of the diastereomeric derivatives of alprenolol and metoprolol in human plasma. This detection system gave excellent sensitivity and made possible the selective detection of the derivatives of the drugs in plasma. Fig. 8 demonstrates the relative fluorimetric response obtained with different excitation wavelengths. The alprenolol derivatives gave maximal response with an excitation wavelength of 198 nm, while 193 nm was the maximum for the metoprolol derivatives. No interference from endogenous compounds in human plasma was observed in the chromatograms using the excitation wavelengths above.

Determination of (R)- and (S)-alprenolol and metoprolol in human plasma

The plasma concentrations of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol were determined using the method described under Experimental.

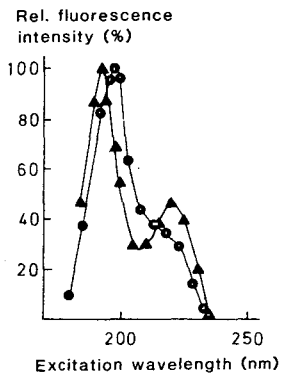


Fig. 8. Relative fluorimetric response at different excitation wavelengths. (•), Alprenolol-L-Leu; (▲), metoprolol-L-Leu.

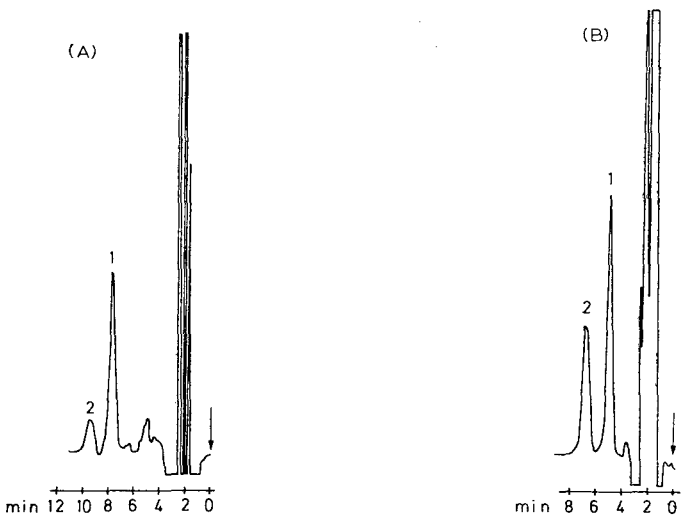


Fig. 9. Separation of (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol extracted from human plasma. (A) 1 = (*S*)-Alprenolol-L-Leu (6.6 ng/ml), 2 = (*R*)-alprenolol-L-Leu (1.6 ng/ml). Conditions as in Fig. 6. (B) 1 = (*S*)-Metoprolol-L-Leu (22.5 ng/ml), 2 = (*R*)-metoprolol-L-Leu (14.2 ng/ml). Mobile phase: 30% acetonitrile in phosphate buffer pH 3.0. Column and flow-rate as in Fig. 6.

Fig. 9A and B demonstrate the separation of the (*R*)- and (*S*)-isomers of the two drugs as their diastereomeric derivatives. The plasma samples were obtained from healthy male volunteers 1.12 and 1.25 h after oral administration of a 100-mg Aptin tablet and a 50-mg Seloken tablet, respectively. Standard curves used for quantitation of the isomers in plasma were constructed by plotting the peak height against the sample concentrations. The standard curves were linear in the studied concentration ranges 0.5—48.5 and 0.5—52 ng/ml for alprenolol and metoprolol, respectively; the curves are shown in Fig. 10. Correlation coefficients were in all cases better than 0.9992.

Standard samples were extracted after addition of drug-free plasma to avoid differences in the recoveries of the drugs from original plasma and standard samples due to protein binding.

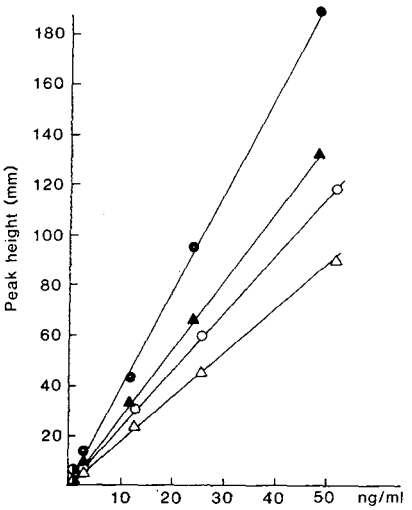


Fig. 10. Standard curves for alprenolol and metoprolol. (•), (*S*)-alprenolol-L-Leu; (▲), (*R*)-alprenolol-L-Leu: (○), (*S*)-metoprolol-L-Leu; (△), (*R*)-metoprolol-L-Leu.

The reproducibility of the method was studied at three different concentrations and the results are summarized in Table II.

The minimum detectable concentrations were 0.2 and 0.4 ng/ml for alprenolol and metoprolol, respectively. The signal-to-noise ratio was 3:1 for each compound at these concentrations.

The column used for this study has a high stability with the mobile phases used. No significant change in the separation efficiency has been observed during continuous use of the column for more than 5 months.

TABLE II

REPRODUCIBILITY OF (*R*)- AND (*S*)-ALPRENOLOL AND (*R*)- AND (*S*)-METOPROLOL DETERMINATIONS IN HUMAN PLASMA

The relative standard deviation (S.D.) calculated for $n = 6$.

Alprenolol		Metoprolol	
Sample conc. (ng/ml)	Relative S.D. (%)	Sample conc. (ng/ml)	Relative S.D. (%)
0.5	9.4	0.5	9.8
12.1	6.7	12.5	6.5
48.5	4.9	52.0	5.1

Pharmacokinetics

To test the applicability of the methods for studies in patients alprenolol and metoprolol were given to two subjects.

The plasma concentrations of (*R*)- and (*S*)-alprenolol in one subject (A) who received a single oral 100-mg dose of racemic alprenolol is shown in Fig. 11. The concentration of (*S*)-alprenolol was much higher than that of (*R*)-alprenolol This subject (subject 7 in ref. 13) had earlier received alprenolol and, as in this

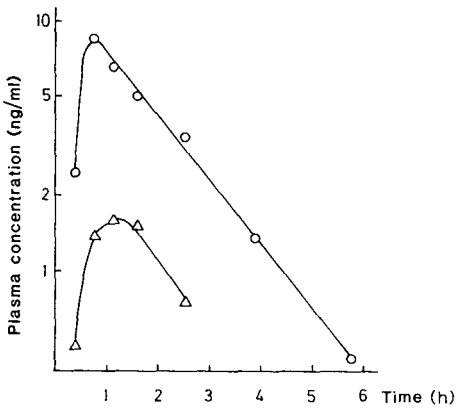


Fig. 11. Plasma concentrations of (*R*)-alprenolol (△) and (*S*)-alprenolol (○) in one subject (A) receiving a 100-mg commercial tablet.

study, had low plasma concentrations of the drug. The same subject has also been shown to achieve unusually high plasma concentrations of the active metabolite 4-hydroxyalprenolol. It is likely that most of accumulated 4-hydroxyalprenolol is the (*R*)-form, which should be inactive. Thus studies estimating relative activities of alprenolol and its active metabolites [14] should reach higher precision if one also considers the separate kinetics of the (*R*)- and the (*S*)-isomers. The above subject also had lower availability of (*R*)- than of (*S*)-propranolol after a single oral dose (40 mg) of racemic propranolol (subject B, Fig. 3 in ref. 2).

In the present study this subject also received a single oral dose (50-mg tablet) of racemic metoprolol. As shown in Fig. 12, subject A also had a lower plasma concentration of (*R*)-metoprolol and thus has lower plasma concentrations of the (*R*)-isomers of three different beta-receptor blocking drugs, probably indicating that his liver oxidizes the (*R*)-isomers of these drugs faster than the (*S*)-isomers. Fig. 12 also shows that another subject (B) had similar con-

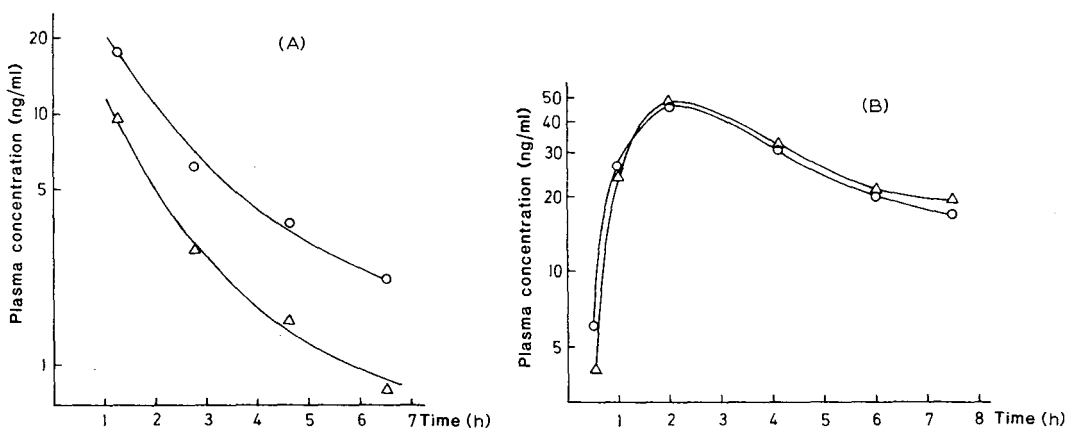


Fig. 12. Plasma concentrations of (*R*)-metoprolol (△) and (*S*)-metoprolol (○) in two subjects (A and B) receiving 50-mg commercial tablets.

centrations of (*R*)- and (*S*)-metoprolol after receiving racemic metoprolol. There seems to be an individual variation in the concentration ratios of the (*R*)- and the (*S*)-isomers. We have recently observed this for propranolol [15].

In conclusion, our HPLC method has enabled simultaneous determination of the (*R*)- and (*S*)-isomers of alprenolol and of metoprolol after giving clinial doses of commercially available tablets. The results show that the isomers can have different kinetics. Therefore it is worthwhile to investigate if this is important clinically.

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DETERMINATION OF THE ANTIDEPRESSANT AGENT CITALOPRAM AND METABOLITES IN PLASMA BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of citalopram [1-(3-(dimethylaminopropyl)-1-(4-fluorophenyl)-5-phthalancarbonitrile] and its two main metabolites (the methylamino and amino derivatives). The compounds were extracted from alkaline plasma with diethyl ether. The combined ether layers were evaporated after addition of 50 μl of 0.1 *N* HCl. The residual extracts were purified with diethyl ether and 20 μl were injected into a Spherisorb ODS 5-μm column with acetonitrile—0.6% phosphate buffer pH 3 (55:45, v/v) as the mobile phase. Using a fluorescence detector the detection limits are 1 ng/ml of plasma for citalopram and the methylamino metabolite and 0.5 ng/ml for the amino metabolite.

INTRODUCTION

Citalopram (I, Fig. 1), a new bicyclic antidepressant agent, is a potent and selective serotonin re-uptake inhibitor [1, 2]. It is demethylated in vivo to its methylamino (II) and amino (III) metabolites (Fig. 1). In urine, another metabolite, the N-oxide of citalopram (V) (Fig. 1) has been observed [3, 4].

A drug/methylamino metabolite ratio of 1:2 in steady-state plasma samples was found. The pharmacokinetics of citalopram in man have been reported using an analytical procedure that includes extraction from alkaline plasma with hexane and separation of the drug from its metabolites through thin-layer

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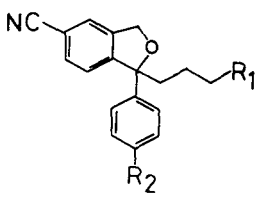

Fig. 1. Formulae of the compounds investigated.

	R_1	R_2
I: LU 10-171 (citalopram)	$-N(CH_3)_2$	F
II: LU 11-109 (methylamino metabolite)	$-NHCH_3$	F
III: LU 11-161 (amino metabolite)	$-NH_2$	F
IV: LU 10-202 (internal standard)	$-N(CH_3)_2$	Cl
V: LU 11-305 (N-oxide)	$-\overset{\uparrow}{\underset{O}{N}}(CH_3)_2$	F

chromatography of the evaporated hexane extracts. The drug and the methylamino metabolite were then determined fluorimetrically after ion-pair formation with 9,10-dimethoxyanthracene-2-sulphonate. The detection limits were 20 ng of I and 50 ng of II in 2-ml plasma samples [3].

This paper describes a simple, selective and sensitive high-performance liquid chromatographic (HPLC) method for the determination of I and II, and in addition the amino metabolite (III), from steady-state plasma samples using a reversed-phase column and fluorescence detection.

EXPERIMENTAL

Standards and reagents

Citalopram (Lu 10-171 (I) hydrobromide, Lu 11-109 (II) oxalate, Lu 11-161 (III) oxalate, Lu 11-305 (V) hydrochloride and the internal standard Lu 10-202 (IV) hydrobromide were supplied from Lundbeck & Co. (Copenhagen, Denmark).

Methanolic stock solutions (0.5%, w/v) of the hydrobromides (I and IV) and the oxalates (II and III) were stable at room temperature for at least two months. Working standard solutions A and B were prepared in water. Solution A: I (1 μg/ml), II (1 μg/ml), III (0.2 μg/ml). Solution B: IV (1 μg/ml). The solutions were stable for at least 14 days when stored at 4°C. All reagents were analytical-reagent grade and aqueous solutions were prepared using glass-distilled water. Acetonitrile (HPLC grade) and diethyl ether were supplied by Rathburns Chemicals (Walkerburn, Great Britain); 1 *N* NaOH was ether-washed; 0.6% (w/v) KH_2PO_4 was adjusted to pH 3 with H_3PO_4.

Glassware

Conical glass-stoppered centrifuge tubes (10 ml) were silanized with 5% dimethyldichlorosilane in toluene for 24 h, then washed with methanol and acetone. Evaporating tubes were rinsed with methanol in an ultrasonic bath and dried just before use.

Chromatography

HPLC analyses were performed on a Perkin-Elmer Series 2/2 liquid chromatograph with a Perkin-Elmer 3000 fluorescence spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.) operating at an excitation wavelength of 240 nm, slit 15 nm, and an emission wavelength of 296 nm, slit 20 nm. Expansion was in the range ×5 to ×100. Using UV detection at 240 nm a Perkin-Elmer spectrophotometer Model LC 55 was used. The column was Spherisorb ODS 5 μm (25 cm × 3 mm I.D.) with a MPLC™ guard column of RP-18 (3 cm × 4.6 mm I.D.). The chromatographic system was operated at room temperature.

The mobile phase used was 55% (v/v) acetonitrile in potassium dihydrogen phosphate (0.6%, w/v) adjusted to pH 3 with phosphoric acid, at a constant flow-rate of 1.3 ml/min.

Extraction procedure

To 1 ml of plasma (either patient plasma or spiked blind plasma) were added 75 μl of working standard solution B followed by 50 μl of 1 *N* NaOH to bring the solution to a pH of about 10. The mixture was extracted twice with 3-ml portions of diethyl ether by mechanical shaking for 15 min. After centrifuging for 10 min at 3200 *g*, the combined ether layers were transferred to 10-ml evaporation tubes containing 50 μl of 0.1 *N* HCl. The diethyl ether was evaporated under a stream of nitrogen in a 40°C water-bath.

The residual extracts were purified by whirlmixing with 0.5 ml of diethyl ether for 10 sec and centrifuged at 625 *g*. The ether layers were then removed and discarded; 15—20 μl of the residual extracts were injected onto the column.

Calibration curves

Preliminary steady-state plasma level determinations of I, II and III from patients given citalopram showed that the concentration ratio of I/III was about 7:1 and that of I/II about 1:1. The concentration ranges to be covered appeared to be from the detection limits upwards to about 200 ng/ml for I and II and to about 50 ng/ml for III. Spiked plasma samples were therefore prepared by adding 25, 50, 75, 100, 150 and 200 μl of working standard solution A to 1 ml of blank plasma; these were worked-up as described under extraction procedure. The calibration curves were calculated using regression analysis.

Recovery studies

A 1-ml volume of plasma spiked with 50 μl of working standard solution A was extracted as described above. The internal standard was added after extraction by substituting the 0.1 *N* HCl solution in the evaporating tubes with 50 μl of a 1 μg/ml solution of IV in 0.1 *N* HCl. Peak height ratios of these extracts were compared to peak height ratios of a standard aqueous solution of I, II and IV at 1 μg/ml and III at 0.2 μg/ml injected directly.

The procedure was repeated using a single extraction with 5 ml of diethyl ether.

Preliminary studies

A chromatogram of a mixture of citalopram, the methylamino and amino

metabolites, the N-oxide of citalopram, the internal standard, caffeine, nitrazepam, levomepromazine and alimemazine is shown in Fig. 2a. A UV detector operating at 240 nm was used. The order of elution is given in Table I. The hypnotics nitrazepam, levomepromazine and alimemazine, together with caffeine, represent possible interfering substances from patient plasma. Caffeine and nitrazepam, however, both eluted ahead of citalopram and its metabolites, while levomepromazine and alimemazine, not separating on the column, were eluted with a longer retention time.

The sample which gave the chromatogram in Fig. 2a was further detected with the fluorescence detector coupled in series with the UV detector, resulting in the chromatogram shown in Fig. 2b. The only peaks which appeared were due to citalopram, the internal standard, and metabolites II, III and V.

The N-oxide (V) could not be detected either in extracts of plasma spiked with 200 ng/ml (Fig. 2c), or in extracts of plasma samples from steady-state patients (Fig. 3), and it is hardly extracted from alkaline solution with ether.

A chromatogram of an extract from 2 ml of blank plasma revealed very few absorbing endogenous plasma constituents using the UV detector (Fig. 4a). Thus, due to the absence of interfering peaks, the method might well be used for analysis with this detector. However, on comparing the peak heights in Fig. 2a and b, when the UV detector was operating at maximum sensitivity (0.02 a.u.f.s.) while the fluorescence signal could still be expanded ten times, it could

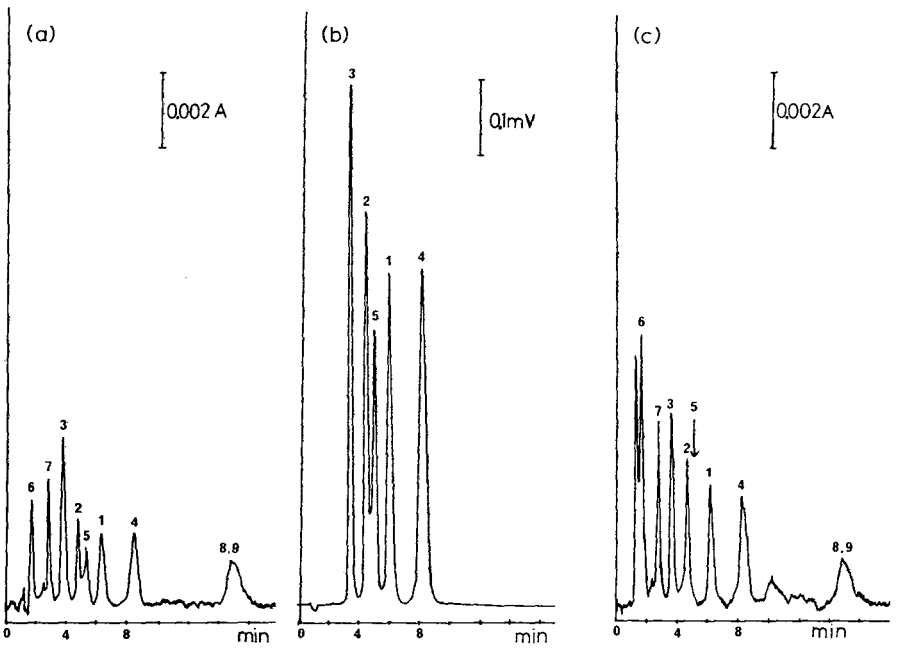

Fig. 2. Chromatograms of about 40 ng of caffeine (6), nitrazepam (7), the amino metabolite of citalopram (3), the methylamino metabolite (2), the N-oxide (5), citalopram (1), internal standard (4), alimemazine (8) and levomepromazine (9) injected on the column and detected with (a) the UV detector and (b) the fluorescence detector, (c) chromatogram of an extract of 1 ml of plasma spiked with 200 ng of the same compounds as in (a) and (b) and detected with the UV detector.

TABLE I

ELUTION OF CITALOPRAM, METABOLITES AND SOME POSSIBLE INTERFERING SUBSTANCES

Conditions: Spherisorb ODS 5 μm, acetonitrile—0.6% phosphate buffer pH 3 (55:45, v/v), 1.3 ml/min

Drug	Chromatographic peak number*	Retention time (min)	Retention time relative to I.S.
Caffeine	6	1.6	0.19
Nitrazepam	7	2.8	0.33
Amino metabolite	3	3.7	0.44
Methylamino metabolite	2	4.6	0.55
N-Oxide	5	5.2	0.62
Citalopram	1	6.3	0.75
Internal standard (I.S.)	4	8.4	1.00
Levomepromazine	9	15.0	1.79
Alimemazine	8	15.0	1.79

*Figs. 2—4.

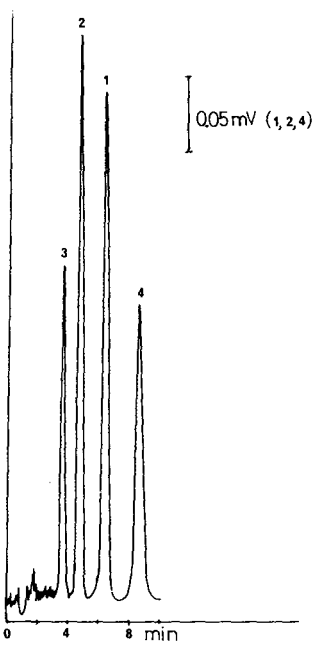

Fig. 3. Chromatogram of an extract of a steady-state patient plasma containing 340 nmol/l citalopram (1), 293 nmol/l methylamino metabolite (2) and 26 nmol/l amino metabolite (3). The amino metabolite was detected with a sensitivity range five times the sensitivity range used for the detection of 1, 2 and 4 (internal standard). The patient had been given a dose of 70 mg citalopram daily.

be concluded that the fluorescence detector gave a much better response with a detection limit of 0.2—0.5 ng while the detection limit of the UV detector could be estimated to be about 10—15 ng. The method was therefore developed using the fluorescence detector.

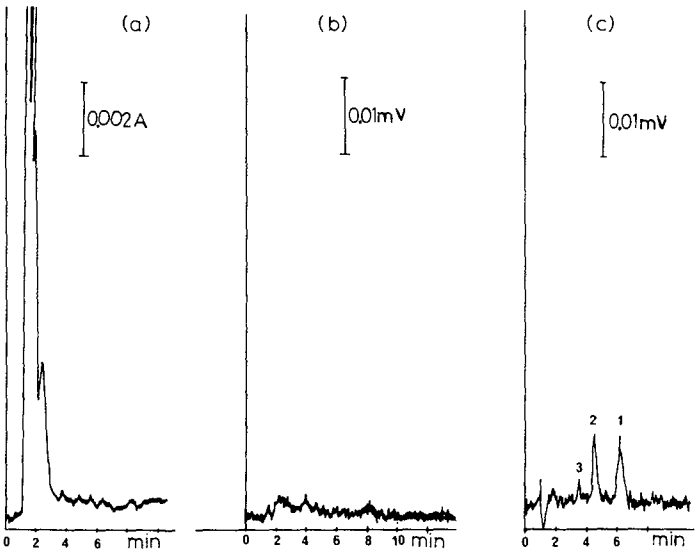

Fig. 4. Chromatograms of extracts of blank plasma: (a) 2 ml of plasma detected with the UV detector; and (b) 1 ml of plasma detected with the fluorescence detector. (c) Chromatogram of an extract of plasma containing 2.5 ng/ml citalopram (1) and methylamino metabolite (2), and 0.5 ng/ml amino metabolite (3), detected with the fluorescence detector.

RESULTS AND DISCUSSION

From the chromatogram of a plasma blank extract (Fig. 4b) it can be concluded that very few fluorescent endogenous plasma constituents are extracted. A chromatogram of an extract of plasma containing I and II at 2.5 ng/ml and III at 0.5 ng/ml is shown in Fig. 4c. The detection limits were 1—2 ng/ml for citalopram and the methylamino metabolite and 0.5—1 ng/ml for the primary amino metabolite.

The equations of the calibration curves were: citalopram, $x = 1.85y + 1.25$, $r^2 = 1.00$; methylamino metabolite, $x = 1.60y + 1.38$, $r^2 = 1.00$; amino metabolite, $x = 1.14y + 1.56$, $r^2 = 0.99$; x = nmol/l, y = percentage peak height relative to internal standard, number of observations = 49.

Relative standard deviations ($n = 9$ for each point) were $< 4\%$ for I, $< 3\%$ for II and $< 5\%$ for III. Eight aliquots of a plasma pool from several patients were analysed. The results showed a somewhat lower standard deviation than in the calibration graphs where the day-to-day variation is incorporated.

The mean recovery from plasma was better than 83% for the two metabolites and better than 79% for citalopram. A single extraction step with 5 ml of diethyl ether gave a lower recovery (Table II).

A series of plasma samples from patients who had received citalopram for several weeks was analysed in duplicate together with the standards. It was possible to analyse 8—10 patient samples during the day.

The ratio of I/II was found to be between 0.4 and 2.5 with a mean of 1.6, and confirmed earlier results [4]. The ratio I/III was between 2.0 and 10.7 with a mean of 7.3. The results revealed that the mean steady-state concentration of III was below the detection limit of the UV detector. Thus, in studying metab-

TABLE II

ANALYSIS OF PLASMA

Compound	Within-run analyses of 8 aliquots of a plasma pool		Recovery [mean (%) ± S.D., n = 10]	
	Mean (nmol/l) ± S.D.	Relative S.D. (%)	Extraction A*	Extraction B**
Citalopram	185 ± 4	2.1	84 ± 5	62 ± 5
Methylamino metabolite	122 ± 3	2.3	88 ± 5	79 ± 5
Amino metabolite	21 ± 0.5	2.6	87 ± 5	78 ± 7

*Extraction A: 2 × 3 ml diethyl ether.
**Extraction B: 1 × 5 ml diethyl ether.

olites of citalopram, fluorescence detection gives a very sensitive and selective method. The method may also be used in studying the pharmacokinetics of the drug.

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DETERMINATION OF AMDINOCILLIN IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of amdinocillin (formerly mecillinam) in human plasma and urine. The assay is performed by direct injection of a plasma protein-free supernatant or a dilution of urine. A 10-μm μBondapak phenyl column with an eluting solvent of water—methanol—1 *M* phosphate buffer, pH 7 (70:30:0.5) was used, with UV detection of the effluent at 220 nm. Azidocillin potassium salt [potassium-6-(D-(−)-α-azidophenyacetamido)-penicillanate] was used as the internal standard and quantitation was based on peak height ratio of amdinocillin to that of the internal standard. The assay has a recovery of 74.4 ± 6.3% (S.D.) in the concentration ranges of 0.1—20 μg per 0.2 ml of plasma with a limit of detection equivalent to 0.5 μg/ml plasma. The urine assay was validated over a concentration range of 0.025—5 mg/ml of urine, and has a limit of detection of 0.025 mg/ml (25 μg/ml) using a 0.1-ml urine specimen per assay.

The assay was applied to the determination of plasma and urine concentrations of amdinocillin following intravenous administration of a 10 mg/kg dose of amdinocillin to two human subjects. The HPLC and microbiological assays were shown to correlate well for these samples.

INTRODUCTION

Amdinocillin [I], 6-β-{[(hexahydro-1H-azepin-1-yl)-methylene]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (Fig. 1) developed by Leo Pharmaceuticals Products, Hälsingborg, Denmark is one of a new class of semisynthetic β-amidino penicillins [1]. The structure of the compound differs from the classical penicillins by possessing an amidino group at the C-6 position of the penicillanic acid moiety instead of an acylated amino group. It has high activity against gram negative organisms, such as *Escherichia coli* [2].

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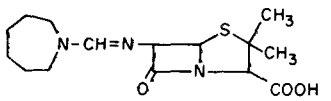
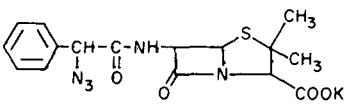
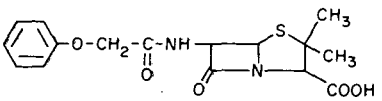
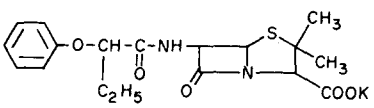
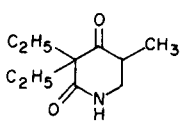
Compound	Name	Structure
I	Amdinocillin, 6-β-{[(hexahydro-1H-azepin-1-yl)-methylene]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0]heptane-2-carboxylic acid	
II	Azidocillin potassium salt, potassium-6[D-(−)-α-azidophenyl-acetamido]-penicillanate	
III	Penicillin V, 6-phenoxyacetamido-penicillanic acid	
IV	Propicillin, α-phenoxypropyl-penicillin	
V	Methyprylon, 2,4-dioxo-3,3-diethyl-5-methylpiperidine	

Fig. 1. Chemical names and structures of compound I to V.

Routine determination of [I] for pharmacokinetic studies [3] in plasma and urine are often performed by microbiological assay [4]. A thin-layer cylinder-plate method for differentiation of amdinocillin pivoxil and amdinocillin has also been reported [5].

Spectrophotometric methods based on the formation of strongly UV absorbing (325—345 nm) imidazol-5-(4H)-one derivatives of amdinocillin by reaction of [I] with imidazole [6] or glycine [7] and mercuric chloride have been reported for use in aqueous solutions [6] and for studies involving the degradation of [I] [7]. The glycine reaction which required the β-lactam ring and the amidino side chain is highly specific for [I] in the presence of degradation products and other penicillins.

High-performance liquid chromatographic (HPLC) assays to measure [I] as the intact compound for stability determination [8] and as the imidazol-5-(4H)-one derivative formed with imidazole and mercuric chloride in biological fluids have been reported [9]. The HPLC assay in biological fluids utilizes post-column derivatization with air segmentation and is specific for penicillins with

an intact β-lactam ring, however, penicilloic acids of penicillins (i.e., ampicillin and amoxycillin) with free amino groups also react to some extent [9]. HPLC procedures have also been reported for the analysis of intact [I] in urine [10, 11] and plasma [11], and the pharmacokinetics of [I] in human study [12].

The present HPLC assay for plasma and urine utilizes reversed-phase chromatography on a μphenyl column with UV detection at 220 nm and is similar to that previously described [11]. Sample preparation for plasma has been simplified by elimination of the evaporation step. In addition, clean extracts are obtained by the inclusion of a diethyl ether wash of the acetonitrile protein-free filtrate prior to HPLC analysis. The urine assay also performed on the μphenyl column utilizes direct analysis of a diluted aliquot of the sample similar to that previously described [10,11]. The internal standard used in the plasma and urine assays is azidocillin potassium salt [II] (Fig. 1). The assay was applied to the determination of plasma concentrations and the urine excretion of [I] in two human subjects following intravenous administration of 10 mg of [I] per kg. The HPLC data obtained in these studies correlated fairly well with those of the bioassay [13], demonstrating that the two procedures are equivalent.

EXPERIMENTAL

Column

The column used was a prepacked 30 cm × 4 mm I.D. stainless-steel column containing 10-μm μBondapak phenyl packing (Waters Assoc., Milford, MA, U.S.A.).

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector and a guard column with Bondapak phenyl/corasil as packing material (Waters Assoc.). An LDC Model Spectromonitor III (Laboratory Data Control, Riviera Beach, FL, U.S.A.) variable-wavelength UV detector was used for quantitation at 220 nm. A back-pressure coil (approximately 3 m × 1.5 mm O.D. × 0.3 mm I.D. Altex PTFE tubing) was connected to the sample outlet of the flow-cell. The mobile phase used for isocratic reversed-phase chromatography in both the plasma and urine assays was a mixture of water—methanol—1 *M* phosphate buffer, pH 7 (70:30:0.5). Changes in retention time or resolution of [I] and [II] caused by the endogenous substances contained in the sample extracts may require small changes (approximately 5%) in the ratio of methanol and water in the mobile phase. The chromatographic system was operated at ambient temperature, with a flow-rate of 2.0 ml/min at a head pressure of $2.3 \cdot 10^3$ p.s.i. (15.9 MPa). Under the above conditions, the retention times of compounds [I] and [II] were approximately 4.7 ($k' = 2.0$) and 6.7 min ($k' = 3.3$), respectively. The injection of 0.05 μg of [I] and 0.25 μg of [II] per 10 μl yielded peaks of nearly 50% full scale response, respectively, at a detector sensitivity of $1 \cdot 10^{-2}$ a.u.f.s. The chart speed on the 10-mV Hewlett-Packard, Model 7132A strip chart recorder was 0.635 cm/min.

Standard solutions

Plasma. Weigh out 10.00 mg of compound [I] ($C_{15}H_{23}N_3O_3S$, mol. wt. = 325.43, m.p. = 146°C with decomposition) into a 10-ml volumetric flask and dissolve in 10 ml water. This stock solution containing 1.0 mg [I] per ml (stock solution A) should be prepared fresh daily. Weigh out 10.00 mg of compound [II] internal standard (azidocillin potassium salt; potassium-6-[D-(−)-α-azido-phenyl-acetamido]-penicillanate, $C_{16}H_{16}N_5O_4SK$, mol. wt. = 413.5, m.p. = 194°C with decomposition; supplied by Beecham Pharmaceuticals, Chemotherapy Research Center, Betchworth, Great Britain), into a 10-ml volumetric flask and dissolve in 10 ml of water. This stock solution contains 1.00 mg [II] per ml (stock solution D). These stock solutions are used to prepare 10-ml working intermediate solutions B and C containing 100 μg/ml and 10 μg/ml of compound [I], and E containing 100 μg/ml of compound [II], respectively, in water. Aliquots of 10, 20 μl of C, 5, 10, 20 μl of B, and 5, 10, 20 μl of A, respectively, and 25 μl of E (equivalent to 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 or 20.0 μg of [I] and 2.5 μg of [II]) are added to 0.2 ml of control plasma to establish a standard calibration curve for the direct quantitation of the concentration of [I] in the unknowns and for the determination of percent recovery. Aliquots (25 μl) of solution E are added to each unknown plasma as the internal standard [II] in the analysis.

An external standard is prepared by transferring aliquots of 0.5 ml of working standard B and 2.0 ml of internal standard E into a separate 10-ml volumetric flask and diluting to volume with water to yield a solution containing 5 μg [I] plus 20 μg [II] per ml. A 10-μl aliquot of this solution (equivalent to 0.05 μg of [I] and 0.2 μg of [II]) is injected for calculation of percent recovery in plasma and to verify the performance of the HPLC system.

Urine. Weigh out 50.00 mg of compound [I] into a 10-ml volumetric flask and dissolve in 10 ml of control human urine. This stock solution containing 5.00 mg [I] per ml of urine (solution A′) should be prepared fresh daily. Serial dilutions of stock solution A′ are made in control human urine to yield solutions B′, C′, D′, and E′ containing 1 mg, 0.2 mg, 0.05 mg, and 0.025 mg [I] per ml of urine, respectively. Weigh out 10.00 mg of compound [II] (internal standard) into a 10-ml volumetric flask and dissolve in 10 ml of water to yield a stock solution containing 1.0 mg [II] per ml (solution F′). The above standards are used to establish a calibration curve for the quantitation of the unknowns in urine.

Reagents

All reagents are of analytical grade purity and are prepared in deionized distilled water. Phosphate buffer (1.0 *M*, pH 7.0) is prepared by mixing 390 ml 1 *M* KH_2PO_4 (136.1 g/l) and 610 ml $K_2HPO_4 \cdot 3H_2O$ (228.2 g/l). Mix well and adjust to pH 7 with 1 *M* phosphoric acid as needed. Other reagents used include diethyl ether, anhydrous (Mallinckrodt, St. Louis, MO, U.S.A.), acetonitrile and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). The mobile phase is filtered through a Millipore filter, type HA, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.) and deaerated prior to use.

Sample handling

Due to the reported instability of [I] in frozen plasma at −25°C [11] it is imperative that the blood specimens be spun down immediately after collection to separate the plasma. The plasma must be stored at 0°C (in an ice bath) if the analysis is to be performed that day (i.e., within 12 h after collection), otherwise the plasma samples must be stored at −70°C (Revco Ultra low Temperature Freezer, Model 800, Rheem Refrigeration Products) to minimize degradation [9]. The urine samples are stored under identical conditions, also due to the reported instability of [I] in the urine at −17 to −20°C [9,10]. The plasma and urine samples stored at −70°C were thawed gradually by keeping the samples in an ice bath with repeated mixing on a vortex-mixer prior to assay. If the volume of the urine sample was greater than 20 ml, the sample was immersed in water (20—25°C) until thawed [10].

Assay procedure

Plasma. Into a 15-ml centrifuge tube, add 0.2 ml of the unknown plasma, 25 μl of internal standard solution E (2.5 μg of [III]) and 0.4 ml of acetonitrile to deproteinate the plasma. Mix for 10—20 sec on a vortex-mixer (Vortex-Genie; ACE Scientific Supply, Linden, NJ, U.S.A.) and then centrifuge the sample in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC Corporation, Needham, MA, U.S.A.) at 0—5°C for 5 min at approximately 2400 rpm (1400 *g*). Transfer the supernatant with a disposable pasteur pipet into a clean tapered 15-ml stoppered centrifuge tube (PTFE No. 13 stopper), and wash the supernatant with 5 ml of diethyl ether (fresh or less than one week old) by shaking for 5 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.). Centrifuge the sample for 5 min and aspirate off the diethyl ether. Immerse the tube containing the aqueous phase in an ice bath prior to HPLC analysis of a 20-μl aliquot (Fig. 2). Along with the samples, process a 0.2-ml specimen of control plasma and eight 0.2-ml specimens of control plasma to which 10, 20 μl of solution C, 5, 10, or 20 μl of solution B, and 5, 10, 20 μl of solution A, respectively, and 25 μl of solution E to each specimen (equivalent to 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20 μg of compound [I] and 2.5 μg of compound [II] each per 0.2 ml plasma, respectively), are added. These standards are used to establish a calibration curve for the direct quantitation of [I] in the unknowns, and for the determination of percent recovery.

Urine. Into a 15-ml centrifuge tube, add 0.1 ml of unknown urine and 0.2 ml of internal standard F′ (200 μg of [II]). Dilute to 1.0 ml with water, mix well and immerse the tube in an ice bath prior to the HPLC analysis of a 10-μl aliquot. Along with the samples, process 0.1-ml specimens of control urine to which are added 0.1-ml aliquots of each of the standards A′, B′, C′, D′, and E′, and 0.2 ml of solution F′, respectively (equivalent to 500, 100, 20, 5, and 2.5 μg of [I] and 200 μg [II]). Dilute these calibration samples to 1.0 ml with water, mix well and immerse the tubes in an ice bath. A 10-μl aliquot of each of these samples is injected to establish a calibration curve for the direct quantitation of the concentration of [I] in the unknowns (Fig. 3).

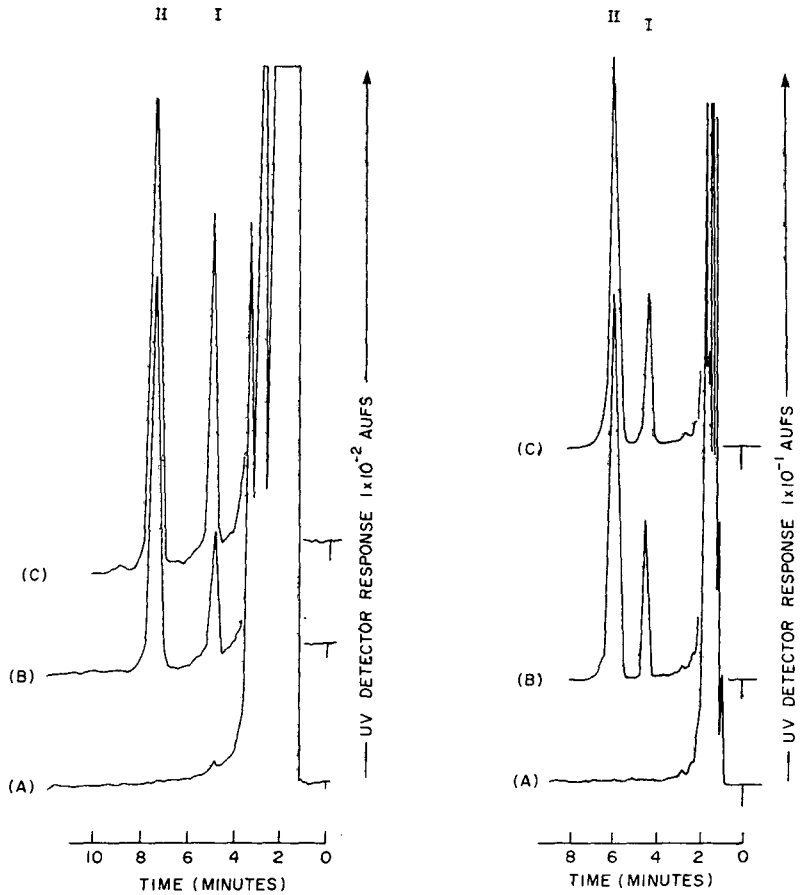


Fig. 2. Chromatograms of HPLC analysis of plasma supernatants of (A) control plasma, (B) control plasma containing 0.5 μg [I] and 2.5 μg [II] per 0.2 ml added authentic standards, and (C) patient plasma 10 min post 10 mg/kg intravenous dose of [I]. Column, Waters, 10 μm μBondapak phenyl; mobile phase, water—methanol—1 *M* phosphate buffer, pH 7 (68:32:0.5) at a flow-rate of 2 ml/min.

Fig. 3. Chromatograms of HPLC analysis of (A) control human urine, (B) control urine containing 0.2 mg [I] per ml and 0.2 mg [II] per ml added authentic standards, and (C) patient urine 4—6 h post 10 mg/kg intravenous dose of [I]. Column, Waters, 10 μm μBondapak phenyl; mobile phase, water—methanol—1 *M* phosphate buffer, pH 7 (70:30:0.5) at a flow-rate of 2 ml/min.

Calculations

The concentration of [I] in μg/ml of plasma or mg/ml of urine in each respective unknown is determined by interpolation from calibration curves of internal standards processed along with the unknowns using the peak height ratio (peak height of [I] to peak height of [II] vs. concentration of [I]). The percent recovery of [I] in plasma is calculated from the ratio of peak height of [I] for the external standard and processed standard (assuming the final volume of aqueous phase after diethyl ether wash to be equal to 150 μl).

RESULTS

Statistical validation of the method in plasma and urine

Plasma. The intra-assay linearity and precision of the method was evaluated in plasma over a concentration range of 0.5—100 μg [I] per ml. Triplicate samples at each concentration in the range of 0.1—20.0 μg of compound [I] were added to 0.2 ml of plasma, and taken through the analytical procedure. The data shown in Table I are best described by a power series equation of the form $y = A\ X^B$, ($y = 0.246\ X^{0.977}$) with a correlation coefficient (r) of 0.9996 indicating the high degree of linearity of the method. The method demonstrated excellent precision over the concentration range investigated with mean coefficient of variation of 1.5%. The percent recovery of compound [I] from the plasma was 74.4 ± 6.3% (S.D.) and the sensitivity limit was 0.5 μg [I] per ml.

Inter-assay assay linearity and precision data for the method were accumulated from the clinical studies. The data showed a mean coefficient of variation of 3.5% (Table II).

Urine. The intra-assay linearity and precision of the method were evaluated

TABLE I

LINEARITY AND INTRA-ASSAY PRECISION OF THE HPLC ASSAY OF COMPOUND [I] IN PLASMA

Compound [I] concentration added (μg/ml)	Mean concentration found ± S.D. ($n = 3$)	Coefficient of variation (%)	Recovery* (%)
0.50	0.53 ± 0.00	0.1	81.3
1.00	0.98 ± 0.02	2.2	79.3
2.50	2.55 ± 0.08	3.3	85.8
5.00	4.94 ± 0.13	2.7	72.1
10.00	9.88 ± 0.05	0.5	67.2
25.00	24.63 ± 0.41	1.7	70.2
50.00	50.50 ± 0.19	0.4	69.4
100.00	105.51 ± 0.94	0.9	70.2
Mean ± S.D.		1.5	74.4 ± 6.3

*The recovery at each concentration represents the mean of three replicate analyses.

TABLE II

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND [I] IN PLASMA

Concentration added (μg/ml)	n	Mean concentration found ± S.D.	Coefficient of variation (%)
0.50	4	0.53 ± 0.01	2.3
1.00	4	0.97 ± 0.03	2.7
2.50	4	2.43 ± 0.13	5.3
5.00	4	5.08 ± 0.23	4.6
10.00	5	9.73 ± 0.38	3.9
25.00	4	24.17 ± 1.37	5.7
50.00	4	51.08 ± 1.43	2.8
100.00	4	103.45 ± 1.14	1.1
Mean			3.5

TABLE III

LINEARITY AND PRECISION OF THE HPLC ASSAY OF COMPOUND [I] IN URINE

	Compound [I] concentration added (mg/ml)	*n*	Mean concentration found ± S.D.	Coefficient of variation (%)
Intra-assay	0.025	3	0.024 ± 0.001	3.3
	0.050	3	0.052 ± 0.002	3.9
	0.200	3	0.209 ± 0.005	2.4
	1.000	3	1.005 ± 0.041	4.1
	5.000	3	5.020 ± 0.030	0.6
	Mean			2.8
Inter-assay	0.025	2	0.024 ± 0.001	3.1
	0.050	3	0.049 ± 0.001	2.7
	0.200	2	0.198 ± 0.006	3.2
	0.500	2	0.501 ± 0.006	0.1
	1.000	2	0.965 ± 0.006	0.1
	5.000	3	5.040 ± 0.050	1.1
	Mean			1.7

in urine, using direct injection of triplicate samples of diluted urine in the concentration range of 0.025—5.0 mg [I] per ml. The data are again best described by a power series equation of the form $y = A\ X^B$ ($y = 2.10\ X^{0.973}$), with a correlation coefficient (r) of 0.9997 and a mean coefficient of variation of 2.8% (Table III) with a sensitivity limit of 25 μg/ml.

Inter-assay linearity and precision data for the method were accumulated from the clinical studies. The data showed a mean coefficient of variation of 1.7% (Table III).

Stability evaluation of [I] in plasma

The maximum stability of [I] in aqueous solution has been reported to be at pH 4.5—5.5 [1]. The compound was reported to be unstable in frozen plasma at −25°C, although stability of [I] for a period of three weeks was apparently attained if the acetonitrile protein-free filtrates of plasma were stored at −25°C [11]. Other investigators reported only minimum degradation of [I] in plasma if the sample were stored at −70°C [9]. In this study, the stability of [I] in plasma was evaluated at three concentrations following storage at −70°C for a period of 50 days.

A stock pooled sample was prepared by adding three 100-μl aliquots of stock solution A (containing 1.0 mg [I] per ml) to three separate 9.9-ml aliquots of fresh control human plasma, mixing well, and combining (total 30.0 ml) to yield a pooled stock containing 10 μg [I] per ml. This pooled plasma stock sample was used to prepare two other pooled samples containing 7.5 and 5 μg [I] per ml, by transferring 7.5 and 5 ml of the stock pool A, and diluting to 10 ml with control (drug-free) human plasma. These three pooled plasma samples were then subdivided into 1-ml aliquots in 15-ml polypropylene tubes and stored at −70°C for a specific time interval prior to analysis.

TABLE IV

STABILITY OF [I] IN PLASMA STORED AT −70°C

All assays were performed on triplicate samples (n = 3).

Day	Amount found (μg) (Amount added = 5.00 μg)	Amount found (μg) (Amount added = 7.50 μg)	Amount found (μg) (Amount added = 10.00 μg)
0	5.08 ± 0.16	7.60 ± 0.16	10.36 ± 0.13
6	5.05 ± 0.34	8.07 ± 0.07	10.44 ± 0.29
21	4.86 ± 0.22	7.48 ± 0.30	9.83 ± 0.29
33	5.49 ± 0.36	8.14 ± 0.07	10.43 ± 0.25
50	5.72 ± 0.04	8.32 ± 0.22	10.59 ± 0.38

TABLE V

PLASMA CONCENTRATION (μg/ml) OF TWO HUMAN SUBJECTS FOLLOWING INTRAVENOUS ADMINISTRATION OF A 10 mg/kg DOSE OF [I] MEASURED BY HPLC AND MICROBIOLOGICAL ASSAY

Sample time	Subject I		Subject 2	
	HPLC	Microbiological assay [13]	HPLC	Microbiological assay [13]
Infusion + 5 min	42.2	42.9	4.2	3.3
Infusion + 10 min	55.6	60.4	6.5	5.9
0 h	38.1	37.3	79.2	86.0
5 min	N.S.*	N.S.	34.6	34.3
10 min	N.S.	N.S.	27.5	30.2
15 min	39.0	36.3	22.0	22.2
30 min	24.5	24.3	16.3	15.4
1 h	13.7	10.0	7.9	6.8
1.5 h	7.7	3.9	4.4	3.5
2 h	N.S.	3.2	2.3	1.9
3 h	1.4	1.4	N.S.	0.9
4 h	0.5	0.7	N.S.	0.6
6 h	N.S.	N.S.	0.4	0.4
HPLC vs. microbiological		r = 0.994 (n = 9)		r = 0.999 (n = 11)

*N.S. = no specimen.

Aliquots (0.2 ml) of each of the pooled samples were analyzed in triplicate by the described procedure on the days indicated in Table IV. The data (Table IV) for the stability of [I] in plasma (physiological pH of ca. 7.4) stored at −70°C showed no discernible degradation over a 50-day period. The data indicate that plasma specimens must be frozen at −70°C immediately on collection and remain at that temperature until analyzed. Any deviation from these storage conditions will result in significant degradation of [I] and consequently invalidate any data generated from these samples.

Application of the method to biological specimens

Plasma concentrations of [I] were measured by HPLC in two human subjects following intravenous doses of 10 mg [I] per kg body weight. The data (Table V) show an excellent correlation of the HPLC assay with the microbiological procedure [13] for the two sets of samples (r = 0.994 and 0.999) demonstrating that the two procedures are equivalent.

The urinary excretion profiles of [I] were also measured by HPLC in the same subjects. The percent of dose recovered as unchanged [I] in the 0—24 h excretion period accounted for 66.6% and was in good agreement with data previously reported using a microbiological assay [3]. A good correlation (r = 0.986) was obtained for the determination of the concentrations of [I] by the HPLC and microbiological assays.

DISCUSSION

The high polarity of highly ionized amphoteric zwitterionic compounds such as [I] precludes their direct extraction from biological fluids into an organic solvent, consequently analysis can only be performed following the formation of a protein-free filtrate of plasma or a dilution of urine. The analysis of these aqueous samples is only possible with reversed-phase HPLC. Preliminary experiments utilizing the acetonitrile protein-free filtrate procedure previously described [11], showed that soluble endogenous compounds derived from the plasma sample often interfere with the measurement of [I] or [II]. In addition, the stability of [I] under the conditions of evaporation was questionable.

The introduction of the diethyl ether wash of the protein-free filtrate effectively removes the interfering endogenous compounds and also extracts the acetonitrile to leave an aqueous phase of approximately 0.15 ml which is suitable for direct injection. This wash step also eliminated the problematic and time-consuming evaporation step. The concentrations of drug present in the urine is well in excess of 20 μg/ml and thus no interference from endogenous compounds is noted.

Azidocillin (potassium salt), [II], was selected as the internal standard for the analysis of [I] in plasma and urine due to its chemical stability and compatible chromatographic properties. Two other antibiotics, penicillin V [III], and propicillin [IV], and the hypnotic methyprylon [V] (Fig. 1) were also found to be suitable for use as internal standards for the assay of [I] with minor changes in the mobile phase composition (Table VI). Compound [III] which showed excellent chromatographic behaviour (Fig. 4) was not selected as the internal standard due to the high ionic strength (0.01 M) phosphate buffer required, which showed deleterious effects on column performance over long periods of use. During the initial stages of development, propicillin [IV] was used very successfully as the internal standard. However, the disadvantage using

TABLE VI

INTERNAL STANDARDS FOR THE ANALYSIS OF COMPOUND [I]

Compound	Composition of mobile phase, water—methanol—1 M phosphate buffer, pH 7	Flow-rate (ml/min)	k'	k' [I]
II, Azidocillin (potassium salt)	70:30:0.5	2.0	3.3	2.0
III, Penicillin V	65:35:1.0	1.5	2.4	1.6
IV, Propicillin	70:30:0.1	2.0	3, 3.4	1.8
V, Methyprylon	70:30:0.1	2.0	2.6	1.8

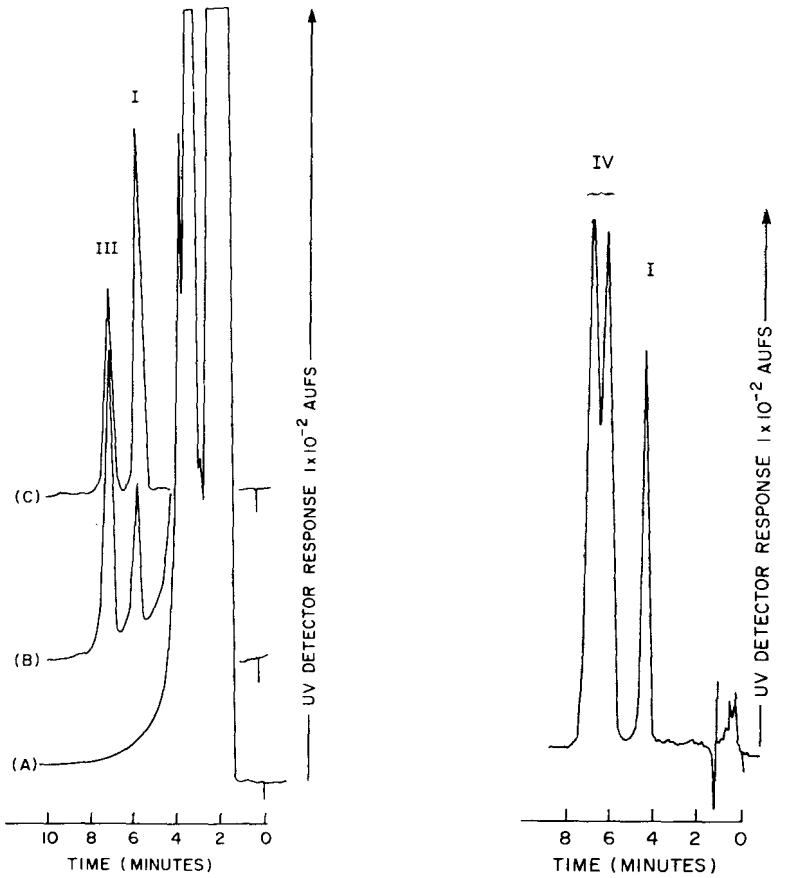


Fig. 4. Chromatograms of HPLC analysis of plasma supernatants of (A) control plasma, (B) control plasma containing 0.5 μg [I] and 25 μg penicillin V [III] per 0.2 ml added authentic standards, and (C) 100 ng [I] and 1000 ng [III] authentic standards. Column, Waters, 10 μm μBondapak phenyl; mobile phase, water—methanol—1 *M* phosphate buffer, pH 7 (65:35:1.0) at a flow-rate of 1.5 ml/min.

Fig. 5. Chromatogram of HPLC analysis of 100 ng authentic standard [I] and 100 ng propicillin [IV] on a μBondapak phenyl column and mobile phase of water—methanol—1 *M* phosphate buffer, pH 7 (70:30:0.1) at a flow-rate 2 ml/min.

this particular penicillin is the presence of two distinct peaks due to the diastereoisomers in the analytical standard (Table VI, Fig. 5), which complicated the measurement of peak height of this compound. The average peak height ratio of the two isomer peaks is 1.04 which is in good agreement with phase solubility data which indicated that the L-isomer was present to the extent of 57% [14]. An exact correlation with phase solubility is difficult due to lack of information pertaining to the ratio of UV absorptivities of the two isomers. Methyprylon [V] is an excellent internal standard (Table VI) for the analysis of urinary [I] and has been used in several preliminary investigations. However, since the compound is extracted into the diethyl ether wash of the protein-free filtrate of plasma, it cannot be used in the plasma assay.

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THIN-LAYER CHROMATOGRAPHIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF METABOLITES OF THE WEAK CARCINOGEN, 7-METHYLBENZ[*c*]ACRIDINE

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SUMMARY

The metabolism of [^{14}C]7-methylbenz[*c*]acridine, a weakly carcinogenic polycyclic aza aromatic hydrocarbon, was studied in rats in vivo, and in rat hepatocytes and hepatic microsomes. Evidence for oxidation of the methyl group and excretion of 7-hydroxymethylbenz[*c*]acridine and benz[*c*]acridine-7-carboxylic acid in rat bile was obtained by thin-layer chromatography. A reversed-phase high-performance liquid chromatographic separation of metabolites was developed and chromatographic profiles of metabolites found in vivo and formed in vitro are presented. Several synthetic potential oxidation products were used to characterise the chromatographic profiles and evidence for the in vitro formation of 7-hydroxymethylbenz[*c*]acridine and 5,6-dihydro-5,6-dihydroxy-7-methylbenz[*c*]acridine was obtained.

INTRODUCTION

Studies with polycyclic aromatic hydrocarbons (PAH) such as benzo[*a*]-pyrene (BP) and 7-methylbenz[*a*]anthracene have indicated that metabolites of these substances, rather than the parent compound are responsible for their observed mutagenic and carcinogenic effect [1—3]. Many aza aromatic compounds are found as tobacco smoke and tar constituents [4] and occur as environmental air pollutants [5—7], and some of these have been shown to be tumorigenic [8] and mutagenic [9,10]. We are engaged in the study of the metabolism and mode of action of these compounds and have subjected 7-methylbenz[*c*]acridine to intensive examination. For metabolic studies on the PAH thin-layer chromatography (TLC) [11—13], gas chromatography (GC) [14—16], and high-performance liquid chromatography (HPLC) [17,18] have all been used. TLC techniques have the disadvantage that separation of isomeric dihydrodiols and phenols of PAH is usually poor, and GC techniques used with dihydrodiol and arene oxides may result in chemical alteration despite initial

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derivatization. HPLC systems used for PAH metabolites comprise reversed-phase octadecylsilane stationary phases with methanol—water or acetonitrile—water gradients as eluents. Amongst PAH metabolites examined are those of 3-methylcholanthrene [19], 7,12-dimethylbenz[*a*]anthracene [20], benz[*a*]-anthracene [21], dibenz[*a*,*h*]anthracene [22], 5-methylchrysene [23] and BP [17,18]. We report herein an examination of the metabolites of 7-methylbenz[*c*]acridine (7MBAC) formed by the rat in vivo or in vitro by HPLC using a reversed-phase gradient.

EXPERIMENTAL

Chemicals

[^{14}C]7-Methylbenz[*c*]acridine [24], *trans*-5,6-dihydroxy-5,6-dihydro-7-methylbenz[*c*]acridine, 5-hydroxy-7-methylbenz[*c*]acridine, 7-methylbenz[*c*]-acridine-5,6-oxide [25], 7-hydroxymethylbenz[*c*]acridine [26] and benz[*c*]-acridine-7-carboxylic acid [27] were synthesised according to literature methods. 9-Hydroxy-7-methylbenz[*c*]acridine and 11-hydroxy-7-methylbenz-[*c*]acridine were prepared in this laboratory [28].

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP and β-glucuronidase containing arylsulphatase (from *Helix pomatia*) were purchased from Sigma (St. Louis, MO, U.S.A.). Penicillin, streptomycin, foetal calf serum, tryptose phosphate broth and Leibovitz L-15 medium were purchased from Flow Laboratories (Sydney, Australia).

Chromatography

Reversed-phase HPLC. An Altex Model 334 gradient liquid chromatograph fitted with 10-μm RP-8 column (25 cm × 4.6 mm I.D.; Browlee Labs., Santa Clara, CA, U.S.A.) was used with a 3.5 cm × 4.6 mm precolumn of Corasil C_{18} Bondapak (37 μm, Waters Assoc., Milford, MA, U.S.A.). The column system was enclosed within a water jacket at 40.0 ± 0.1°C, and was eluted with a methanol—water gradient comprised of three sequential linear ramps. They were (1) 20—30% methanol over 15 min, (2) 35—70% methanol over 70 min and (3) 70—100% methanol over 5 min. A flow-rate of 1.2 ml min^{-1} and UV detection at 254 nm were used. One-minute fractions were collected and assayed for radioactivity by liquid scintillation counting in ACS cocktail (10 ml, Amersham Australia, Sydney, Australia).

Thin-layer chromatography. This was conducted using 0.25-mm Merck Kieselgel GF_{254} plates with visualisation by fluorescence or fluorescence quenching. The silica gel was scraped at 1-cm intervals and radioactivity was determined to confirm the presence of metabolites. Alternatively a Nuclear Chicago Actigraph III was used for radiochromatogram scans.

Gas chromatography—mass spectrometry. A Finnigan 9500 gas chromatograph interfaced with a Finnigan 3200 mass spectrometer and Finnigan 6100 data system was used. Extracts were derivatized with Trisil (Pierce, Rockford, IL, U.S.A.) for 1 h at 60°C before GC. For acidic fractions the temperature program after 2 min at 200°C was 10°C min^{-1} to 280°C while for neutral fractions a gradient of 4°C min^{-1} was used. Gas-Chrom Q (100—120 mesh) with 3%

OV-101 and a methane [for chemical ionization mass spectrometry (CIMS)] flow-rate of 20 ml min^{-1} were used.

Microsomal metabolism

Hepatic microsomes were prepared from male Wistar rats (100—150 g) according to a published method [29]. Incubation mixtures (3 ml) contained magnesium chloride (9 μmol), NADP (1.5 μmol), glucose-6-phosphate (12.5 μmol), glucose-6-phosphate dehydrogenase (1 unit), microsomal protein (0.2 mg/ml), potassium phosphate buffer, pH 7.4 (300 μmol) and [^{14}C]-7MBAC (200 nmol). Incubations were carried out in diffuse light at 37°C under air with constant shaking for 10 min. The reaction was stopped by cooling the incubation flasks in ice. The pooled contents of four flasks were extracted with ethyl acetate (3 volumes). The extract was treated with anhydrous sodium sulphate and the solvent evaporated under reduced pressure. The residue was dissolved in a methanolic solution of the available synthetic standards and analysed by HPLC.

Isolated hepatocyte metabolism

Isolated hepatocytes were prepared by slight modifications of the method of Jones et al. [30]. Washed liver slices from three male 50-day-old Wistar rats were digested with collagenase—hyaluronidase (0.05%:0.06%) in Hank's buffered salt solution (lacking calcium and magnesium salts) containing 20 m*M* HEPES buffer (pH 7.4) for 90 min at 37°C. The isolated hepatocytes were washed twice in Hank's solution before resuspending in culture medium.

Incubation mixtures (10 ml) contained $1.2 \cdot 10^7$ hepatocytes, 100 μ*M* [^{14}C]-7MBAC and 0.2% v/v dimethylformamide. Mixtures were incubated for 20 min at 37°C in diffuse light, under air, then centrifuged (15 sec, 500 *g*) to separate the cells from the incubation medium. The cells were homogenised in a Potter Elvejhem mortar and portions of both the incubation medium and the cell homogenate were extracted three times with ethyl acetate. Pooled extracts were treated as above for microsomes.

In vivo metabolism

Male Wistar rats (250—300 g), anaesthetised with urethane (1.25 g/kg), were cannulated at the bile duct and injected via the femoral vein with [^{14}C]-7MBAC (2 mg/kg) dissolved in 20% dimethylsulphoxide (DMSO)—water. Bile was collected for 6 h and then hydrolysed with β-glucuronidase at 37°C under nitrogen for 18 h. The bile was adjusted to pH 2.75 with concentrated hydrochloric acid and the solution was extracted with ethyl acetate (2 × 3 volumes). The ethyl acetate extracts were treated with anhydrous sodium sulphate and the solvent evaporated under reduced pressure after marker amounts of synthetic standards were added where appropriate. The residue was redissolved in ethyl acetate or DMSO and aliquots were analysed by TLC or HPLC respectively.

RESULTS AND DISCUSSION

Preliminary experiments with TLC of the rat bile extracts indicated that

TABLE I

BILIARY 7MBAC METABOLITES IDENTIFIED BY TLC

Solvent system	R_F standard	R_F metabolite	Percentage of dose*
7-Hydroxymethylbenz[c]acridine			
Methanol—toluene (1:9)	0.42	0.42	4.6
Ethyl acetate—hexane (1:3)	0.23	0.23	
Acetone—hexane (7:13)	0.42	0.42	
Benz[c]acridine-7-carboxylic acid			
Methanol—chloroform (3:7)	0.31	0.31	6.5
Acetic acid—ethanol—ethyl acetate (1:12:19)	0.50	0.50	
Ethanol—chloroform (2:3)	0.40	0.40	

*Excreted in 5-h bile after two 1-mg doses of [^{14}C]-7MBAC and determined by integration of radiochemical TLC scans.

both the alcohol, 7-hydroxymethylbenz[*c*]acridine (7-hydroxy MBAC), and benz[*c*]acridine-7-carboxylic acid were present in amounts of about 4—6% of an intravenous dose of 7MBAC in a neutral and acidic fraction respectively of the ethyl acetate extract. This conclusion was supported by cochromatography of marker compounds with radioactivity in three systems (Table I) and the proportions were determined by integration of radiochemical scans. Regions of the thin-layer chromatograms corresponding to the alcohol and the carboxylic acid were examined by gas chromatography—mass spectrometry (GC—MS) after derivatization as the trimethylsilyl ether and ester respectively. For the acidic metabolite a small peak emerged from the column with a retention time of 8.90 min, corresponding to that of the trimethylsilyl ester of the authentic carboxylic acid. The methane CI mass spectra of the derivatized metabolite and acid were in close agreement. Ions present were found at *m/e* 346 (100%, the base peak, quasimolecular ion, MH^+); 374 (20%, $M + C_2H_5^+$); 330 (11%, $MH^+ - CH_4$); 256 (12%, MH^+ — trimethylsilanol); and 230 (18%, 256 $- C_2H_2$). These fragmentation pathways from the protonated molecular ion or quasimolecular ion, MH^+, have not been proven by accurate molecular weight determinations since the spectrometer available was a low-resolution instrument. They are, however, reasonable postulates based on the expected loss of a neutral molecule from an even electron ion [31]. For the neutral metabolite a peak was not clearly visible in the gas chromatogram (Fig. 1) but selected ion monitoring at *m/e* 332, 360 and 316 showed pronounced peaks at a retention time of 7.30 min. The ions monitored were chosen to correspond to the quasimolecular ion MH^+, $M + C_2H_5^+$ and $MH^+ - CH_4$, respectively, of the trimethylsilyl derivative of 7-hydroxy MBAC, and the identity of the 7.30-min peak as the alcohol derivative was confirmed by its mass spectrum. Close correspondence of the metabolite derivative spectrum with that of authentic 7-hydroxy MBAC derivative was seen. Ions were present at *m/e* 360 (26%); 332 (100%); 316 (22%); 270 (22%, $M + C_2H_5^+$ — trimethylsilanol); and 242 (89%, MH^+ — trimethylsilanol). Other peaks seen in the limited mass scans probably correspond

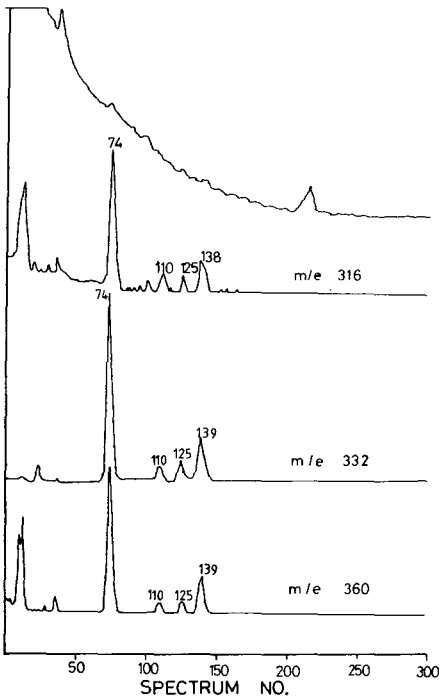
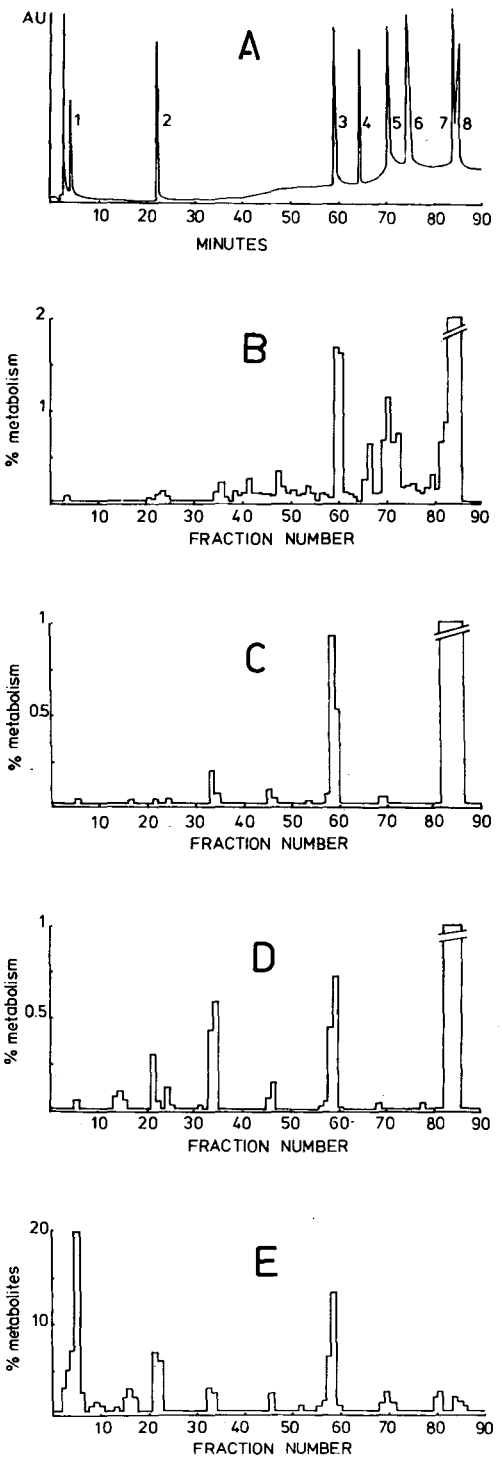


Fig. 1. Selected ion monitoring of neutral bile metabolites extracted from TLC plates in region corresponding to 7-hydroxymethylbenz[*c*]acridine standard.

to phenol derivatives which cochromatographed with 7-hydroxy MBAC on TLC. Extracts of the earlier TLC fractions (lower R_F values) only gave peaks on GC—MS selection ion scans at *m/e* values of 332 and 316 suggesting that possible dihydrodihydroxytrimethylsilyl derivatives of 7MBAC had decomposed by loss of trimethylsilanol during GC [16] or fragmented normally during MS examination. The available K region dihydrodiol, however, behaved normally on GC after trimethylsilylation and gave a single GC peak with ions at values expected for a bis-trimethylsilyl ether derivative at *m/e* 450, (20%, $M + C_2H_5^+$); 422 (100%, MH^+); 406 (80%, $MH^+ - CH_4$); 360 (15%, $M + C_2H_5^+$ — trimethylsilanol); and 332 (90%, MH^+ — trimethylsilanol). Efforts to analyse liver microsomal metabolites by TLC and GC—MS similarly gave evidence for the formation of several dihydrodiols by the appearance of *m/e* peaks at 332 presumably by loss of trimethylsilanol from bis-trimethylsilyl derivatives.

The limited ability of TLC to separate the metabolites from 7MBAC led us to examine HPLC as an analytical method. Using the standards synthetically available a gradient was developed which offered the possibility of analysing for the carboxylic acid in the presence of the essentially neutral dihydrodiols and phenols (Fig. 2A). The separation of the 5- and 9-phenols (peaks 5 and 6) was increased very considerably by the use of a higher temperature (40°C) for the analytical column. At ambient temperatures (22—28°C) the two peaks were incompletely separated, and the retention times of all the standards were more variable. The gradient separation has the disadvantage that the 11-phenol emerges from the column after the unchanged hydrocarbon and is incompletely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A
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C
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% metabolism
1
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D
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20
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E
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FRACTION NUMBER

separated from it. Consequently this method cannot be used to identify and determine 11-hydroxy-7-methylbenz[*c*]acridine as a 7MBAC metabolite where unchanged parent hydrocarbon is present (Fig. 2B, C and D).

The radiochemical histograms (Fig. 2B—E) illustrate the value of this method to separate 7MBAC metabolites. Liver microsomes (Fig. 2B) convert the aza hydrocarbon principally to metabolites cochromatographing with the 7-hydroxy MBAC, and other radioactivity was present in regions corresponding to the 5,6-dihydrodiol and phenols. When hepatocytes were used to metabolise 7MBAC, the profiles of the metabolites inside and outside the cell (Fig. 2C and D, respectively) were different. 7-Hydroxy MBAC appeared to be the major metabolite inside, while outside considerable amounts of more polar metabolites were found. Amongst these, the 5,6-dihydrodiol and possibly benz[*c*]-acridine-7-carboxylic acid were present. Phenolic type metabolites were almost absent. When bile was collected from rats which had received intravenous [^{14}C]7MBAC, and treated with β-glucuronidase and arylsulphatase to cleave conjugates, the metabolites obtained gave substantially different HPLC profiles (Fig. 2E). The parent compound at a retention time of 84 min was absent as would be expected, and there was some resemblance to the profile of hepatocyte 7MBAC metabolites found outside the cells (Fig. 2D), especially in the 10—60-min region of the chromatogram. The presence of the 7-hydroxy MBAC and benz[*c*]acridine-7-carboxylic acid as major metabolites is clear and the 5,6-dihydrodiol at a retention time of 22 min was also found. Only small amounts of phenols were present. In all profiles there were unidentified peaks of radioactivity which emerged from the column with retention times less than 60 min (the retention time of the alcohol).

The metabolite peaks corresponding to the alcohol and K-diol obtained from microsomes of rats have mass spectra in agreement with these assignments. Others, for which no synthetic standards are available, await structure confirmation. Based on the HPLC cochromatography alone there is no certainty that peaks of radioactivity are chemically homogeneous and further work on these metabolites is in progress to establish their homogeneity, spectral characteristics and identity. It is probable, based on results obtained with 7,12-dimethylbenz[*a*]anthracene [32], that multiply oxidised metabolites arising by further metabolism of the alcohol are produced. The appearance of liver preparation profiles containing several metabolites is similar to observations with other polycyclic aromatic compounds [17,18], and is consistent with observations that 7MBAC oxidation by liver microsomes of variously treated rats is closely paralleled by BP oxidation [33].

The metabolic fractionation described is expected to provide information on the comparative profiles of metabolites of 7MBAC produced by variously

Fig. 2. HPLC of (A) [^{14}C]7-methylbenz[*c*]acridine metabolism standards, (B) metabolites produced by hepatic microsomes from Wistar rats, (C) metabolites produced from hepatocytes found inside and (D) outside cells, and (E) metabolites extracted from rat bile after β-glucuronidase—arylsulphatase hydrolysis. Standards are (1) benz[*c*]acridine-7-carboxylic acid, (2) *trans*-5,6-dihydro-5,6-dihydroxy-7-methylbenz[*c*]acridine, (3) 7-hydroxymethylbenz[*c*]acridine, (4) 7-methylbenz[*c*]acridine-5,6-oxide, (5) 5-hydroxy-7-methylbenz[*c*]-acridine, (6) 9-hydroxy-7-methylbenz[*c*]acridine, (7) 7-methylbenz[*c*]acridine, (8) 11-hydroxy-7-methylbenz[*c*]acridine.

treated animals and will allow testing of these metabolites in the test of Ames et al. [34] to determine which metabolites are responsible for the S9 fraction mediated mutagenicity of 7MBAC [35]. This work is in progress.

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Note

Fluorescence detection of cystine by *o*-phthalaldehyde derivatisation and its separation using high-performance liquid chromatography

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The *o*-phthalaldehyde (OPA)—2-mercaptoethanol (MCE) derivatisation of primary amines [1] is ideally suited to the detection of amino acids in physiological fluids using liquid chromatography. This derivatisation step may be successfully performed using either a post-column [2] or pre-column [3] technique. The production of fluorescent derivatives when reacting OPA—MCE with primary amines is by the formation of 1-alkylthio-2-alkylisoindoles [4]. Since the reaction only proceeds in the presence of MCE, cystine is reduced to its monomer, cysteine. One disadvantage of the OPA—MCE reaction is that cysteine, unlike other amino acids containing primary amines, yields a derivative with minimal fluorescence [1]. This problem may be overcome by oxidising cystine or cysteine to cysteic acid, which when reacted with OPA—MCE yields a derivative with fluorescent properties comparable to those of other amino acid derivatives [1]. Because the conditions for these two reactions are vastly different, one being oxidising and the other reducing, it is difficult to obtain between-sample reproducibility of amino acid derivatisation with OPA—MCE following oxidation of cystine and cysteine.

This paper reports a simple procedure to overcome this problem by pretreating cysteine with iodoacetic acid before OPA—MCE derivatisation. This produces a cysteine derivative with a fluorescence intensity similar in magnitude to other amino acid OPA—MCE derivatives. This technique permits rapid chromatographic separation of cysteine OPA—MCE derivative using high-performance liquid chromatography (HPLC) and its quantification in biological samples.

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EXPERIMENTAL

Apparatus

The HPLC system used comprised an Altex pump and a 150 mm × 4.6 mm I.D. column pre-packed with 5-μm diameter Ultrasphere ODS (Anachem, Luton, Great Britain) and a Schoeffel FS970 fluorescence detector (Kratos, Manchester, Great Britain) using an excitation wavelength of 230 nm and an emission cut-off filter of 417 nm. The analytical column was fitted with a pre-column 70 mm × 2 mm I.D., packed with 25—37 μm diameter CO:PELL ODS (Whatman, Maidstone, Great Britain). Injections were made using a Rheodyne valve fitted with a 20-μl loop. Quantification of analytes was performed using an SP4100 integrator (Spectra-Physics, St. Albans, Great Britain).

Reagents

Unless otherwise stated all chemicals were analytical grade obtained from BDH (Poole, Great Britain). All solvents were filtered prior to use through a Millipore 0.5-μm filter (Millipore, London, Great Britain). Iodoacetic acid and MCE were obtained from Aldrich (Gillingham, Great Britain) and the OPA was Sepramar grade from BDH. The OPA—MCE was prepared in accordance with Lindroth and Mopper [3]. All amino acids were obtained from Sigma London (Poole, Great Britain).

Five standard solutions were prepared containing 50, 100, 150, 200 and 250 μM cystine by diluting in water, a 10 mM solution of cystine in 0.1 M hydrochloric acid.

Chromatographic conditions

Optimum chromatographic conditions were established as follows: isocratic solvent, 50 mM disodium hydrogen phosphate buffer (pH 7.4 ± 0.1)—acetonitrile (89:11).

Derivatisation

The following volumes of reagents were used to optimise the derivatisation conditions. To 100 μl of each cystine standard were added 200 μl of 200 μM homocysteic acid (internal standard), 500 μl of 70 mM perchloric acid (containing varying concentrations of MCE ranging from 2.5 mM to 125 mM), 200 μl of 3 M sodium hydroxide. After mixing, 200 μl of a solution of iodoacetic acid of varying concentrations ranging from 10 mM to 100 mM was added. To 200 μl of this solution were added 10 μl of OPA—MCE reagent and immediately 20 μl were injected onto the column using a filled-loop technique.

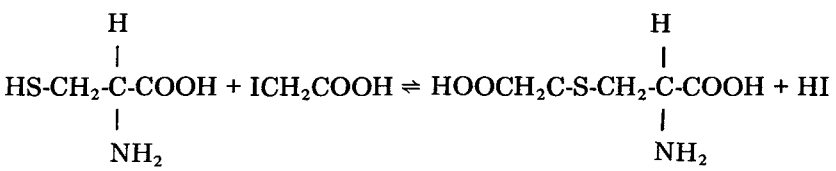
The procedure used when serum or urine was assayed was as follows. To 100 μl of serum or urine were added 200 μl of 200 μM homocysteic acid (internal standard) and 500 μl of protein precipitant (25 mM MCE in 70 mM perchloric acid solution). After centrifugation at 12,000 g for 2 min, 400 μl of the supernatant were added to 100 μl of 3 M sodium hydroxide. To 200 μl of this solution were added 100 μl of OPA—MCE reagent and 20 μl immediately injected onto the column using a filled-loop technique.

Quantitation

Peaks on the chromatograms were identified by their retention times and analyte concentrations calculated by proportions using peak area ratio with the internal standard.

RESULTS AND DISCUSSION

The measurement of cystine in physiological samples is important diagnostically in aminoacidopathies. When cystine is reacted with OPA—MCE reagent, because of the reducing conditions, the derivative produced is that of cysteine. The reason cysteine, compared with other primary amines, yields a weakly fluorescent OPA—MCE derivative, although not fully understood, does depend on the presence of sulphydryl groups on the cysteine molecule. By blocking the sulphydryl groups with iodoacetic acid the amino acid forms a strongly fluorescent isoindole product.



Cysteine Iodoacetic acid Cysteine derivative

Initially an excess of iodoacetic acid (100 m*M*) was employed to find the

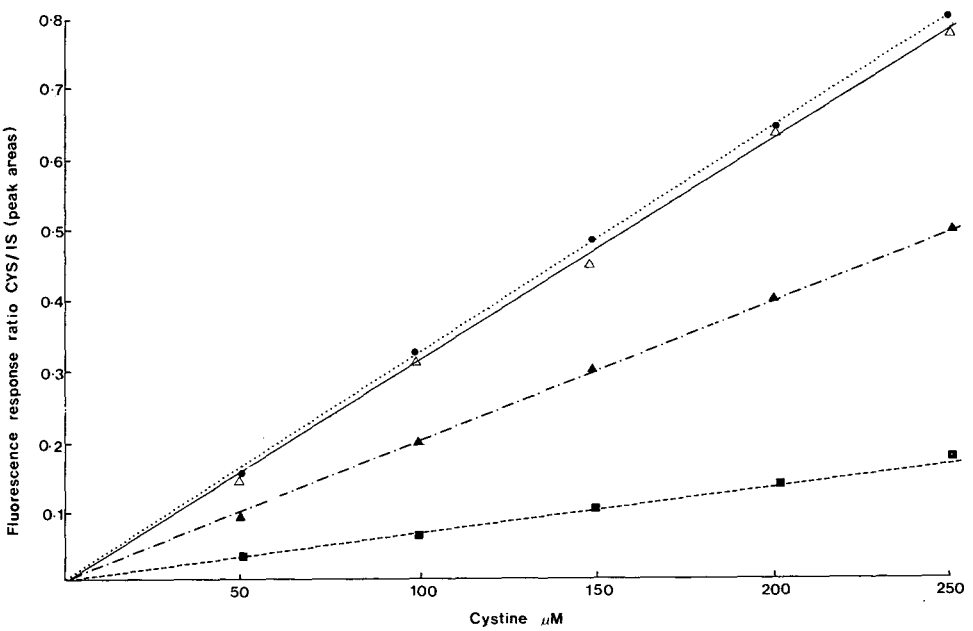


Fig. 1. The effect of varying concentrations of iodoacetic acid and cystine on the relative fluorescence of the cysteine OPA—MCE (CYS) derivative and internal standard (IS). Each point represents a mean of duplicate injections. The derivatisation procedure was performed as described in the text with the following concentrations of iodoacetic acid: •. . . . . •, 100 m*M*; △——△, 50 m*M*; ▲ • — • — ▲, 25 m*M*; ■ — — — ■, 10 m*M*.

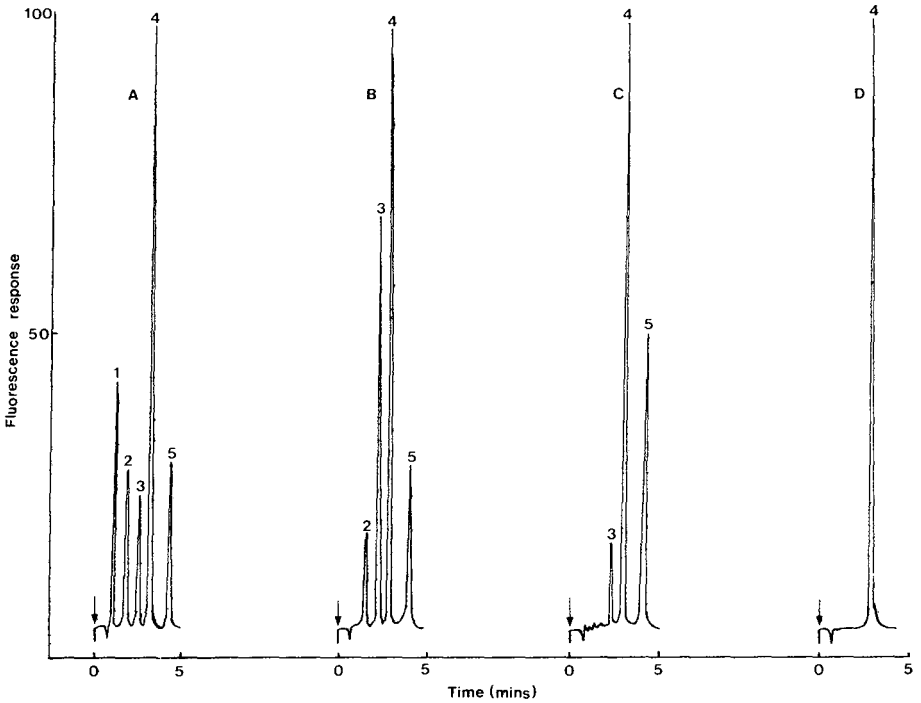


Fig. 2. Chromatograms of the following samples after derivatisation using the method described in the text: A, 100 μM standard solution of each amino acid in water; B, plasma with a cystine concentration estimated to be 99 μM; C, urine with a cystine concentration estimated to be 178 μM; D, internal standard, homocysteic acid. Peaks: 1, phosphoserine; 2, aspartic acid; 3, glutamic acid; 4, internal standard; 5, cystine. Arrow indicates the time of injection.

optimal MCE concentration required for the reduction of cystine. With the dilutions employed this was established as 25 mM MCE in 70 mM perchloric acid. Fig. 1 shows the effect of increasing concentrations of iodoacetic acid on the fluorimetric response of the cysteine derivative. Maximum fluorescence was obtained using 50 mM iodoacetic acid, and the method was linear up to 250 μM cystine.

Fig. 2 shows the chromatograms obtained with serum, urine and a standard cystine solution. The mean cystine concentration of a serum sample injected 20 times was found to be 106 μM with a standard deviation of ± 3.6 μM (coefficient of variation ± 3.4%).

The use of different reagents such as iodoacetamide and acrylonitrile, to block the sulphydryl groups, also enables the formation of different cysteine OPA—MCE derivatives to suit other chromatographic conditions. The fluorescence response of the cysteine OPA—MCE derivative described is linear with different concentrations of cystine allowing quantitative, precise and rapid estimations using this technique.

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Note

Simple method for the determination of homovanillic acid and vanillylmandelic acid in urine by high-performance liquid chromatography

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The levels of homovanillic acid (HVA) and vanillylmandelic acid (VMA) in urine are important indices in the diagnosis of neuroblastoma and phaeochromocytoma [1, 2]. Various techniques have been used for their determination such as spectrophotometry [3, 4], paper chromatography [5] and thin-layer chromatography [6, 7]. These procedures are, however, time consuming and unreliable. Gas chromatography with packed columns [8—13] and glass capillary columns [14] and mass fragmentography [15, 16] have also been used. These methods are specific and sensitive, but complicated for routine use.

A practical method for the simultaneous determination of HVA and VMA in urine by high-performance liquid chromatography (HPLC) was first proposed by the authors of this paper [17, 18]. A column of porous poly-

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styrene was used in a gradient elution mode with increasing pH of the eluent. Absorbance at 280 nm was monitored for quantitative evaluation. Other workers used chemically bonded reversed-phase packings [19, 20]. However, these methods required gradient elution with an increasing ratio of acetonitrile in the eluent in order to obtain rapid elution. Methods based on HPLC with electrochemical detection have been tried, but they can not determine both acids simultaneously [21—24]. HPLC with post-column derivatization was successful in the analysis of VMA, but not of HVA [25, 26].

In this present work, a simplified HPLC method for the simultaneous determination of HVA and VMA in urine is presented. A column of hydroxymethylated porous polystyrene and isocratic elution are used. The reliability of the method was tested by gas chromatography—mass spectrometry (GC—MS).

EXPERIMENTAL

Materials

The column packing material was Hitachi Gel No. 3011-O (Tokyo, Japan) which consists of spherical porous particles of a hydroxymethylated styrene—divinylbenzene copolymer with an average particle diameter of 5 μm.

HVA, VMA, hippuric acid, vanillic acid, *p*-hydroxyphenylacetic acid, ferulic acid and *p-n*-amylbenzoic acid were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 3,4-Dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylacetic acid (DOPAC), vanillyllactic acid (VLA) and vanillylpyruvic acid (VPA) were from Sigma (St. Louis, MO, U.S.A.). Caffeic acid was from Nakarai Chemicals (Kyoto, Japan). All other chemicals were of reagent grade and were obtained from Wako Pure Chemical (Osaka, Japan).

High-performance liquid chromatography

A high-pressure liquid chromatograph Jasco Trirotor-II (Japan Spectroscopic Co., Tokyo, Japan) equipped with a variable-wavelength UV detector Uvidec-100-II (Japan Spectroscopic Co.) was used. Absorbance was continuously recorded at 280 nm. The packing materials, dispersed in water—acetonitrile (4:1, v/v), were packed into stainless-steel tubing at a pressure of about 150 kg/cm^2 by the slurry packing technique.

HVA and VMA were extracted from urine according to the method described previously [27]. To 5 ml of urine were added 1 ml of 1 *M* citric acid—hydrochloric acid buffer (pH 1.0) and 1.5 g of sodium chloride. After the resulting mixture had been extracted with 10 ml of ethyl acetate by shaking for 5 min, 8 ml of the organic phase were taken and evaporated to dryness under reduced pressure. The residue was dissolved in 0.4 ml of methanol and 4-μl aliquots of the solution were analyzed under the conditions shown in Fig. 1.

Gas chromatography—mass spectrometry [28]

Extraction and derivatization of urinary HVA and VMA for GC separation were performed by the methods described by Addanki et al. [12] and Muskiet et al. [13]. Five milliliters of urine were acidified to pH 1.0 with 6 *N* hydrochloric acid using a pH meter; 1.5 g of sodium chloride were added and the

solution was extracted three times with 5 ml of ethyl acetate by shaking for 3 min. To the combined ethyl acetate extract 30 μg of *p-n*-amylbenzoic acid were added as an internal standard (I.S.) and the solution was evaporated to dryness under reduced pressure. To obtain the trimethylsilyl derivatives, the dried residue was reacted at 70°C for 30 min with 100 μl of bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane. One microliter of the solution was injected into the gas chromatograph of the GC—MS—computer system and analyzed under the conditions described in the legend to Fig. 4.

Mass spectra were obtained at selected *m/z* and each substance was quantitated from its peak height ratio to *p-n*-amylbenzoic acid.

RESULTS

Using the present HPLC technique, HVA and VMA were well separated from catecholamine-related phenolic acids, hippuric acid, *p*-hydroxyphenylacetic acid, caffeic acid and vanillic acid as shown in Fig. 1. VPA was not eluted under the present conditions. The relationship between peak height and amount injected into the chromatograph was linear over the range of 10—300 ng for both HVA and VMA.

A typical chromatogram of urine from a normal subject is shown in Fig. 2. The recoveries of HVA and VMA were 72% and 68%, respectively. The coefficient of variation of the peak height was 2% for 10 mg of both acids per liter of urine. The minimum detectable concentration was 0.4 mg/l.

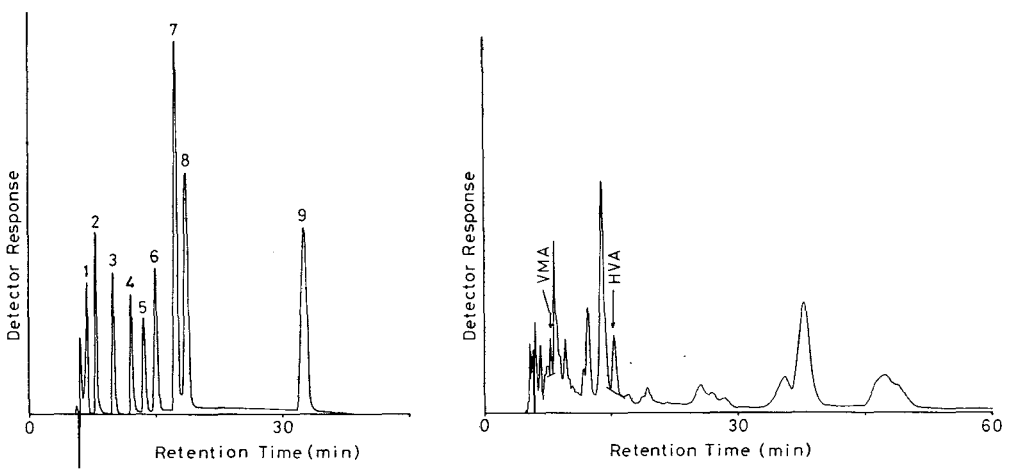

Fig. 1. Liquid chromatogram of a standard mixture of phenolic acids. Five microliters of a mixture (30—60 μg/ml) were applied to the chromatograph. Column: stainless-steel tubing (500 × 4.6 mm I.D.) packed with Hitachi Gel No. 3011-O (particle diameter about 5 μm) at 60°C. Eluent: 0.05 *M* tartaric acid—sodium hydroxide buffer (pH 3.20)—acetonitrile (4:1, v/v). Flow-rate: 1.0 ml/min. Detector: 280 nm, 0.04 absorbance units corresponded to full scale. Peaks: 1 = DOMA; 2 = VMA; 3 = DOPAC and VLA; 4 = hippuric acid; 5 = *p*-hydroxyphenylacetic acid; 6 = HVA; 7 = caffeic acid; 8 = vanillic acid; 9 = ferulic acid.

Fig. 2. Liquid chromatogram of a normal urine. For conditions see legend to Fig. 1.

The chromatogram shown in Fig. 3 was obtained from the urine of a patient with neuroblastoma. The concentrations of VMA and HVA in this sample were 7.4 ± 0.1 mg/l (8.4 ± 0.2 μg/mg of creatinine) and 15.5 ± 0.3 mg/l (17.7 ± 0.3 μg/mg of creatinine), respectively; these values represent the mean and range of duplicate determinations. Fig. 4 shows a mass chromatogram of the same urine. VMA and HVA were quantitated as 7.6 mg/l and 17.5 mg/l, respectively.

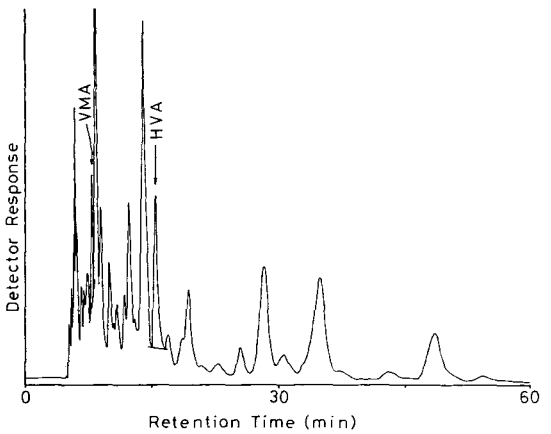

Fig. 3. Liquid chromatogram of urine from a patient with neuroblastoma. For conditions see legend to Fig. 1.

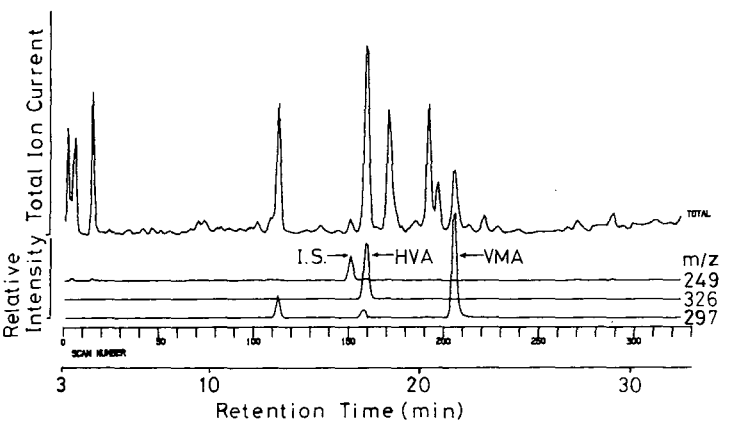

Fig. 4. Total ion current and mass chromatogram at selected *m/z* of the urine of a neuroblastoma patient (see Fig. 3). A Shimadzu—LKB 9000 GC—MS combination with a glass column of Gas-Chrom Q, 100—120 mesh (3 m × 3 mm I.D.) coated with 1% OV-101 was equipped with a computer system (GC—MS PAC 300D, Shimadzu Seisakusho). The column temperature was programmed from 130° C to 220° C at 3° C/min. The injector was kept at 250° C. The helium flow-rate was 30 ml/min. The mass spectrum in the range of *m/z* 150—430 was taken by continuous scanning as an electron energy at 20 eV, accelerating voltage 3.5 kV and ionizing current of 60 μA. The ion source and the slit separator were kept at 270° C and 250° C, respectively. The interval time of the continuous scanning was 5 sec.

DISCUSSION

The affinity of HVA for porous polystyrene such as Hitachi Gel No. 3010 is much stronger than that of VMA. Therefore, the HPLC spearation method reported previously [17, 18] required gradient elution. The difference in affinity between HVA and VMA is smaller for porous polystyrene with hydrophilic hydroxymethyl groups such as Hitachi Gel No. 3011-O. HVA and VMA are eluted, therefore, from such a gel column with a single eluent. In order to obtain satisfactory resolution, the particle diameter of No. 3011-O has to be 5 μm.

Under the conditions described in this work, HVA was not separated adequately from 5-hydroxyindole-3-acetic acid (5-HIAA). However, since 5-HIAA is not well extracted by ethyl acetate at pH 1.0 [8], the normal amount of 5-HIAA excreted in urine (2—5 mg/day) does not interfere with the determination of HVA. Vanillic acid, a metabolite of dietary vanillin, caused high apparent HVA values in the colorimetric assay of Ruthven and Sandler [29]. It did not interfere with HVA in the present method. Caffeic acid, a metabolite of chlorogenic acid contained in coffee, which affected the determination of HVA in the previously described method [18], was completely separated from HVA.

Due to the simplicity of the procedure, the present HPLC method seems promising for routine analysis of HVA and VMA in urine.

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Note

Determination of adenosine in normal human plasma and serum by high-performance liquid chromatography

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Adenosine (ADO), an ubiquitous nucleoside, has many different physiological actions. It is formed by the hydrolysis of adenosine 5′-phosphate (AMP) by 5′-nucleotidase. Subsequently ADO is either reconverted to AMP by adenosine kinase or metabolized to inosine (INO) by adenosine deaminase, an enzyme present in all tissues and red blood cells [1]. The final product of degradation of ADO is uric acid. ADO exerts a vasodilatory effect upon most vascular beds, especially the coronary system [2—4]; however, it exhibits vasoconstrictor properties in the kidney [5, 6]. ADO has been demonstrated to have a neurotransmitter role in the central nervous system [7, 8]; it also inhibits the release of norepinephrine from peripheral nerve terminals [5, 9]. In the adipose tissue, ADO has an insulin-like action [10]. A deficiency of adenosine deaminase leads to high levels of ADO in plasma and tissues which results in an immunodeficiency syndrome [11].

Very few procedures have been developed for the analysis of ADO in normal human plasma. A fluorimetric assay method was developed by Gardiner [12]. In the procedure INO and ADO were enzymatically converted to hypoxanthine and estimated by the rate of appearance of hydrogen peroxide after the addition of xanthine oxidase. A radioimmunoassay has been developed by Schrader et al. [13], which utilizes antibodies directed against an antigen containing ADO. Interfering adenosine deaminase activity was removed from the antisera by treatment with DEAE-cellulose. A few high-performance liquid chromatographic (HPLC) procedures have been developed for ADO assay in plasma and serum. Pfadenhauer and Tong [14] used a boronate affinity column for the isolation of ADO from plasma. The collected eluate was lyophilized to dryness and the reconstituted residue was analyzed by HPLC using a reversed-phase column with phosphate buffer, pH 3.5 as the mobile phase. Circulating

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levels of ADO in six healthy volunteers ranged from 18 to 149 ng/ml with an average of 62 ng/ml. Two other procedures have been developed for the analysis of ADO by HPLC in human serum by Brown et al. [15], and Hartwick and Brown [16]. Both procedures used reversed-phase columns with phosphate buffers at pH 5.8 containing 10% methanol as the mobile phase. Free ADO was not detectable in normal human serum but it was measured in serum samples from a patient suffering from adenosine deaminase deficiency.

An HPLC assay procedure is described here for the analysis of ADO in normal human plasma. 2′-Deoxycoformycin (2′-DCF), a potent inhibitor of adenosine deaminase activity, is added to the samples to inhibit the conversion of ADO to INO. A reversed-phase radial compression column is used for rapid separation of ADO from plasma ultrafiltrate.

EXPERIMENTAL

Materials

INO, ADO and adenosine deaminase were obtained from Sigma (St. Louis, MO, U.S.A.); [^{3}H]ADO (49 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.SA.), and heparin (1000 units/ml) was procured from Upjohn (Kalamazoo, MI, U.S.A.). The 2′-DCF was a gift from Developmental Therapeutics Program, Chemotherapy, National Cancer Institute (Bethesda, MD, U.S.A.). Stock solutions were prepared by dissolving 0.1 mg 2′-DCF in 10 ml normal saline (final concentration, 10 ng/μl).

Instrumentation

A Waters Assoc. (Milford, MA, U.S.A.) 202 high-performance liquid chromatograph with a U6K injector and a radial compression column unit was used with a 11.5 cm × 0.8 cm, 10-μm C_{18} μBondapak column. The guard column contained 10-μm C_{18} μBondapak reversed-phase packing. A variable-wavelength detector (Instrumentation Specialties, Lincoln, NB, U.S.A.) set at 254 nm was used for detection.

Operating procedures and conditions

The mobile phase consisted of 0.05 *M* NaH_2PO_4 (in distilled water) adjusted to pH 7.4 and degassed for 30 min. Standards were prepared by dissolving ADO and INO (final concentration 20.0 μg/ml) in the mobile phase. The column was at ambient temperature (approximately 22°C) and the flow-rate was set at 8.0 ml/min (14 MPa). Sample volumes of 180 μl were injected per analysis.

Sample collection

Blood samples were drawn via venipuncture in the morning from normal adult male and female volunteers, into 7-ml vacutainer tubes containing 10 μl of heparin (10 units) and 140 μl of 2′-DCF (1400 ng). The samples were immediately cooled in ice and the tubes were centrifuged at 2500 rpm (1400 *g*) for 15 min; then 3.5 ml of plasma were transferred to an ultrafiltration cone (CF-50, Amicon, Lexington, MA, U.S.A.) and centrifuged at 1400 *g* for 2 h. An aliquot (0.8 ml) of the ultrafiltrate was dried under a stream of air and reconstituted with 200 μl of the mobile phase.

The recovery of ADO was determined by adding 0.05 μCi of [^{3}H] ADO to blank plasma prior to ultrafiltration. After 2 h of centrifugation, [^{3}H] ADO was measured in the ultrafiltrate and in the residue remaining in the cone (Beckman 255 Scintillation Spectrometer, counting efficiency for ^{3}H $\approx$ 40%). The recovery of ADO was found to be essentially quantitative.

RESULTS AND DISCUSSION

Fig. 1a shows a chromatogram of ADO and INO isolated from normal human plasma containing 2′-DCF. The retention times for INO, 2′-DCF and ADO were 104, 207 and 400 sec, respectively. In chromatograms of plasma samples which did not contain 2′-DCF, the ADO peak was not observable (Fig. 1b) and the INO peak was more pronounced. Positive identification was achieved by peak superimposition, i.e. by adding INO and ADO standards (200 ng) to ultrafiltrate samples and observing increased peak height at the corresponding retention times. Identification was also confirmed by the absence of the ADO peak and the enhancement of the INO peak in the samples that did not contain 2′-DCF or in samples to which adenosine deaminase (5—20 units) had been added.

The limit of detection (2:1 signal-to-noise) of this procedure is 10 ng of ADO. Repetitive injections of standards gave good reproducibility of retention times (S.D. = ± 2%) and peak heights (S.D. = ± 2.3%). Standard curves were

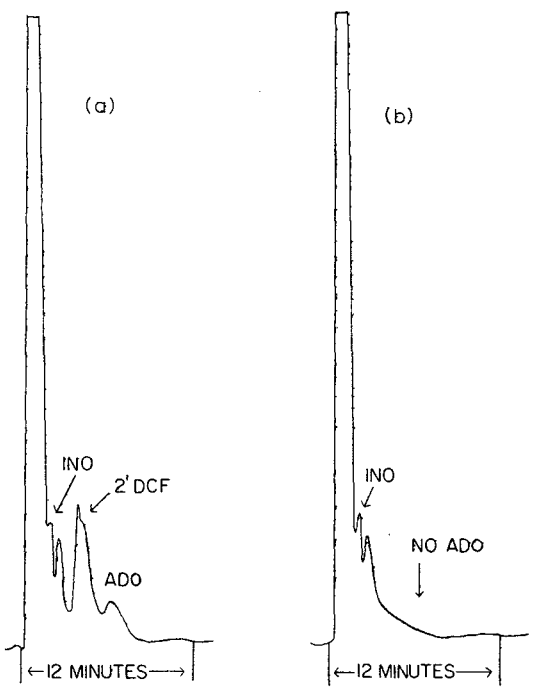

Fig. 1. (a) Chromatogram of INO and ADO from normal human plasma. The peak at 207 sec is that of 2′-DCF. Blood was collected in the presence of heparin and 2′-DCF. (b) Chromatogram of an extract of normal human plasma obtained from blood collected in the presence of heparin and absence of 2′-DCF. Note that only INO is detectable.

linear in the range of 20—1000 ng ADO and the day-to-day reproducibility varied less than 3.2% (S.D.). The working portion of the standard curve is in the range of 30—300 ng ADO.

After approximately 110—135 injections of plasma samples, the column resolution starts to decrease and this deterioration is noted even with the use of a guard column. The retention time of ADO increases considerably and the ADO peak becomes too broad to be quantitated. If 5% methanol is added to the mobile phase, the ADO peak becomes sharp with a retention time of 400 sec but the INO peak can not be resolved from the solvent front. At this stage the column can be used for only 10—20 injections before it is deteriorated beyond use.

Variations were not found in the levels of endogenous ADO in blood samples collected and allowed to stand for 15 or 30 min prior to workup. If release and uptake of ADO occurs by red blood cells, it does not appear to affect the final determination in a 30-min time span. The effect of short-term local ischemia, obtained by use of a tourniquet, upon ADO levels was studied. Blood samples were collected immediately and 2 min after the tourniquet had been applied; no significant difference was noted between the levels of ADO in the two samples.

In the absence of added 2′-DCF, ADO was not detected in any plasma sample. The use of 200 ng of the inhibitor per ml blood collected provided maximal inhibition of adenosine deaminase; concentration of 2′-DCF as high as 2500 ng/ml blood gave the same values of ADO.

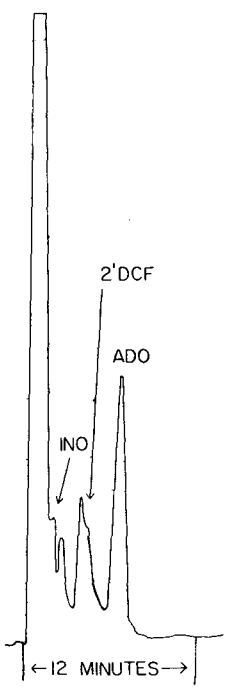

Fig. 2. Chromatogram of an extract of normal human serum obtained from blood collected in the presence of 2′-DCF and the absence of heparin. The sample clotted in the collection vial producing a very large quantity of ADO.

Clotting could not be prevented in the blood collection vials or ultrafiltration cones without the use of heparin. Fig. 2 presents a chromatogram of a sample which clotted in the collection vial; it shows very large amounts of ADO to be present in the serum, probably due to the breakdown of ATP which occurs in the normal clotting process.

The ADO levels in plasma and serum of normal adult male and female volunteers are presented in Tables I and II, respectively. Although the number of people tested is too small for a definitive conclusion, there appears to be no significant difference between the two sexes. The ADO levels reported in this study are slightly higher than those published by previous investigators [14]; this is probably due to the use of the adenosine deaminase inhibitor in these experiments. It is observed that a large amount of ADO is present in serum probably as a result of the normal clotting process. This could lead to falsely increased levels of ADO and limit the clinical usefulness of the method. Heparin prevents clotting from taking place and it is felt that plasma determination would be of greater clinical utility since it is more likely to represent true circulating levels of ADO.

Previous publications have reported the measurement of ADO only in patients with adenosine deaminase deficiency [15, 16] and ADO was not

TABLE I

ADENOSINE LEVELS IN PLASMA OF NORMAL ADULT VOLUNTEERS

Subject	Age	Sex	Plasma (ng ADO per ml)
1	30	M	81
2	24	M	121
3	30	M	150
4	32	M	281
5	33	M	142
6	32	M	134
7	32	F	132
8	28	F	136
9	23	F	173
10	25	F	73
11	34	F	62
Mean ± S.D.			135 ± 38

TABLE II

ADENOSINE LEVELS IN SERUM OF NORMAL ADULT VOLUNTEERS

Subject	Age	Sex	Serum (ng ADO per ml)
1	30	M	1278
3	30	M	1611
10	24	F	830
12	24	F	2330
Mean ± S.D.			1512 ± 458

detectable in normal human plasma. It has been noted in this study that the elapse of time between sample collection and centrifugation was sufficient to allow breakdown of ADO to INO beyond the limit of detection of this HPLC procedure. Only by the use of the adenosine deaminase inhibitor (2′-DCF), at the time the blood was drawn, were levels of ADO quantitated in normal human plasma.

CONCLUSIONS

This HPLC method for the determination of ADO levels in the plasma of normal human subjects is simple, rapid and reproducible. The use of 2′-DCF to prevent degradation of ADO is necessary. This procedure can be applied to study ADO in different physiological and pathological conditions in man.

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Note

Quantitation of physiological α-tocopherol, metabolites, and related compounds by reversed-phase high-performance liquid chromatography

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Vitamin E, α-tocopherol, has been linked to a variety of seemingly unrelated pathological conditions in man. For example, in childhood, bronchopulmonary dysplasia, retrolental fibroplasia and hemolytic anemia have all been associated with a vitamin E deficient state [1]. High dose vitamin E has been used therapeutically in the treatment of non-congenital spherocytic hemolytic anemia secondary to glucose-6-phosphate deficiency [2].

At the present time, colorimetric, fluorometric, hemolytic estimate, paper chromatographic, gas chromatographic [3], and high-performance liquid chromatographic (HPLC) [4—8] methods have been used in the assay of α-tocopherol. In addition, some HPLC methods are capable of separating the naturally occurring vitamin E analogues α-, β-, γ- and δ-tocopherols.

Recently, we have investigated the potential protective effect of vitamin E in relation to anthracycline induced cardiomyopathy [9] and carcinogenicity [10]. In the study of these drug nutrient interactions the metabolism and distribution of α-tocopheryl acetate (major component of commercially available vitamin E) becomes an important topic. The HPLC system we describe was used to separate α-tocopherol, α-tocopheryl acetate, α-tocopheryl quinone, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, and γ-tocopherol. The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid was used as a reference compound for retention time of water soluble metabolites.

EXPERIMENTAL

Reagents

Glass-distilled methanol and hexane were obtained from Burdick and Jackson

Labs. (Muskegon, MI, U.S.A.). α-Tocopheryl acetate was purchased from Sigma (St. Louis, MO, U.S.A.). Calbiochem-Behring (La Jolla, CA, U.S.A.) supplied the pure α-tocopherol. The α-tocopheryl quinone, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, and [^{14}C]α-tocopheryl acetate were the gifts of Hoffmann-La Roche (Nutley, NJ, U.S.A.).

Apparatus and samples

A Glenco HPLC system I (Houston, TX, U.S.A.) equipped with a Whatman Partisil 10-μm ODS 250 mm × 4.6 mm reversed-phase column was the base instrument used in isocratic elutions. A Laboratory Data Control (Riviera Beach, FL, U.S.A.) chromatographic system consisting of two Constametric III pumps and Gradient Master programmer were used in gradient elutions. The gradient system was equipped with a Custom LC Spherisorb 5 μm ODS 150 mm × 4.6 mm reversed-phase column. Ultraviolet detection at 275 or 285.5 nm was obtained on a variable-wavelength UV detector (Laboratory Data Control). Electronic integration of peak areas was obtained with a digital integrator CSI-38 (Columbia Scientific Industries, Austin, TX, U.S.A.). Fractions used in scintillation counting were collected with a fraction collector (LKB Instrument, Rockville, MD, U.S.A.) and counted with a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.). A Brinkman homogenizer was used in tissue processing.

Plasma and tissue samples assayed in this system were obtained from Sprague-Dawley (SD) rats. [^{14}C]α-Tocopheryl acetate was administered intraperitoneally (i.p.) daily to SD rats at a dose of 300 mg/kg (1.8 g/m^2) for one to four consecutive days according to experimental protocol. Plasma samples were also obtained from patients receiving high doses of α-tocopherol orally.

Procedures

For routine plasma analysis the isocratic system was used with a mobile phase of 87% methanol and 13% filtered distilled water. Eluent flow-rate was 1.30 ml/min with UV monitoring at 285.5 nm and 0.01 a.u.f.s. Analyses of tissue samples were usually performed using a convex gradient with an initial mobile phase of 88% methanol, 12% water increasing for 20 min to 100% methanol at a flow-rate of 2.0 ml/min. In tissue chromatograms, where quantitation of minimal amounts of α-tocopheryl quinone is a priority, UV monitoring was at 275 nm and 0.01 a.u.f.s. In elutions from which fractions were collected, chromatographic conditions were the same and fractions were collected every 40 sec at 0.8 ml per fraction.

Tissue and plasma samples were extracted in a similar manner in preparation for HPLC analysis. Plasma was extracted directly. Tissue samples were prepared for extraction by homogenizing 1 g of tissue in 4 ml of normal saline. One ml of tissue homogenate was extracted for routine analysis. Usually, 200 μl of plasma were added to a 12-ml conical centrifuge tube. Two hundred μl of double-distilled ethanol and 500 μl of hexane were then added to the tube. The contents were mixed and centrifuged at 9000 *g* for 10 min at 4°C. Four hundred μl of the hexane supernatant were removed and dried under nitrogen. At the time of HPLC analysis the sample was reconstituted in 100 μl of a mixture of 87% methanol and 13% propanol, centrifuged, and injected.

For the extraction of 1 ml of plasma or tissue the volume of ethanol and hexane added was proportionally increased. Mixing and centrifugation procedures were similar to those used for 200 μl of plasma.

Calibration and reproducibility

Calibration curves were constructed for both isocratic and gradient elutions. The isocratic elution calibration was performed for plasma analysis using a 200-μl sample size. Gradient elution was calibrated using a 1-ml sample size appropriate for tissue analysis. Saline, for the 200-μl sample size, and 4% bovine serum albumin in saline, for the 1-ml sample, were spiked with known amounts of standard.

Extraction methods were as outlined in the Procedures section. Table IA represents the results of the 200-μl calibration curve for the compounds listed using isocratic elution and monitoring at 285.5 nm. α-Tocopheryl acetate was not analysed because the compound is absent from plasma. Table IB represents the 1-ml calibration curves using gradient elution and monitoring at 275 nm. For both sections two samples were assayed at each concentration and four different concentrations were used. The lower limit of detectability and correlation coefficient were calculated from a least-squares linear regression. The lower limit of detectability was designated as 20,000 area units, where assay variability became excessive (n=6, C.V.=19%). Mean percentage recovery for both tables was determined by comparing the midpoint values of the extraction regression lines to midpoint values on regression lines from direct injection standard curves. The correlation coefficient for all direct injection curves was $>0.996, p<0.001$.

Assay reproducibility was determined using UV monitoring of α-tocopherol.

TABLE I

CALIBRATION CURVE DETECTION LIMITS, LINEARITY, AND RECOVERY

	Calibration range (mole/l)	Lowest detectable concentration (mole/l)	Correlation coefficient	Mean percentage recovery
A. 200-μl sample volume				
α-Tocopherol	$5\cdot10^{-6}$—$5\cdot10^{-5}$*	$3.7\cdot10^{-6}$	0.997	65
α-Tocopheryl quinone	$2.5\cdot10^{-5}$—$2.5\cdot10^{-4}$	$7.5\cdot10^{-6}$	0.999	58
γ-Tocopherol	$5\cdot10^{-6}$—$5\cdot10^{-5}$	$3.9\cdot10^{-6}$	0.997	63
B. 1-ml sample volume				
α-Tocopheryl acetate	$2\cdot10^{-6}$—$5\cdot10^{-5}$	$1.3\cdot10^{-6}$	0.999	64
α-Tocopherol	$2\cdot10^{-6}$—$5\cdot10^{-5}$	$1.2\cdot10^{-6}$	0.995	57
α-Tocopheryl quinone	$2\cdot10^{-6}$—$5\cdot10^{-5}$	$6.7\cdot10^{-7}$	0.999	44
γ-Tocopherol	$2\cdot10^{-6}$—$5\cdot10^{-5}$	$2.1\cdot10^{-6}$	0.998	60

*Eight data points are used in each calibration curve.

Two hundred μl of a plasma sample were assayed repeatedly using the extraction and sample preparation procedure outlined previously: ten values were determined on five separate days. The following values were obtained shown in integrated unit area: mean 212,741; standard deviation, 21,526 (C.V.=10%).

A colorimetric procedure [9] was also used in analysis of plasma samples from patients and the results compared to our HPLC values. From a comparison of nineteen samples the mean value for the colorimetric assay was $7.1 \cdot 10^{-5}$ mole/l α-tocopherol and from the HPLC assay $8.9 \cdot 10^{-5}$ mol/l, a 20% higher value. By linear regression analysis of the two assays, r=0.96 with a slope of 1.09. One possible explanation for the higher HPLC values is overcompensation for carotene interference in the colorimetric assay.

RESULTS AND DISCUSSION

Fig. 1A represents a typical chromatogram of a patient plasma sample analysed using isocratic elution. Small quantities of α-tocopheryl quinone and γ-tocopherol can be detected in human plasma along with α-tocopherol. Of 24 patient plasma samples assayed by HPLC, γ-tocopherol was detected at a quantitatible level in five patients. The range of γ-tocopherol concentration was $3.7 \cdot 10^{-6}$ to $1.1 \cdot 10^{-5}$ mol/l representing from 3 to 24% of the total tocopherol detected. In the same group of samples a quantitatible level of α-tocopheryl quinone ($1.6 \cdot 10^{-5}$ mol/l) was found only in the patient with the highest level of α-tocopherol ($2.5 \cdot 10^{-4}$ mole/l). α-Tocopheryl acetate was not detectable in any plasma samples from experimental animals (or patients).

Fig. 1B is an analysis of liver from a rat treated with four i.p. doses of [^{14}C]α-tocopheryl acetate assayed using the gradient elution. Gradient elution dramatically increases the selectivity of our system without increasing analysis time substantially. Gradient elution also alleviates problems associated with late eluting materials present in some types of tissues. α-Tocopheryl acetate, γ-tocopherol, α-tocopherol, and α-tocopheryl quinone are detectable in the chromatogram by UV absorption, and radioactivity was present in areas corresponding to peaks 1, 3, and 4. A different liver sample was monitored simultaneously at 254 and 285.5 nm and the area ratio of 254:285.5 absorbance for peak 1 was compared to the ratio for known α-tocopheryl quinone. The ratios were equal within 5%, further verifying peak identity.

In general, α-tocopherol, α-tocopheryl quinone, α-tocopheryl acetate, and γ-tocopherol were detectable in liver, kidney, and heart samples from animals treated with α-tocopheryl acetate. In plasma and mammary fat only α-tocopherol and γ-tocopherol were detected by UV absorbance or radioactivity. Table II represents in part, data from our distribution study using the HPLC technique. Treated animals received four i.p. doses of [^{14}C]α-tocopheryl acetate at 300 mg/kg and were killed at the times indicated, after the last dose. Control animals were of comparable age and dietary regimen. Plasma samples were analyzed with an isocratic system and monitored at 285.5 nm. Heart samples were analyzed using a gradient system at 275 nm. Fractions were collected from the eluent and counted using liquid scintillation counting. By comparing the amount of compound detected by UV absorbance to the amount detected from radioactivity, a specific activity indicating radioactive

TABLE II

ANALYSIS OF RAT HEART AND PLASMA

Concentration (concn.) values are given in μg/ml ± S.D., for plasma and μg/g ± S.D. for heart. Specific activity (S.A.) is in mC ± S.D. *n* = number of samples analyzed.

No. of injections	Injected S.A.	Days after last injection	Plasma			Heart				
			α-Tocopherol			α-Tocopherol			α-Tocopheryl acetate concn.	α-Tocopheryl quinone concn.
			n	concn.	S.A.	*n*	concn.	S.A.		
0	—	—	6	23.1±5.2	—	2	26.0±5.9	—	B.Q.*	B.Q.
4	44.8	2	3	20.0±4.2	22.0±4.3	1	50.4	3.9	3.2	1.7
4	35.7	7	1	6.7	2.8	1	28.9	4.8	9.7	B.Q.
4	35.7	21	1	23.6	3.4	2	40.0±13	5.6±2.6	10.2±8.2	1.5
4	35.7	70	2	34.8±9.5	13.3±0.9	2	33.3±3.2	16.4±2.5	B.Q.	1.7±0.5

*B.Q. = Below reliable level of quantitation: <2.8 μg/g for α-tocopheryl acetate and <1.3 μg/g for α-tocopheryl quinone.

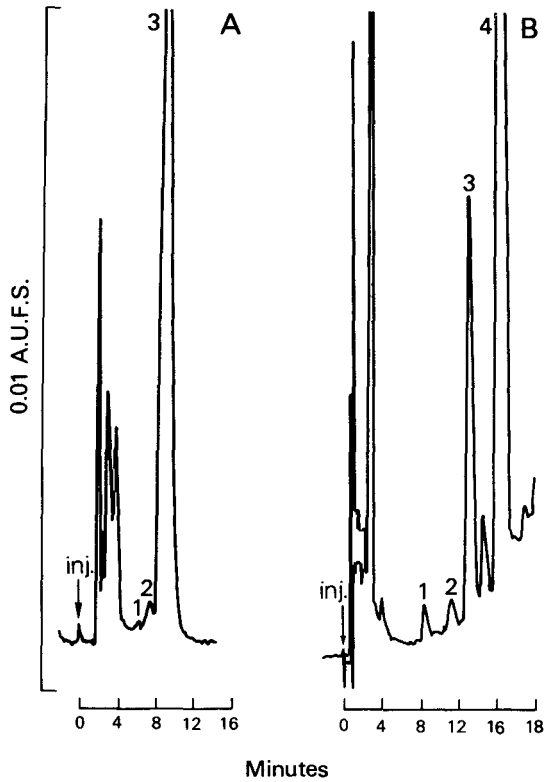

Fig. 1. (A) Injection (50 μl) of an extraction of 200 μl of plasma from a patient receiving high doses of α-tocopherol orally. Peaks: (1) α-tocopheryl quinone, 0.28 μg; (2) γ-tocopherol, 0.11 μg; (3) α-tocopherol, 3.53 μg. Conditions: Whatman Partisil ODS 10 μm 250 mm × 4.6 mm column; mobile phase, methanol—water (87:13); flow-rate, 1.30 ml/min; detection at 285.5 nm. (B) Injection (25 μl) of an extraction of 1 ml of liver homogenate containing 0.22 g of tissue from a rat receiving α-tocopheryl acetate. Peaks: (1) α-tocopheryl quinone, 0.04 μg; (2) γ-tocopherol, 0.2 μg; (3) α-tocopherol, 1.8 μg; (4) α-tocopheryl acetate, 24 μg. Conditions: Custom LC Spherisorb ODS 5 μm 150 mm × 4.6 mm; mobile phase, gradient elution from methanol—water (88:12) to 100% methanol in 20 min; flow-rate 2.0 ml/min; detection at 275 nm.

dilution was calculated. This dual detection method enables us to quantitate both the total amount of a compound and to determine what portion of the total pool has been replaced by radioactive material. From the data in Table II we see changes in the tocopherol pool that would not be readily apparent if quantitation were by UV absorption or radioactivity individually. This type of dynamic analysis may prove useful in determining rate of tissue uptake of administered compounds and may allow detection of otherwise non-detectable changes in distribution or metabolism.

The HPLC system outlined has been successfully used to quantitate α-tocopheryl acetate and its metabolic products in human plasma and in animal tissues and plasma. These types of separations and quantitations are invaluable in studying the metabolism and distribution of the compounds. With further refinement of techniques it is hoped that we can achieve the ability to monitor

tocopherol levels in other tissues along with testing the capabilities of our system in separating the remaining tocopherol analogues.

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Note

Separation of vitamin B_6 compounds by paired-ion high-performance liquid chromatography

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It is well known that vitamin B_6 [i.e. pyridoxine(3-hydroxy-4,5-bis-(hydroxymethyl)-2-methylpyridine, PN) pyridoxal (PL), pyridoxamine (PM) and their phosphorylated derivatives] is required for growth and development of normal and neoplastic tissues, in general, and that pyridoxal-5'-phosphate (PLP) is the coenzyme of many classes of enzymes [1,2]. The principal urinary metabolite of all forms of vitamin B_6 is 4-pyridoxic acid (PIC) which has no biological activity [3]. The vitamin's effects on tryptophan metabolism, i.e. increased xanthurenic acid excretion in the vitamin depleted state after tryptophan loading, with similar observations during pregnancy and in women receiving oral contraceptive pills coupled with observation of low plasma PLP in these two groups of women [4,5] has prompted investigators to seek methods for the separation and quantitation of the vitamin B_6 compounds in biological materials. Vanderslice et al. [6] have recently reviewed vitamin B_6 methodology and concluded that the best method is separation by high-performance liquid chromatography (HPLC). In fact, these workers reported the separation of all vitamin forms including PIC using a thermostated (55°C) anion-exchange column and two buffer systems at pH 10 and pH 2.5. Separation was completed in approximately 115 min. In a more recent report from the same laboratory [7], the separation and quantitation of the vitamin forms was reported using a dual system of two anion-exchange columns thermostated at 50°C and 18°C, respectively, and the two buffer systems, reported previously [6]. Quantitation was effected using a 650/40 Perkin-Elmer fluorescence spectrophotometer. An effective run with the dual column system required approximately 100 min.

In order to study the metabolic interconversions of vitamin B_6 in vivo investigators have employed radioactively labeled pyridoxine precursor and

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ion-exchange (gravity) chromatography as well as high-voltage electrophoresis for separation of the vitamin metabolites [8—12]. Ion-exchange (gravity) chromatography requires long time periods and does not differentiate well between phosphorylated vitamin B_6 forms, metabolites that might coelute, or between overlapping peaks. For instance, recently we synthesized pyridoxine-5′-phosphate (PNP) [13] which eluted as a single peak on a Dowex H^+ column [12] but was further resolved into three peaks upon subsequent chromatography by HPLC. Although the latter method (electrophoresis) requires much less time to run samples it suffers from the fact that only labeled samples of 5 μl can be effectively run and only when all the vitamin B_6 standards are included with each sample in sufficient amounts for further identification following the completed run [9]. The method also does not differentiate PIC from the other vitamers [9]. Because of these and other undesired features of these methods (i.e. use of buffers; during sample concentration buffer salt is also concentrated, which subsequently interferes with the identification of unknown metabolites) we resorted to ion-pairing HPLC for studying vitamin B_6 metabolism in vivo. This communication reports on a relatively quick method based on ion-pairing for the separation and identification of vitamin B_6 tissue metabolites including PIC. The short period required for a run (<40 min) and the relatively easy separation of the vitamers from the ion-pairing agent (thus avoiding salt interference with identification) make the method more unique and advantageous over other procedures.

EXPERIMENTAL

The apparatus is an HPLC Waters Assoc. system equipped with a Model U6K universal liquid injector, Model 440 UV absorbance detector with a 313-nm filter and Model 6000A solvent delivery system. A 30 × 0.39 cm μBondapak C_{18} column, particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.) was used.

Solvents

(A) 10% 2-propanol with 0.09% glacial acetic acid. (B) 10% 2-propanol with 0.09% glacial acetic acid with a mixture of counter-ions (seven-carbon and eight-carbon alkyl chains). The counter-ions were of sodium-1-heptanesulfonate and sodium-1-octanesulfonate at 0.004 *M* concentration.

The solvents were prepared with HPLC water, filtered through 0.45-μm filter and degassed before use.

Standards

The standards used were: pyridoxine hydrochloride, pyridoxal hydrochloride, pyridoxamine dihydrochloride, pyridoxal-5′-phosphate hydrochloride, pyridoxamine-5′-phosphate hydrochloride, 4-pyridoxic acid and 3-hydroxypyridine and were obtained in the purest state available from Chemical Dynamics Corp. (S. Plainfield, NJ, U.S.A.) or from Sigma (St. Louis, MO, U.S.A.). Pyridoxine-5′-phosphate was prepared as described by Peterson and Sober [13], purified by cation-exchange chromatography (Dowex H^+) [12] and further by HPLC.

Standard solutions. Standard solutions of 1 mg/ml were prepared in HPLC water (10 mg of the standard compound were dissolved in 10.0 ml of pure water).

Working standards. The working standard solutions of 20 ng/μl were prepared by diluting 200 μl of the standard solution to 10.0 ml with solvent B.

Procedure

The eight standards were separated using solvents A and B at a flow-rate of 0.7 ml/min. The column was equilibrated by passing three column volumes (ca. 25 ml) of solvent B. An aliquot of the mixture of the eight standards is injected and elution proceeds for the first 5 min using solvent B. At exactly 5 min, by simply switching to the solvent A channel, elution with solvent A is effected for the remainder of the run. The column is then again equilibrated with solvent B and ready for another run.

Extraction of vitamin B_6 metabolites

Vitamin metabolites are extracted using perchloric acid as described previously [8,12]. The sample is neutralized to pH 4.2 with 3 *M* potassium hydroxide, allowed to stand overnight in the cold and afterwards filtered through Whatman No. 1 filter paper. The filtrate is cleared by centrifugation at 25,000 *g* for 16 h and 4°C and (if desired) chromatographed on a Dowex H^+ column [8,12]. Material isolated from this column is further chromatographed, if desired, using HPLC and ion-pairing as described in this report. If necessary, the sample could be concentrated by freeze drying prior to HPLC, however. Albeit, labeled vitamers can directly be chromatographed by HPLC after acid extraction.

RESULTS AND DISCUSSION

Fig. 1 is a typical chromatogram showing the separation of seven vitamin B_6 metabolites and of 3-hydroxypyridine used here as a marker. The order of elution and retention time was as follows: PNP, 3.7 min; PLP, 5.0 min; PIC, 6.7 min; pyridoxamine-5′-phosphate (PMP), 9.8 min; PL, 21.3 min; 3-hydroxypyridine (marker), 24.4 min; PN, 28.8 min; PM, 38.8 min. The entire run requires less than 40 min and can further be shortened by increasing, after the elution of PMP or of PL (a) the flow-rate, (b) the concentration of 2-propanol in solvent A, or both.

Fig. 2 shows linear dose—response graphs for all the vitamin B_6 vitamers including PIC. A 313-nm filter was used to record the absorbance of the metabolites. PIC does not absorb well at 254 nm and requires large amounts for detection. However, at the higher concentrations the PIC peak spreads and interferes with the separation of the phosphorylated forms (PLP and PMP). On the other hand, although PN and PNP themselves do not absorb well at 313 nm and do require large amounts for detection, they nevertheless have non-spreading, sharp peaks which do not interfere with the separation of the other metabolites (Fig. 1).

The advantage of the present method over other procedures is (a) its simplicity, (b) the relatively short time required for each run (<40 min), (c) the

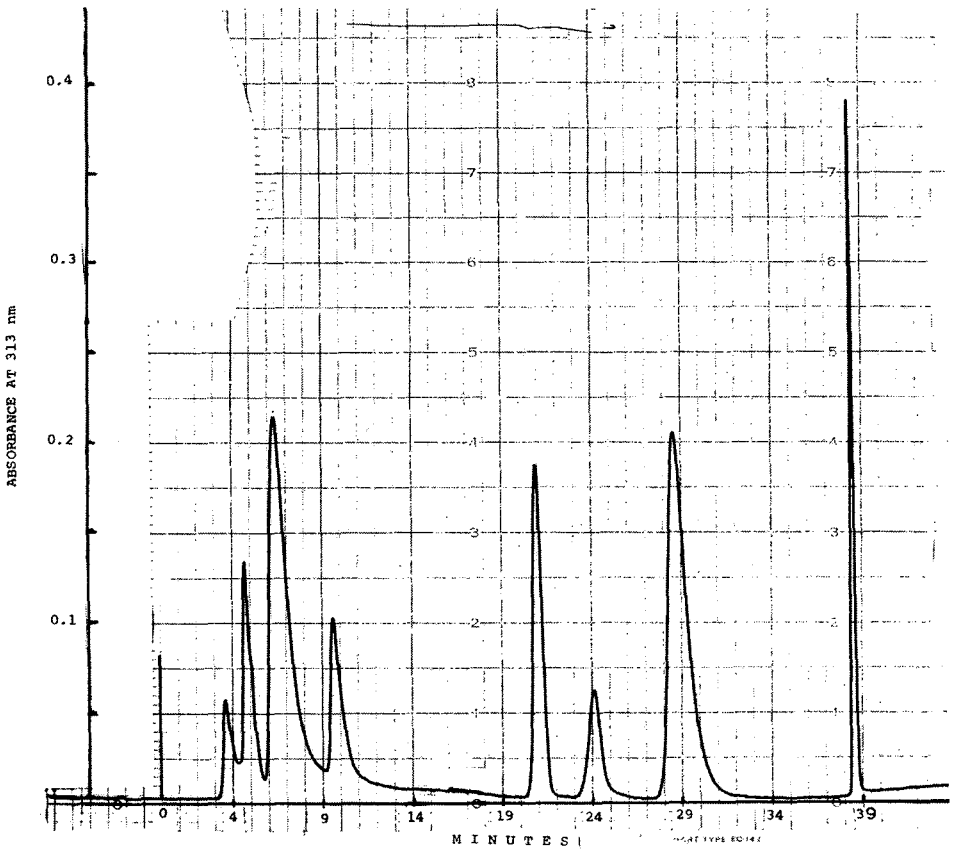

Fig. 1. Separation of vitamin B_6 metabolites and 4-pyridoxic acid by ion-pairing HPLC. Injection time is indicated at time ○. Chart speed was 0.5 cm/min. The order of elution and the retention times were: PNP, 3.7 min; PLP, 5.0 min; PIC, 6.7 min; PMP, 9.8 min; PL, 21.3 min; 3-hydroxypyridine, 24.4 min; PN, 28.8 min; PM, 38.8 min. The amounts of each standard (in the mixture) injected, were: PNP, ca. 6000 ng; PLP, 396 ng; PMP, 400 ng; PIC, 400 ng; 3-hydroxypyridine, 1200 ng; PN, 3000 ng; PL, 400 ng; PM, 140 ng. A typical chromatographic run is shown.

absence of buffers and of salt interference with subsequent metabolite identification, and (d) the ease of separation and subsequent recovery of the metabolites from the alkyl sulfonate counter-ion. As mentioned above, the actual run can also be further shortened. In addition, it would seem feasible that the separation of the vitamers could be quantitated using similar equipment employed by other research workers [6,7] which at this time is not available to us.

As our research endeavor is presently oriented to studying the metabolic transformations of labeled pyridoxine in tumor cells by (subsequent) detection of labeled metabolites attempts to quantitate the vitamers in tissues were not made. Albeit, the synthesis of PIC from labeled pyridoxine precursor by tumor cells [12,14] was recently verified using ion-pairing HPLC. A homogenate was prepared from minimum deviation transplantable Morris hepatoma No. 7777 in 0.05 *M* ammonium formate, pH 7.5 buffer. Samples

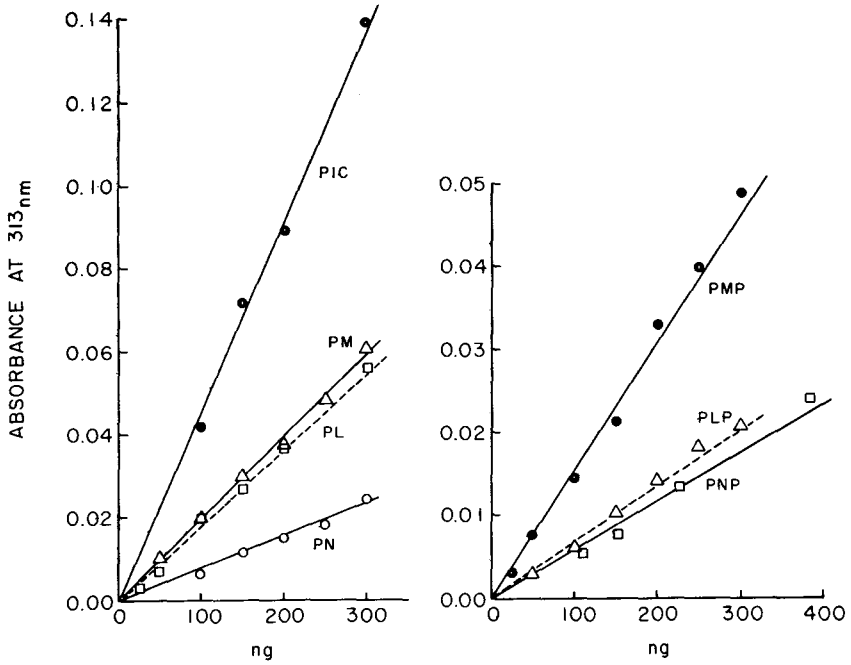

Fig. 2. Typical dose—response curves of PN, PL, PM, PIC, and of the phosphorylated vitamin forms PMP, PLP and PNP. The ordinate (graph on the right) has been expanded. All curves are representative showing a linear relationship between dose injected and absorbance at 313 nm.

were incubated (37°C, 60 min) with shaking in the presence of pyridoxine hydrochloride labeled with tritium at carbon atom No. 6 (Amersham, Arlington Heights, IL, U.S.A.). Vitamin B_6 metabolites were acid extracted [8,12] and chromatographed on Dowex H^+ [12]. Positive identification of PIC was however not possible because of peak overlapping with phosphorylated vitamin forms. Therefore, tubes containing radioactivity were concentrated by freeze-drying and subsequently analyzed by HPLC, as described in this report. Absorbance was continuously monitored at 313 nm. Eluted fractions were collected every 30 sec. Radioactivity was measured in a Tri-Carb liquid scintillation spectrometer, as described previously [12,14]. Fig. 3 demonstrates the positive identification of 4-pyridoxic acid produced by the tumor homogenate (from the labeled pyridoxine precursor) as the radioactivity closely follows the absorbance of the authentic standard. In this instance, the results demonstrate the existence of enzymes in these tumor cells able to effectively utilize pyridoxine subsequently degrading to PIC. Although PIC has been identified as a catabolic vitamin product in rats [10], its presence and importance in hepatoma cells (Fig. 3) to our knowledge has not been reported or assessed.

The HPLC separation technique utilizing heptane- and octanesulfonates as the counter-ions has been successfully employed in studies on vitamin B_6 metabolic transformations by tumor cells [15]. Radioactively labeled precursor was administered to tumor bearing rats and subsequently the vitamers were extracted and separated as described in the Experimental section. The sen-

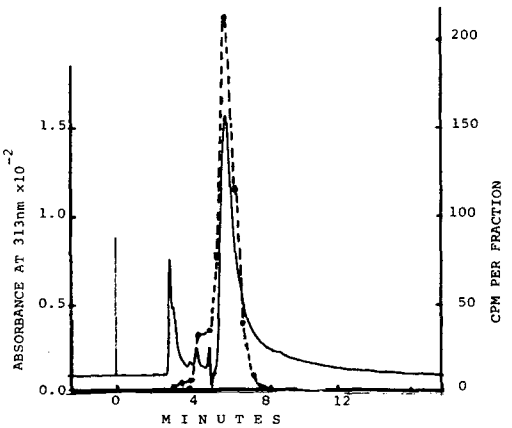

Fig. 3. Identification of radioactively labeled 4-pyridoxic acid (PIC) by ion-pairing HPLC. The radioactivity of the vitamer extracted from the tumor tissue coincides with the absorbance of the authentic PIC sample.

sitivity of the method is indeed extremely high as it is quite possible to detect and measure labeled metabolites even when the counts per minute are quite low. Since radioactivity can be derived only from the administered labeled precursor the method is in addition quite precise especially when the optical density of the unknown labeled vitamin B_6 metabolite coincides with that of the authentic standard sample (i.e. Fig. 3).

ACKNOWLEDGEMENTS

This work was supported by grant 1R01 CA 28140 awarded by the National Cancer Institute, DHEW. Our thanks are due to Dr. Harold P. Morris for a tumor donor animal given to us gratis and for his continued interest and support.

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Note

Determination of deacetylmetipranolol in body fluids by gas chromatography—chemical-ionization mass spectrometry

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(First received January 27th, 1981; revised manuscript received July 28th, 1981)

Metipranolol [1-4(acetoxy-2,3,5-trimethyl-phenoxy)-3-isopropyl-amino-propan-2-ol, Fig. 1a] is a β-adrenoreceptor blocking drug, known under the name Disorat® or Torrat® (Boehringer-Mannheim, Mannheim, G.F.R.) and is used as an antihypertensive agent. For therapeutic purposes it is usually administered twice a day in 10- or 20-mg oral doses. For pharmacokinetic studies a single dose of 40 mg is given. Metipranolol is very rapidly metabolized to the pharmacologically active metabolite deacetylmetipranolol (DMP). A few minutes after the oral application the metabolite is detected in serum and urine. Metipranolol could not be identified in biological fluids.

A detailed study concerning the pharmacokinetics and pharmacology of metipranolol (MP) in animals and men has been published [1—3]. β-Blocking drugs can be determined specifically with high sensitivity after derivatization with trifluoroacetic anhydride (TFAA) or heptafluorobutyric anhydride by gas chromatography, using an electron-capture detector [4—6]. A very recent publication [7] discusses the determination of biological levels of β-blocking agents using high-performance liquid chromatography.

For the determination of DMP in our laboratory, first a gas chromatographic method was developed on the basis of methods described in the literature [4—6]. This procedure worked well with pure substances. The analysis of DMP in biological materials, however, showed interfering peaks on the chromatogram which made a reliable quantitative measurement of the metipranolol metabolite impossible. Due to the high biological background a gas chromatographic methane chemical-ionization mass spectrometric method was developed in our laboratory. Chemical-ionization mass spectrometry was chosen for its ability to produce stable high-mass molecular ions of greater relative intensities

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than did electron-impact mass spectrometry, allowing the highly specific measurement of DMP with excellent sensitivity.

PRINCIPLE OF ASSAY

To serum, or urine, containing DMP a certain amount of 1,4(acetoxy-2,3,5-trimethylphenoxy)-3-D_6-isopropyl-amino-propan-2-ol (Fig. 1b) as internal standard was added. After extraction under alkaline conditions (pH $\geqslant$ 10) the organic phase is evaporated to dryness. The residue is derivatized with TFAA to give tris-trifluoroacetyl-D_0-DMP [$(TFA)_3$-D_0-DMP] and its hexadeutero analogue [$(TFA)_3$-D_6-DMP], respectively. DMP reacts in positions 1, 2 and 3.

After injection into the gas chromatograph, the mass spectrometer monitors the quasimolecular ions of $(TFA)_3$-D_0-DMP at *m/z* 556 and $(TFA)_3$-D_6-DMP at *m/z* 562 in the multiple ion detection mode. Methane is used both as the carrier gas and as the chemical ionization reactant gas.

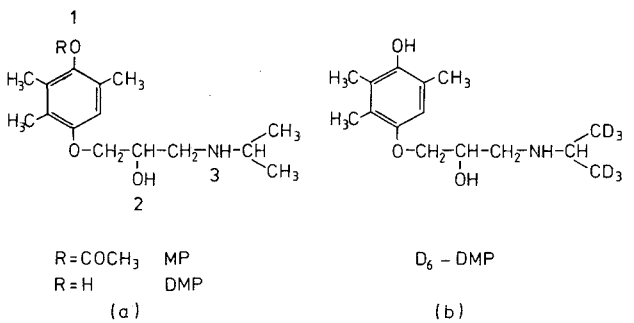

Fig. 1. Structural formulae of (a) metipranolol (MP) and deacetylmetipranolol (DMP) and (b) the internal standard.

EXPERIMENTAL

Materials

DMP and the internal standard D_6-DMP were synthesized in the research laboratories of Boehringer Mannheim and were of analytical grade. The content of D_0-DMP in D_6-DMP was $< 0.01\%$ and did not influence the assay. The solvents (Merck, Darmstadt, G.F.R.) were of analytical grade and were used without further purification. TFAA was purchased from Macherey, Nagel & Co. (Düren, G.F.R.). A solution of the ethyl ester of gallic acid in methanol (100 mg/l) was applied prophylactically to prevent autoxidation during the work-up procedure.

Gas chromatography

A Hewlett-Packard Model 5710A gas chromatograph in conjunction with a Hewlett-Packard autosampler, Model 7671 (36 samples), was used. The autosampler was controlled by a Finnigan autosequencer box and a home-built interface, which enables communication between the two devices. The Finnigan autosequencer box switches the vacuum diverter valve, the filament and the electron multiplier. The total run time for one sample was 6 min.

A glass column (180 cm × 2 mm I.D.) was packed with 3% SE-30 on Chromosorb W HP. The column was conditioned at 320°C overnight. The flow-rate of the carrier gas was adjusted to 20 ml/min. The temperature settings were 250°C for the injector and 210°C for the oven. Under these conditions $(TFA)_3$-DMP eluted with a retention time of approximately 3 min.

Interface

The gas chromatographic column was coupled via a stainless-steel needle valve to the mass spectrometer. The transfer line (glass-lined tubing) was held at a temperature of 250°C.

Mass spectrometer

A Finnigan 3300 quadrupole mass spectrometer in combination with a Finnigan Model 6100 data system was used. The instrument was run in the chemical-ionization mode with methane as reagent gas. The source pressure was maintained at 1 Torr, the electron energy at 100 eV. The emission current was 30 μA.

Extraction conditions

In order to obtain a high extraction efficiency of DMP from serum or urine a series of solvents under various pH conditons was investigated. It was found that a pH value of approximately 10 and rather polar solvents were necessary to extract DMP to an acceptable extent. In addition to that, a saturated sodium chloride solution was added to the biological fluid to enhance the recovery.

Work-up procedure

Into a 50-ml separation funnel (separation funnels were used instead of centrifuge tubes to prevent formation of emulsions) are pipetted 0.5 ml of 0.2 *M* sodium hydroxide, 1 ml of a saturated solution of sodium chloride, 15 μl of antioxidant solution, 4 ml of water, 10 ml of diethyl ether, 0.2 ml of an aqueous solution containing 0.1 mg/l D_6-DMP, 1 ml of serum (urine is diluted 1:5), and 2.5 ml of dichloromethane. The funnel is manually shaken for 1 min. After phase separation the aqueous phase is discarded. The organic phase is collected in a 10-ml centrifuge tube; 8 ml of the organic phase are transferred into another tube and evaporated to dryness under a stream of nitrogen. The residue is dissolved in 150 μl of diethyl ether, transferred into a 300-μl capacity conical vial and evaporated to dryness. After adding 25 μl of acetonitrile and 50 μl of TFAA the bottle is capped and heated for 30 min at 50°C in a metal block. After complete evaporation the sample is taken up in 50 μl of acetonitrile; 5 μl are injected into the chromatograph. The sample solutions in acetonitrile proved to be stable for at least one week.

Evaluation and calculation

Evaluation of the peak areas by electronic integration was either carried out by the Finnigan 6100 data system or by the Hewlett-Packard Lab Data System HP 3354. For calculation of the drug concentration a calibration curve was established together with each series of biological samples, using the following concentrations: serum, 100, 50, and 10 ng/ml; urine, 500, 100, 50, and 10

ng/ml. The plot of area ratios D_0/D_6-DMP against concentrations is linear in the range 2—500 ng/ml.

As an example the linear regression of a typical calibration is given below:

$$C_{D_0\text{-DMP}} = 10{,}164 \cdot A_{rel} - 0.145$$

with $r = 0.99998$.

Equations of this type were used to calculate the serum concentration of DMP (D_0-DMP) as a function of the measured relative area

$$A_{rel} = \frac{A_{D_0\text{-DMP}}}{A_{D_6\text{-DMP}}}$$

RESULTS

The chemical-ionization mass spectrum of $(TFA)_3$-D_0-DMP is shown in Fig. 2. The base peak at *m/z* 442 formed after the elimination of one molecule of trifluoroacetic acid from the quasimolecular ion is shifted in the spectrum of the internal standard by six mass units to higher mass. The ions chosen for the quantitative determination of DMP were the quasimolecular ions at *m/z* 556 and *m/z* 562 for the internal standard.

A mass chromatogram obtained from human plasma containing the internal standard in the amount of 20 ng/ml is shown in Fig. 3a. In none of the investigated blank serum samples were interfering peaks observed. An ion-

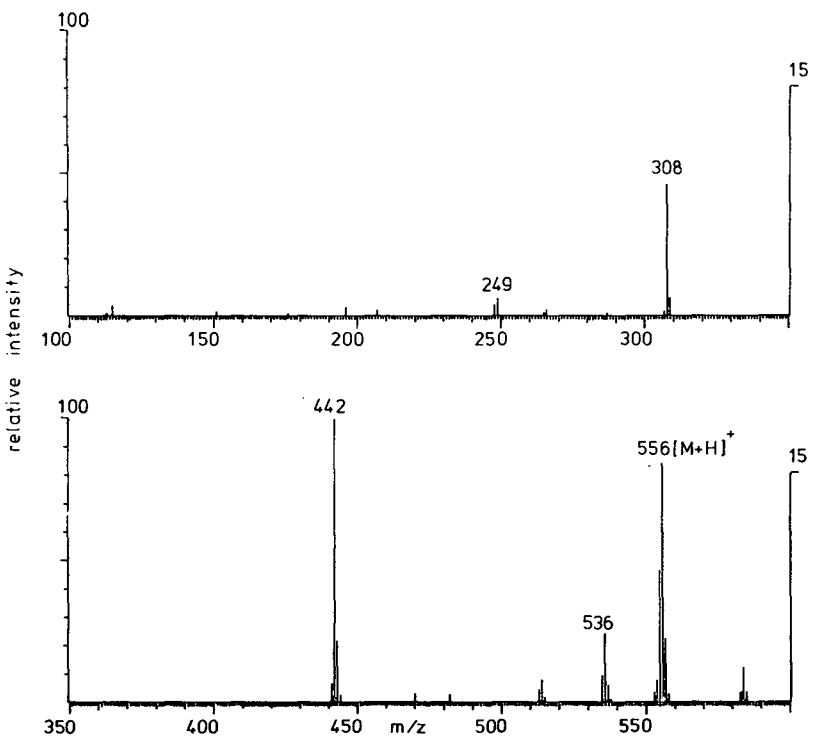

Fig. 2. Chemical-ionization mass spectrum of the TFA derivative of D_0-DMP.

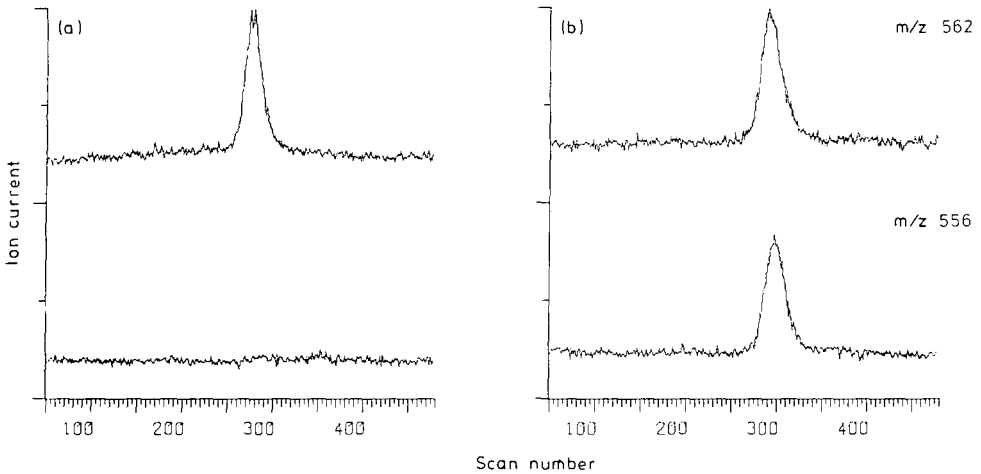

Fig. 3. Selected ion monitoring of derivatized serum extract, recording D_0-DMP (m/z = 556) and D_6-DMP (m/z = 562). (a) Blank spiked with 20 ng/ml D_6-DMP; (b) containing 10 ng/ml D_0-DMP and 20 ng ml D_6-DMP.

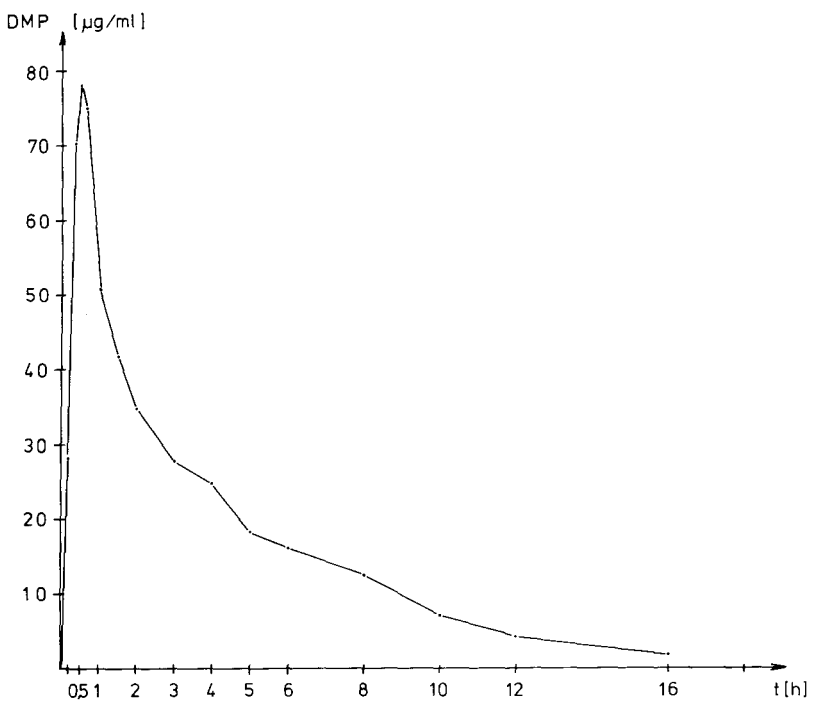

Fig. 4. Serum concentration—time curve of DMP after an oral dose of 40 ng of MP to a healthy volunteer.

monitor recording of a 10 ng/ml DMP human plasma sample spiked with 20 ng/ml D_6-DMP is shown in Fig. 3b.

The detection limit was found to be in the 1—2 ng range using 1-ml sample volumes. In this range the quantitative measurements were still reliable enough

for pharmacokinetic studies. The development of a method to measure concentrations in the range below 1 ng/ml, which seems to be necessary for further kinetic studies applying different mass spectrometric techniques, is proceeding in our laboratories.

An example of a pharmacokinetic profile of DMP after application of an oral dose of 40 mg of metipranolol to a healthy volunteer is presented in Fig. 4. The peak plasma concentration was 78 ng/ml 0.6 h after application.

The assay was routinely applied for more than two years and some thousands of biological samples were analyzed with good results. Under these conditions the average life-time of a column is between 4 and 6 weeks. Cleaning of the ion source was necessary in the same time interval.

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The authors wish to thank Dr. W. Friebe for the synthesis of D_6-DMP. The technical assistance of Mr. J. Klaus is gratefully acknowledged.

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Note

Determination of ethacrynic and tienilic acid in plasma by gas—liquid chromatography—mass spectrometry

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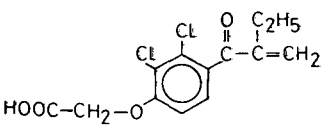
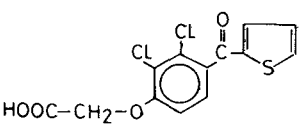
In 1962, Schultz et al. [1] reported that various unsaturated ketone derivatives of aryloxyacetic acids had diuretic activity. Today ethacrynic acid (Fig. 1) is one of the most potent diuretics next to furosemide [2]. Both



ethacrynic acid



tienilic acid

Fig. 1. Phenoxyacetic acids with diuretic activity.

have a very rapid onset of action, with diuresis beginning 20—60 min after oral administration. The duration of action is relatively short.

More recent investigations of Cragoe and Woltersdorf [3] have shown that the importance of the enone structure for SH-group binding capacity has been overestimated; dihydroethacrynic acid also possesses diuretic activity. Another phenoxyacetic acid derivative with diuretic effects is tienilic acid (2,3-dichloro-4-(2-thenoyl)phenoxyacetic acid (Fig. 1). Tienilic acid has a longer duration of action than ethacrynic acid but is less potent. The uricosuric effect of the mercurial diuretics absent in the case of ethacrynic acid is regained with tienilic acid [4, 5].

The major contra-indication of ethacrynic acid is hypertension, but in

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January 1980 the preparation Selacryn was withdrawn from the market on suspicion of causing liver damage. The Indanones MK-196 and MK-473 (Merck Sharp and Dohme) also possess diuretic and uricosuric effects and show very similar pharmacological properties.

Ethacrynic acid has a low rate of biotransformation and only limited amounts are excreted in the form of unstable glutathione, cysteine and acetylcysteine conjugates [6]. Tienilic acid has also a low rate of biotransformation. After oral administration the maximal plasma concentration of the unchanged drug is over twelve times higher than that of the major metabolite, the alcohol arising from reduction of the C=O group [7]. Oxidation of the thienylcarbonyl function to the corresponding acid, dichlorocarboxyphenoxyacetic acid, occurs to only a limited extent [8].

Except for a report using labelled drug no technique has been described for the quantitative estimation of ethacrynic acid in plasma [6]. Desager et al. [9] and Hwang et al. [7] measured tienilic acid by gas chromatography after extraction from plasma and reaction with diazomethane. A similar method for the quantitative determination of MK-196 has been described by Zacchei and Wishousky [10].

The aim of the present study was to develop a specific and sensitive technique for the estimation of phenoxyacetic acids using gas chromatography in conjunction with mass spectrometry.

MATERIALS AND METHODS

Extraction of ethacrynic acid and tienilic acid from plasma

A 1-ml volume of plasma was mixed with a methanolic solution of the sample containing 0.01—0.10 μg of substance, then the internal standard was added (1 μg of ethacrynic acid, or tienilic acid). After addition of 1 ml of 3 *N* hydrochloric acid and 5.0 ml of diethyl ether, the sample was subjected to a 3-min extraction (mechanical shaker, 250 l/min) and then centrifuged for 5 min at 1950 *g*. Four milliliters of the organic phase were evaporated to dryness. The residue was then subjected to derivatisation. For measurement of recovery, various concentrations of the substance were added to plasma (the internal standard was added immediately before derivatisation). The reproducibility of the technique was tested by repeated estimations in the concentration range 0.05—5 μg/ml of plasma.

Derivatisation with pentafluorobenzyl bromide

After evaporation of the ethereal plasma extract, the residue was mixed with 2 ml of a 2% solution of pentafluorobenzyl bromide (EGA Chemie, Steinheim, G.F.R.) in acetonitrile and approximately 10 mg of anhydrous potassium carbonate. After 45 min reaction at 70°C, the solution was evaporated to dryness in vacuo and the residue taken up in 50 μl of acetonitrile. After ultrasonic irradiation 5 μl of this solution were subjected to gas chromatography.

Gas chromatography—mass spectrometry

A Varian gas chromatograph Model 3700 coupled to a MAT 44 mass spec-

trometer was used. The column was glass (2 m × 2 mm) packed with 1% OV-17 on Chromosorb W, 80—100 mesh. The conditions of measurement were: programmed temperature operation from 200 to 300°C at 30°C/min; carrier gas, helium at a flow-rate of about 30 ml/min; injection port temperature, 300°C; line temperature, 300°C; ion source temperature, 250°C; Electron energy, 80 eV (electron-impact mode), 160 eV (chemical-ionisation mode); emission current, 0.8 mA. The investigations using chemical ionisation were carried out using isobutane as reaction gas (purity 99.5%; Messer, Griesheim, G.F.R.) at a pressure of between 330 and 340 μbar.

RESULTS

Derivatisation with pentafluorobenzyl bromide

For derivatisation with pentafluorobenzyl bromide, a reaction temperature of 70°C and reaction time of 45 min were found to give the best results. This confirms the data given by other workers from similar studies [11]. The derivatives were stable for at least 24 h. Using a 2% solution of reagent in acetonitrile, evaporation of the poorly volatile excess pentafluorobenzyl bromide in vacuo was possible.

Gas chromatography

One per cent OV-17 as stationary phase was found to be the most suitable with regard to shape of the peaks and sharpness of separation. Hwang et al. [7] and Desager et al. [9] used OV-17 or OV-225 as stationary phase for the measurement of the methyl derivative of tienilic acid.

Mass spectrometry

In the electron-impact mode, detection of the molecular ion peak was possible for both substances; the relative intensity with the ethacrynic acid derivative was under 1%. A high intensity of *m/e* 180 is due to the reagent. With quantitative measurement in the lower mass regions, interference is likely, especially after plasma extraction. However, using chemical ionisation, the molecular ion peaks became the base peaks. Little, if any, fragmentation occurred (see Figs. 2 and 3). Therefore quantitative measurement could be carried out in the mass region > 480.

Adjustment to constant reaction gas pressure is particularly important with chemical ionisation, since the decrease or increase in sensitivity brought about by a change in reaction gas pressure is not the same for all substances tested. Fig. 4 illustrates the relatively higher sensitivity to variations in pressure shown by ethacrynic acid compared to tienilic acid.

Quantitative estimation of ethacrynic acid and tienilic acid in plasma

After ether extraction the rate of recovery of ethacrynic acid and tienilic acid was 94.5 ± 2.9% and 95 ± 4.2%, respectively. A 3-min extraction achieved an almost total recovery with separation of the ether-extractable impurities. Both Hwang et al. [7] and Desager et al. [9] used the same solvent, but their respective extraction times of 15 min and 3 sec differed considerably. Recovery rates of 98% and 95% were reported.

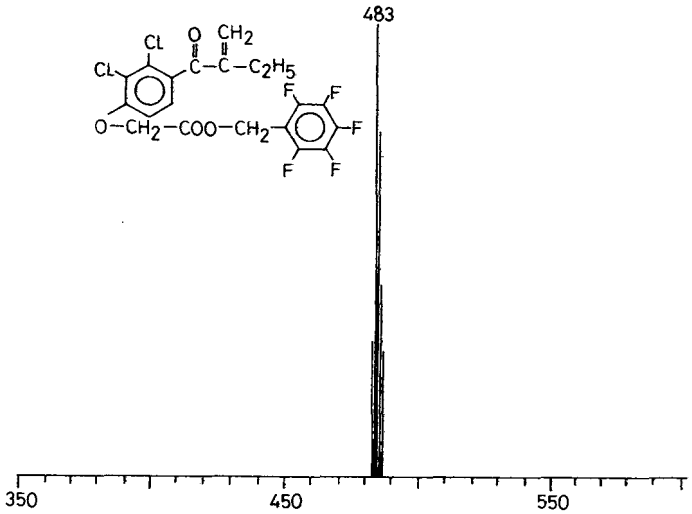

Fig. 2. Mass spectrum of ethacrynic acid after derivatisation with pentafluorobenzyl bromide and gas chromatography (chemical-ionisation mode).

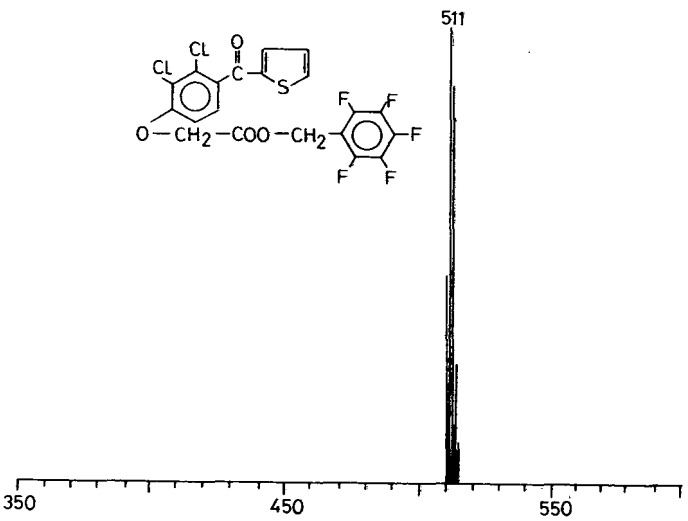

Fig. 3. Mass spectrum of tienilic acid after derivatisation with pentafluorobenzyl bromide and gas chromatography (chemical-ionisation mode).

Linear regression of the calibration graph gave a value of 0.998; the mean standard deviation of the method for measurement in the range 0.05—1 μg/ml plasma amounted to ± 5%. The limit of detection was between 10 and 20 ng/ml of plasma.

Fig. 5 shows a typical gas chromatogram of a spiked plasma sample after derivatisation with pentafluorobenzyl bromide (1 μg/ml tienilic acid and 0.5 μg/ml ethacrynic acid). The quantitative estimation was made by the molecular ions and base peaks, *m/e* 511 and *m/e* 483.

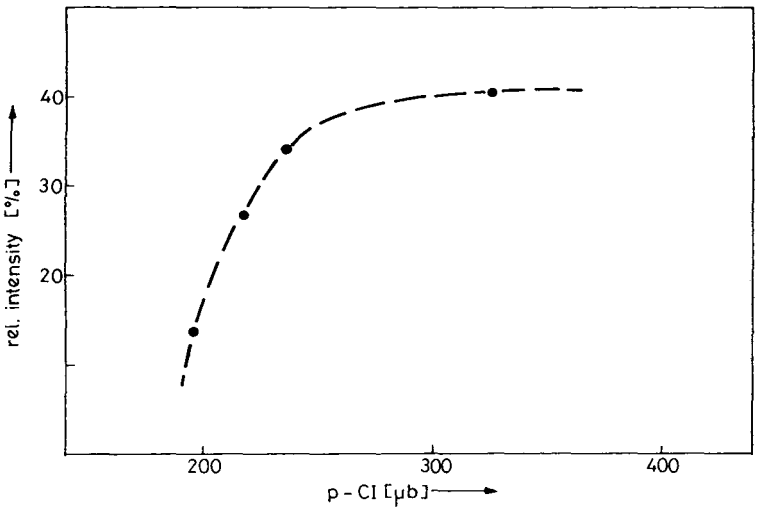

Fig. 4. Influence of reaction gas pressure (in µbar) on the relative intensity of the ethacrynic acid peak (1 µg of tienilic acid vs. 500 ng of ethacrynic acid).

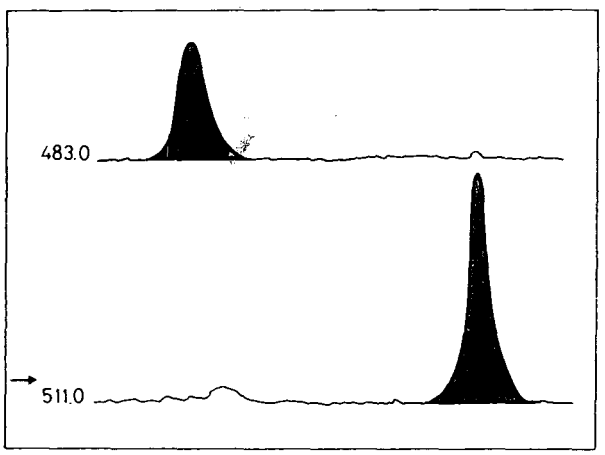

Fig. 5. Quantitative estimation of ethacrynic acid and tienilic acid in plasma.

DISCUSSION

A method has been developed for the gas chromatographic—mass spectrometric measurement of ethacrynic acid and tienilic acid, after derivatisation with pentafluorobenzyl bromide, which can be applied to further phenoxyacetic acids such as MK-196 and MK-473 (Merck Sharp and Dohme). Various reports have appeared in the literature concerning the use of pentafluorobenzyl bromide for derivatisation of organic acids [11—15]. Due to the high reactivity of pentafluorobenzyl bromide, interference can occur through acid impurities, especially from biological material [15]. Greving et al. [14] reported interference in the gas chromatographic measurement by fatty acids, especially palmitic acid. Using chemical ionisation with isobutane, fragmentation of substances during mass spectrometry can be avoided and an inter-

ference-free evaluation of the molecular ion peaks in the mass region > 480 is possible. The practical importance of the technique was demonstrated by the quantitative estimation of ethacrynic acid and tienilic acid from plasma. Measurements of plasma concentrations in the ng/ml and μg/ml range were possible.

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Note

Assay of flunitrazepam, temazepam and desalkylflurazepam in plasma by capillary gas chromatography with electron-capture detection

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Flurazepam, flunitrazepam, and temazepam are benzodiazepine derivatives which are used either as hypnotics, tranquillizers, or in anaesthesia (Fig. 1).

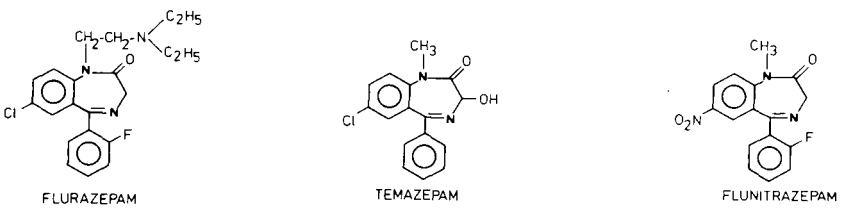

Fig. 1. Structure of flurazepam, temazepam and flunitrazepam.

Flurazepam is very rapidly metabolized in man and only trace amounts of unchanged drug can be measured in plasma [1]. Its major metabolite is the N-desalkyl derivative, which is slowly eliminated from the body and which has psychopharmacological activity similar to that found for flurazepam. This metabolite is probably largely responsible for the hypnotic and persistent sedative action of flurazepam. Temazepam has no active metabolites; flunitrazepam is active as such, but probably also has active metabolites [2]. These drugs have shorter elimination half-lives and are shorter-acting than flurazepam [3, 4].

As it was our aim to perform a comparative pharmacokinetic study in man of several benzodiazepines used as hypnotics (nitrazepam, flunitrazepam, temazepam, triazolam and flurazepam), assay methods had to be developed which were not only rapid but also sensitive enough to measure plasma concentrations for at least three times the elimination half-life of a particular compound after a single therapeutic dose.

Various gas chromatographic methods for the determination of flunitrazepam, desalkylflurazepam and temazepam in plasma have been described. These methods either determine the intact drug or the benzophenone derivative, which is obtained upon acidic hydrolysis of benzodiazepines [5—12]. This latter procedure, however, is not very suitable because it gives rise to serious loss of specificity. Recently high-performance liquid chromatographic procedures have been developed for the determination of benzodiazepines in plasma [13, 14]. All methods described, however, are generally rather time consuming and often do not reach the sensitivity needed for a pharmacokinetic study in man after a single therapeutic dose (sensitivity requirements for plasma are desalkylflurazepam 1 ng/ml, flunitrazepam 0.1 ng/ml, and temazepam 5 ng/ml).

Another disadvantage of previously published assay methods for benzodiazepines is the use of benzene for extraction from biological fluids. This solvent is very toxic and its use should be prohibited whenever possible.

Recently an assay method for the determination of underivatized nitrazepam was developed in our institute, using gas chromatography with a support-coated open tubular (SCOT) column, a solid injection system and electron-capture detection [15]. This method has proved to be sensitive, specific and rapid. With some modifications it could also be satisfactorily applied for triazolam and the details of that method are described elsewhere [16]. In this paper the application of a similar gas chromatographic method is presented for temazepam, flunitrazepam and desalkylflurazepam in plasma. Temazepam had to be converted into its O-trimethylsilyl derivative (TMS-temazepam) according to the method of Belvedere et al. [6], whereas flunitrazepam and desalkylflurazepam were determined as unchanged drugs.

EXPERIMENTAL

Drugs and chemicals

Flunitrazepam (Rohypnol, Hoffmann-La Roche), desalkylflurazepam, active metabolite of flurazepam (Dalmadorm, Hoffmann-La Roche), nordiazepam, metabolite of diazepam and diazepam (Valium, Hoffmann-La Roche) were kindly supplied by Hoffmann-La Roche (Basle, Switzerland); temazepam (Levanxol, Farmitalia; Normison, Wyeth) was obtained from Wyeth (Amsterdam, The Netherlands).

For the preparation of the standard solutions, ethanol (p.a. grade) (E. Merck, Darmstadt, G.F.R.) was used; the other organic solvents were freshly distilled (J.T. Baker, Deventer, The Netherlands). For the derivatization of temazepam, N,O-(trimethylsilyl)acetamide (BSA) (Chrompack, Middelburg, The Netherlands), was used.

Extraction procedure

To 1.0 ml of plasma in a centrifuge tube (in the case of temazepam, 0.10 ml of plasma and 0.90 ml of distilled water) were added 50.0 μl of ethanol containing a suitable internal standard (see Table I) and 1.0 ml of 0.2 *M* borate buffer (pH 9.0). After homogenization the sample was extracted twice with 5 ml of pentane—dichloromethane (1:1) for 15 sec on a whirlmixer. Follow-

ing centrifugation for 5 min at 2000 *g* the upper organic layer was transferred to a silanized conical evaporation tube and evaporated to dryness at 50°C under a gentle stream of dry nitrogen. In the case of temazepam, 50 μl of a 40% BSA solution in acetone were subsequently added and evaporated again to dryness (60°C). Finally, the residue was redissolved in 40 μl of ethyl acetate and 2—3 μl of this solution were brought on to the glass-lined needle of the solid injection system [15]. After evaporation of the ethyl acetate, the residue was injected into the gas chromatograph.

Apparatus and chromatographic conditions

A Hewlett-Packard Model 5713A gas chromatograph, equipped with a ^{63}Ni pulse-modified electron-capture detector and a solid injection system was used. A SCOT column (10 m × 0.4 mm I.D.) made of Duran 50 glass was used. The support layer was Tullanox (silanized fumed silica), particle size < 10 μm (Cabot Corp., Boston, MA, U.S.A.), and the stationary phase was 0.5% PPE-21 (Chrompack) and 3% OV-17 (Chrompack). The operating temperatures were injection port 350°C, detector 350°C, and column 215°C. The flow-rate of the carrier gas (helium) was 10 ml/min and that of the auxiliary gas (argon—methane, 95:5) 25 ml/min.

Preparation of calibration graphs

The plasma concentrations of the three benzodiazepines were calculated with the aid of calibration curves, prepared by adding known amounts of the drugs to 1.0 ml (in the case of temazepam, 0.10 ml + 0.90 ml distilled water) of blank plasma. These standard samples were analysed by the described procedure and the ratio of the peak height of the various drugs to internal standard was plotted against the known concentrations of the drugs. The same procedure was followed for the determination of the extraction yield of the three benzodiazepines from plasma at various concentrations except that the compounds used as internal standards (Table I) were now added after extraction (external standards). The ratios obtained were compared with those of standard amounts of the drugs.

TABLE I

INTERNAL STANDARD (I.S.), RETENTION TIMES, STANDARD DEVIATIONS OF CALIBRATION GRAPH AND THE LOWEST MEASURABLE CONCENTRATION

Compound	I.S. (plasma conc.)	Retention time (min)		S.D. of calibration graph (%)	Lowest measurable plasma concentration
		Compound	I.S.		
Desalkylflurazepam	Nordiazepam (25.0 ng/ml)	1.5	1.7	⩽ 2.5	1 ng/ml
TMS-temazepam	Diazepam (25.0 ng per 0.10 ml)	1.7	1.3	⩽ 12.0	0.25 ng per 0.10 ml
Flunitrazepam	Nordiazepam (5.0 ng/ml)	2.2	1.7	⩽ 9.9	0.05 ng/ml

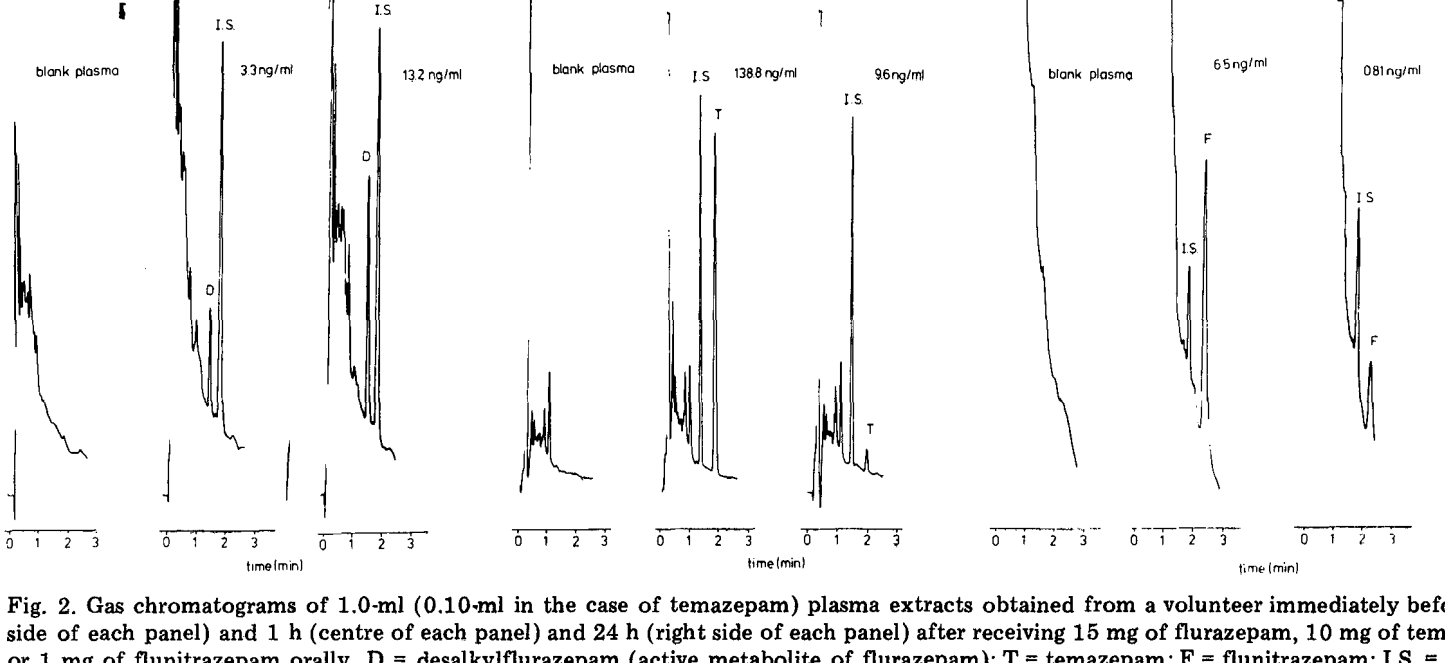

Fig. 2. Gas chromatograms of 1.0-ml (0.10-ml in the case of temazepam) plasma extracts obtained from a volunteer immediately befo side of each panel) and 1 h (centre of each panel) and 24 h (right side of each panel) after receiving 15 mg of flurazepam, 10 mg of tem or 1 mg of flunitrazepam orally. D = desalkylflurazepam (active metabolite of flurazepam); T = temazepam; F = flunitrazepam; I.S. = standard

RESULTS AND DISCUSSION

Assay procedure

Fig. 2 shows gas chromatograms of extracts of plasma samples taken 1 and 24 h after oral ingestion of 15 mg of flurazepam, 10 mg of temazepam and 1 mg of flunitrazepam, as well as gas chromatograms of the corresponding blank extracts. Diazepam was chosen as internal standard for temazepam assay; for both desalkylflurazepam and flunitrazepam, nordiazepam proved to be the most suitable internal standard. Other benzodiazepines were deliberately chosen as internal standards because of the similarity in chemical structure, extraction and chromatographic behaviour. There is a good separation between drug and internal standard, and from the blank chromatograms it appears that no interfering substances are co-extracted.

According to the standard curves there is good linearity between the detector response (peak height drug/peak height internal standard) and the concentration of the compound to be determined in the following ranges: desalkylflurazepam 3.75—25 ng/ml, temazepam 1—25 ng/0.10 ml, and flunitrazepam 0.1—10 ng/ml. The correlation coefficient of such curves was not less than 0.999. Extraction yields determined in the same concentration ranges appeared to be constant and linear with concentration with mean values as follows: desalkylflurazepam 93% (relative standard deviation at each concentration was 6% or less, $n = 4$); temazepam 65% (relative standard deviation at each concentration was 18% or less, $n = 4$) and flunitrazepam 95% (relative standard deviation at each concentration was 12% or less, $n = 4$).

Fig. 3 shows the plasma calibration graphs for the three compounds. For all three benzodiazepines the correlation coeffiecient of each individual curve was greater than 0.999, illustrating the good linearity of the method for each of them. The highest value of the standard deviation observed at each concentration as well as the lowest measurable concentration are given in Table I.

Human experiments

The utility of the present method for the assay of desalkylflurazepam, temazepam and flunitrazepam after a single therapeutic dose was demonstrated in a preliminary study with healthy volunteers. Three healthy female volunteers swallowed 1 mg of flunitrazepam, 15 mg of flurazepam or 10 mg of temazepam with 150 ml of tap water after an overnight fast. The drugs were administered in a cross-over design with intervals of at least three weeks. No food, fluid, or tobacco was allowed for 3 h after drug administration. Blood samples were taken 20, 40, 60, 80 and 100 min and 2, 3, 4, 9, 24, and 48 h after drug administration, for all three drugs, from a forearm vein by means of a flexible venous canula for the first 4 h and subsequently by venous puncture. Additional samples were taken after 6, 12 and 32 h for temazepam, after 6, 32 and 72 h for flunitrazepam, and after 96 and 168 h for flurazepam. Blood clotting was prevented by adding a small drop of heparin solution (5000 I.U./ml) to the samples. After separation, the plasma samples were stored at −20°C until taken for analysis. The plasma concentration profile obtained for one volunteer is shown in Fig. 4. Desalkylflurazepam was already detectable in the first sample taken after flurazepam administration and the concentra-

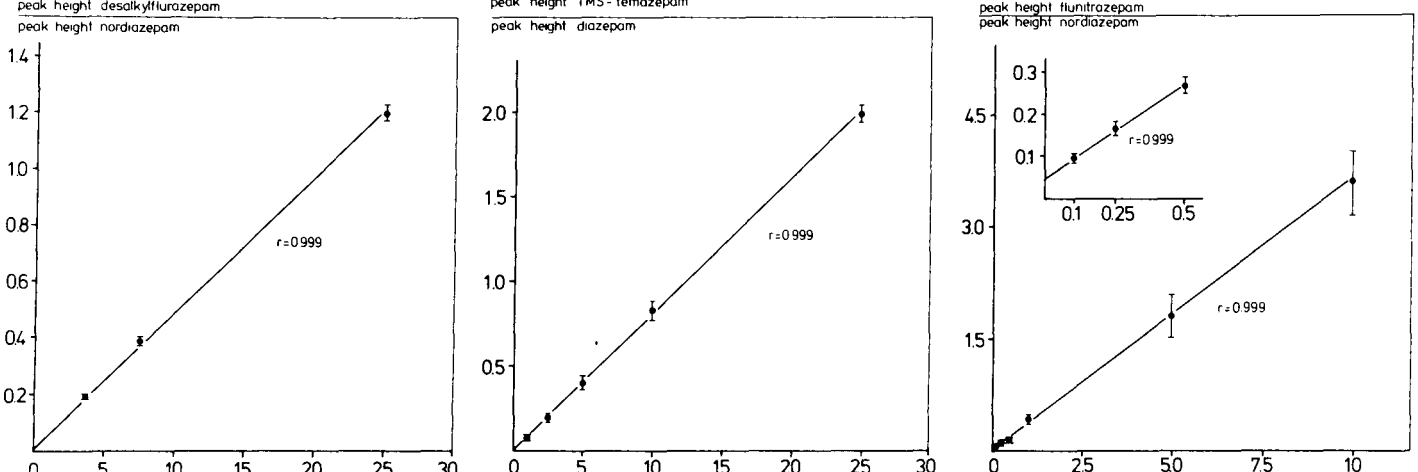

Fig. 3. Calibration graphs of (left) desalkylflurazepam in plasma in the concentration range 3.75—25 ng/ml, (centre) TMS-temazepam in in the concentration range 1—25 ng temazepam per 0.10 ml, and (right) flunitrazepam in the concentration range 0.1—10 ng/ml. Each represents the mean ± S.D. of four observations. In the case of flunitrazepam, the inset indicates, on an expanded scale, the relations tween the detector response and flunitrazepam at low concentrations of flunitrazepam.

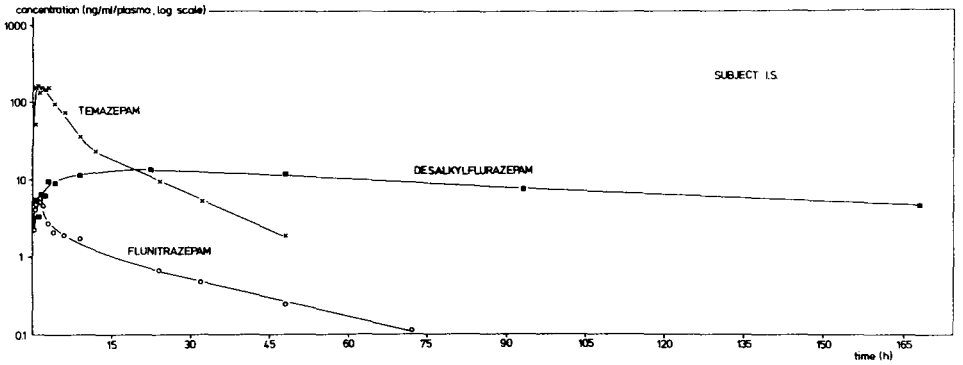

Fig. 4. Plasma level profile on semi-logarithmic scale following oral administration of temazepam (10 mg), desalkylflurazepam (15 mg flurazepam) and flunitrazepam (1 mg) in a healthy volunteer. The drugs were given at intervals of at least 3 weeks.

tion continued to increase slowly until 9—24 h after ingestion. The half-life of desalkylflurazepam was found to be very long (73—97 h) which is in agreement with the values found by Kaplan et al. [1]. Flunitrazepam and temazepam were fairly rapidly absorbed with a mean t_{max} of 1.0 h. After the peak concentration had been reached, there was a rapid decrease in concentration of both drugs, most probably due to distribution of the compounds from plasma into tissues. From the subsequent mono-exponential concentration decay the elimination half-lives could be derived; for temazepam this was 10—21 h and for flunitrazepam 18—49 h (Table II).

TABLE II

PHARMACOKINETIC PARAMETERS* OF DESALKYLFLURAZEPAM (DAF), TEMAZEPAM (Te) AND FLUNITRAZEPAM (FN) FOLLOWING ORAL ADMINISTRATION OF 15 mg DALMADORM, 10 mg LEVANXOL AND 1 mg ROHYPNOL, RESPECTIVELY

Subject	t_{max} (h)			C_{max} (ng/ml)			$t_{½\ el}$ (h)		
	DAF	Te	FN	DAF	Te	FN	DAF	Te	FN
I.S.	24	1.0	1.3	17.4	158.8	5.8	93.0	10.6	18.7
L.S.	9	1.0	1.3	17.5	211.8	6.2	73.4	20.9	48.7
M.R.	24	1.0	0.3	21.7	214.6	6.3	97.1	13.7	17.5

* t_{max} = time of peak concentration, C_{max} = peak concentration, $t_{½\ el}$ = elimination half-life.

CONCLUSIONS

The procedure described for the quantitative determination of desalkylflurazepam, temazepam and flunitrazepam in plasma is rapid and reliable. Furthermore, the sensitivity permits the measurements of the plasma concentrations for at least three times the elimination half-life after therapeutic dosing for all three drugs. From the results of the preliminary study in healthy volunteers it appears that the method is suitable to be applied in pharmacokinetic studies in man after a single therapeutic dose.

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Note

Simple, rapid and sensitive reversed-phase high-performance liquid chromatographic method for thiopental and pentobarbital determination in plasma and brain tissue

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Barbiturates such as pentobarbital and thiopental are used clinically to treat intraoperative intracranial hypertension [1], metabolic coma [2], head injury [3] and cerebral ischemia [4,5]. Because barbiturates complicate determination of both the origin and depth of coma, are used in long-term therapy, and are potent circulatory and respiratory depressants, a rapid and accurate method of quantitating plasma levels is needed to guide therapy. Furthermore, because many of the questions on the therapeutic use of barbiturates such as optimal dose and duration of administration are unresolved, pursuit of these questions in animal models would be greatly facilitated by a method enabling rapid and accurate quantitation of barbiturate levels in brain tissue and blood.

Quantitation of pentobarbital and thiopental in biological fluids have been done mainly by gas—liquid chromatography (GLC) after extensive extraction procedures and derivatization [6—11]. Poor reproducibility and sensitivity usually resulted because of degradation at high temperatures, incomplete derivatization, reversible or irreversible absorption onto the packing materials and peak tailing.

Two high-performance liquid chromatographic (HPLC) methods have been reported for the determination of thiopental in plasma [12,13]. The first method [12] of direct injection of plasma samples into the column is not applicable for tissue samples. It has the advantage of simplicity but suffers the disadvantage of rapid loss of column efficiency. The use of a precolumn prolonged the life of the analytical column, but reproducibility was poor. The second method [13] measures plasma concentrations of the thiopental and lidocaine by a multiple-step extraction procedure. This method has low

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sensitivity and strong interference by plasma constituents. In neither method was quantitation of pentobarbital mentioned.

Our HPLC assay for pentobarbital and thiopental has several advantages over GLC and other HPLC methods. A simple one-step extraction is used to separate the barbiturates from biological fluids and tissues, and the reconstituted sample is directly injected into the chromatograph. Both pentobarbital and thiopental were quantitated with excellent sensitivity and reproducibility.

EXPERIMENTAL

Reagents and materials

Sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL, U.S.A.) and thiopental (Pentothal, Abbott Laboratories) were used. Solvents were of HPLC grade and other chemicals were analytical reagent grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Stock solutions of the barbiturates (1.0 mg/ml) were prepared in 75% methanol. Phosphate buffer was prepared by titrating 0.01 *M* potassium hydrogen phosphate with 0.01 *M* potassium dihydrogen phosphate to pH 7.8.

Apparatus

A Waters Assoc. (Waters Assoc., Milford, MA, U.S.A.) high-performance liquid chromatograph equipped with a constant-flow pump (Model 6000A), a loop-type injector (Model U6K), and a fixed wavelength (254 nm) UV detector (Model 440) was used. A stainless-steel column (30 cm × 3.9 mm) packed with fully porous 10-μm silica particles with a chemically bonded monomolecular layer of octadecylsilane (μBondapak C_{18}) was utilized for barbiturate separation. A microprocessor (Model C-R1A Chrompatopac, Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) was used for peak area integration and calculations.

Chromatographic conditions

The mobile phase consisted of 0.01 *M* phosphate buffer—acetonitrile—tetrahydrofuran (THF) (78:22:4). A flow-rate of 2.0 ml/min was established at about 14 MPa.

Preparation of plasma standards

Various amounts of thiopental and pentobarbital stock solutions were added to human plasma. The concentration ranges of the standards were from 0—30 μg/ml of thiopental and 0—50 μg/ml of pentobarbital. Thiopental served as the internal standard for pentobarbital determination while pentobarbital was the internal standard for the thiopental assay.

Extraction and HPLC assay

Plasma samples. Two μg of thiopental or 4 μg of pentobarbital internal standard in 0.2 ml of plasma, 0.1 ml of 1 *N* hydrochloric acid and 2 ml of chloroform were placed into a 15-ml screw-capped centrifuge tube. The tubes were agitated on a mechanical shaker (Burrell wrist-action shaker, Burrell

Corporation, Pittsburgh, PA, U.S.A.) for 15 min, then centrifuged at 500 *g* for 10 min. The chloroform layer was transferred to a conical test tube, evaporated to dryness at 40°C under a stream of nitrogen, and reconstituted with 200 μl of the mobile phase. A 30-μl aliquot was injected into the HPLC system.

Brain samples. Whole rat brain was weighed and homogenized (Polytron, Brinkman Instruments, Westbury, NY, U.S.A.) in 4 ml distilled water per gram of tissue. Pentobarbital and thiopental (5—20 μg) were added to 4 ml of the homogenate. After mixing with 1 ml of 1 *N* hydrochloric acid, it was extracted, dried under nitrogen and redissolved in 1 ml of mobile phase. A 30-μl aliquot was injected into the HPLC system.

Recovery. The recovery of the barbiturates from plasma and brain tissue was determined by comparing chromatographic peak areas of pentobarbital and thiopental in plasma and brain samples with peak areas obtained by direct injection of equal amounts of the drugs in solvent.

Validation of the assay method for plasma samples

Known amounts of the barbiturates were added to aliquots of plasma to obtain various final concentrations. The samples were assayed in triplicate and the coefficients of variation and relative errors were calculated.

RESULTS AND DISCUSSION

The calibration curves were constructed by plotting the peak area ratios of thiopental or pentobarbital to their internal standards against known concentrations of thiopental and pentobarbital. Excellent linearity was observed ($r > 0.996$) for both barbiturates, but the linear range for thiopental (0—20 μg/ml) was less than for pentobarbital (0—50 μg/ml). Standard curves were constructed by least-square regression for calculation of unknown concentrations.

Under the assay conditions, baseline resolution of pentobarbital and thiopental were obtained with retention times of 9.5 and 12.0 min, respectively (Fig. 1c). If the amount of THF was reduced or eliminated from the mobile phase, the resolution of both barbiturates was less (Fig. 1b) or there was no separation at all (Fig. 1a). THF is thought to form intermolecular hydrogen bonding with the underivatized imino or oxo functional groups of the barbiturates and hence shield the hydrophilic sites. The formation of the less hydrophilic complex by THF greatly increased the resolution and decreased peak tailing.

In plasma, more than 93% of the pentobarbital was extracted in contrast to 82% for thiopental (Table I). The smaller amount of thiopental extracted could be due to the higher lipophilicity or affinity of thiopental to the plasma constituent. However, the ratio of the recovery of these two barbiturates was constant at these concentrations which is essential for using each as the internal standard. Recovery of both barbiturates was substantially less in brain tissue ranging between 46 and 49% for pentobarbital and between 40 and 44% for thiopental. This was probably due to the loss of chloroform by gel formation of the chloroform—homogenate mixture. However, the ratios of percent recovery were similar to that in plasma.

Determination of the precision and accuracy for determination in plasma

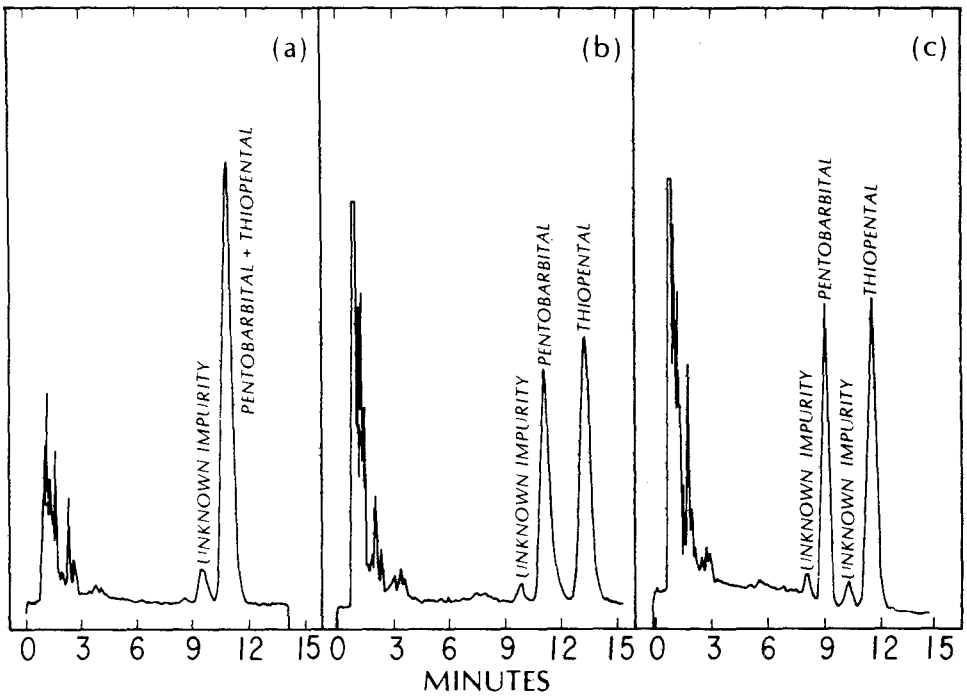

Fig. 1. Chromatograms obtained from plasma samples. The concentrations of pentobarbital and thiopental were equivalent to 20 and 10 μg per ml of plasma. A 30-μl aliquot of the final reconstituted solution was injected. The effluent was detected at 254 nm and 0.01 a.u.f.s. The mobile phases were 0.01 *M* phosphate buffer (pH 7.8)—acetonitrile—THF at ratios of: (a) 78:22:0; (b) 78:22:2, and (c) 78:22:4.

TABLE I

RECOVERY OF THIOPENTAL AND PENTOBARBITAL FROM PLASMA AND BRAIN TISSUE

In all cases $n = 3$. Values given as mean (S.D.).

Concentration (μg/ml)	Pentobarbital	Thiopental	Ratio*
Percent recovery from plasma			
5.0	94.2 (9.1)	83.3 (8.2)	0.88
10.0	93.1 (7.0)	81.9 (7.5)	0.88
20.0	93.4 (5.7)	81.8 (1.7)	0.87
Percent recovery from brain			
5.0	48.9 (1.7)	43.8 (2.0)	0.89
10.0	46.3 (3.6)	40.0 (2.6)	0.86
20.0	47.9 (2.4)	41.2 (3.6)	0.86

*Mean percent of thiopental:pentobarbital.

reveal an overall coefficient of variation of 4.3% for pentobarbital (Table II) and 4.0% for the thiopental (Table III) and average relative errors of 4.7% and 5.3%, respectively.

TABLE II

PRECISION AND ACCURACY OF HPLC ASSAY OF PENTOBARBITAL IN PLASMA

In all cases $n = 3$.

Actual concentration (μg/ml)	Measured concentration [μg/ml, mean (S.D.)]	Coefficient of variation	Relative error (%)
3.80	3.98 (0.045)	1.1	4.7
6.82	7.12 (0.184)	2.6	4.4
14.5	14.0 (0.577)	4.1	3.7
23.9	22.2 (1.71)	7.7	7.4
37.7	37.8 (3.04)	8.0	6.1
51.2	51.9 (1.10)	2.1	2.0
	(overall)	(4.3)	(4.7)

TABLE III

PRECISION AND ACCURACY OF HPLC ASSAY OF THIOPENTAL IN PLASMA

In all cases $n = 3$.

Actual concentration (μg/ml)	Measured concentration [μg/ml, mean (S.D.)]	Coefficient of variation	Relative error (%)
2.15	2.20 (0.145)	6.6	5.4
4.68	4.54 (0.104)	2.3	2.8
9.71	9.83 (0.484)	4.9	4.1
14.5	14.5 (0.737)	5.1	3.9
19.2	19.2 (0.306)	1.6	1.2
28.5	24.3 (0.902)	3.7	14.6
	(overall)	(4.0)	(5.3)

The sensitivity for both barbiturates was about 0.1 μg/ml in plasma and 0.2 μg/g in brain tissue. Because only a small portion of the sample was used for each determination, the sensitivity could be greatly improved by increasing sample size (by using 1 ml of plasma instead of 0.2 ml) or by reconstituting the sample residue in a smaller volume of mobile phase solvent. Since there was no interference by using the crude plasma extract, we estimate that a 10- to 20-fold increase in sensitivity could be obtained. Sensitivity could also be improved by using detector wavelengths of maximum absorbancy. Absorbance for thiopental was higher at 300 and 200 nm than at 254 nm (Fig. 2). UV absorption by pentobarbital was greatly increased by lowering the wavelength to less than 240 nm. Thus, with the use of the variable-wavelength UV detector a higher sensitivity may be obtainable.

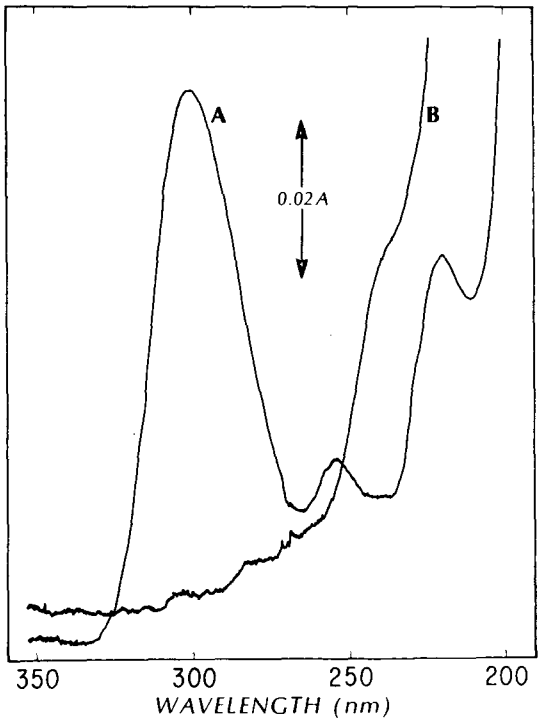

Fig. 2. UV absorption spectra of thiopental (A) and pentobarbital (B). The concentrations were 1.0 and 2.5 μg of thiopental and pentobarbital per ml of mixed solvent of 0.01 *M* phosphate buffer—acetonitrile—THF (78:22:4).

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Note

Determination of a new anti-inflammatory agent, 1-isobutyl-3,4-diphenylpyrazole-5-acetic acid, by high-performance liquid chromatography

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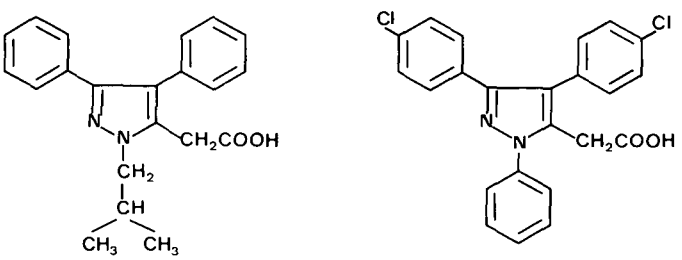
(First received April 22nd, 1981; revised manuscript received July 21st, 1981)

1-Isobutyl-3,4-diphenylpyrazole-5-acetic acid (LM 22070) (Fig. 1a) is a new drug which possesses promising analgesic, antipyretic, and anti-inflammatory properties. Its activity is either the same as (antipyretic action) or inferior to (analgesic and anti-inflammatory activity) that of indomethacin, but it is always markedly superior to that of phenylbutazone. Its ulcerogenic activity in the rat is three times weaker than that of indomethacin [1].

A rapid and sensitive method for the quantitation of LM 22070 in biological fluids was required to determine the pharmacokinetic parameters of this drug in humans. Therefore, a reversed-phase liquid chromatographic (RPLC) method based on ion-suppression chromatography and fluorescence detection was



a b

Fig. 1. (a) Chemical structure of LM 22070 (1-isobutyl-3,4-diphenylpyrazole-5-acetic acid). (b) Chemical structure of internal standard: 1-phenyl-3,4-di-*p*-chlorophenylpyrazole-5-acetic acid.

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developed. 1-Phenyl-3,4-di-*p*-chlorophenylpyrazole-5-acetic acid (Fig. 1b) was used as internal standard.

MATERIALS AND METHODS

Chemicals and reagents

LM 22070 and 1-phenyl-3,4-di-*p*-chlorophenylpyrazole-5-acetic acid (used as internal standard) were kindly supplied by Pharmuka (Gennevilliers, France).

All reagents were of analytical grade and used without further purification: diethyl ether (E. Merck, Darmstadt, G.F.R.), disodium citrate, 1 *N* sodium hydroxide, 0.1 *N* hydrochloric acid, orthophosphoric acid, potassium dihydrogen phosphate, acetonitrile and methanol (Prolabo, Paris, France), enzyme solution containing 100,000 units of β-glucuronidase per ml and 1,000,000 units of arylsulfatase per ml (I.B.F., Gennevilliers, France).

Buffer solutions

Citric acid—0.1 *M* disodium citrate buffer (pH 4.4) and 0.2 *M* phosphate buffer (pH 3) were stored at 4°C.

Stock solutions

LM 22070 stock solution (20 mg per 100 ml) and internal standard stock solution (1 mg per 100 ml) were prepared in methanol. A ten-fold dilution of these two solutions was performed before use.

Chromatography

High-performance liquid chromatographic analyses were performed on a Chromatem 38 liquid chromatograph equipped with an Altex pump and operated at ambient temperature (Touzart et Matignon, Paris, France). A Jobin-Yvon Model J.Y. 3 D spectrofluorescence detector (Jobin-Yvon, Long jumeau, Paris, France) was operated at 274 nm for excitation and 350 nm for emission. The output of the detector was displayed on a recorder Model PE 1286 (Sefram, Paris, France) having a 1-V full-scale range.

The column was 15 cm × 4.6 mm I.D. stainless steel, packed with LiChrosorb RP-8 (5 μm particle size; E. Merck) by a balanced density slurry technique [2]. A 20-μl loop injection valve Model 70-10 (Rheodyne, Berkeley, CA, U.S.A.) was used to introduce samples into the chromatographic system. The efficiency of newly made columns was tested with a standard mixture (benzene—naphthalene—anthracene) using sodium nitrate as an unretained marker. The solvent system was methanol—water (80:20). Under these conditions, the column efficiency was 3700 theoretical plates for benzene, 4200 for naphthalene and 4700 for anthracene.

The mobile phase, acetonitrile—water—0.2 *M* phosphate buffer (pH 3) (65:15:20), was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) under negative pressure, and degassed by ultrasonic vibration. The column was preconditioned with mobile phase until a stable recorder output was obtained. The flow-rate was then adjusted to 1.6 ml/min, generating a pressure of about 120 bars.

Extraction precedures

Plasma. To 1 ml of plasma transferred into a 45-ml screw-capped centrifuge tube, were added 1 ml of citrate buffer, 100 μl of internal standard solution (0.1 mg per 100 ml) and 15 ml of diethyl ether. After agitation and centrifugation (5 min at 3000 *g*), the organic phase was carefully pipetted out into a 15-ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°C. The sample residue was dissolved in various volumes (0.5—3 ml) of mobile phase depending on the expected drug concentration; 20-μl aliquots were injected into the chromatograph.

Urine. In order to determine the total amount of LM 22070 excreted in urine, the conjugate drug must be first hydrolysed. The best conditions for complete hydrolysis consisted of incubating 0.1 ml of urine, 1 ml of citrate buffer (pH 4.4), 100 μl of internal standard dilution (0.1 mg per 100 ml) and 0.1 ml of the enzyme solution at 37°C for 16 h. The sample was then treated as before. The residue was dissolved with 1—2 ml of mobile phase and 20-μl aliquots were injected into the chromatograph.

Calibration, repeatability and accuracy

Calibration curves were obtained by plotting peak area ratios (LM 22070/internal standard) against drug levels from different pools of plasma containing concentrations ranging from 0.2 to 8 μg/ml.

Repeatability and accuracy of the assay were studied by measuring the LM 22070 concentration from various plasma samples spiked with 0.5, 2 and 6 μg/ml of the drug. The absolute analytical recovery from plasma was calculated by comparing the peak areas from plasma extracts containing 0.5, 2 and 6 μg/ml of LM 22070 to peak areas obtained by direct injections of pure standard solutions of the drug.

Calculations

The calculations of plasma and urine concentrations were always made by the internal standard method using peak area ratios.

Clinical study

A pharmacokinetic study of 1-isobutyl-3,4-diphenylpyrazole-5-acetic acid was performed on ten healthy male subjects. Each subject received one single intravenous dose containing 50 mg of the drug. Blood samples (5 ml) were collected before drug administration and 5, 15, 20, 60, 90, 120, 180, and 240 min after dosing. The plasma immediately harvested and frozen until analysis.

RESULTS AND DISCUSSION

The present RPLC method for the quantitative analysis of 1-isobutyl-3,4-diphenylpyrazole-5-acetic acid in plasma and urine involves a simple extraction of an acidified biological sample with diethyl ether, followed by ion suppression chromatography using a fluorescence detector.

Because of its lower polarity, ether is less likely to extract interfering compounds than many chlorinated solvents. So ether extracts of blank plasma were free of endogenous absorbing peaks able to interfere with the assay (Fig. 2A).

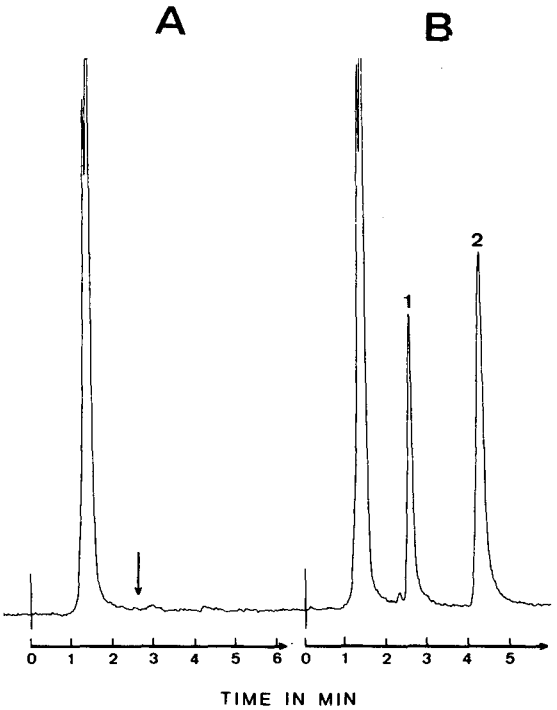

Fig. 2. (A) Chromatogram of a blank plasma. The arrow indicates the retention time of LM 22070. (B) Chromatogram of a plasma extract containing LM 22070 (1) 6 μg/ml and internal standard (2) 0.4 μg/ml.

The drug is a weak acid (pK_a = 5.5), so the buffered (pH 4.4) mobile phase suppresses the solute ionization and reduces peak tailing. In such conditions, good resolution and efficiency were obtained (R_s = 5.6, N = 2990 theorical plates for LM 22070).

Optical fluorescence response under the described chromatographic conditions was achieved by use of an excitation wavelength at 274 nm and an emission wavelength at 350 nm (Fig. 3). The excitation maximum of the internal standard was 273 nm and the emission maximum was 345 nm, under the same conditions.

Fig. 2B shows the chromatogram of an extract from 1 ml of human plasma containing LM 22070 (6 μg/ml) and internal standard (0.4 μg/ml). Under the described chromatographic conditions, the retention times of LM 22070 and internal standard were 2.6 min and 4.3 min, respectively. Fig. 4 shows the chromatogram of an urine extract (0.6 ml).

Because of the large variations in plasma levels during a 24-h kinetic study, it is often necessary to take variable volumes of biological sample for analysis. Thus it was found necessary to maintain a constant ratio of aqueous to organic phase during the initial extraction step, in order to obtain a reproducible recovery. The absolute analytical recovery of LM 22070 from plasma was 97.5% (Table I). It was quite comparable with the absolute analytical recovery of the internal standard (96%).

Estimates of repeatability and accuracy of the method are shown in Table II.

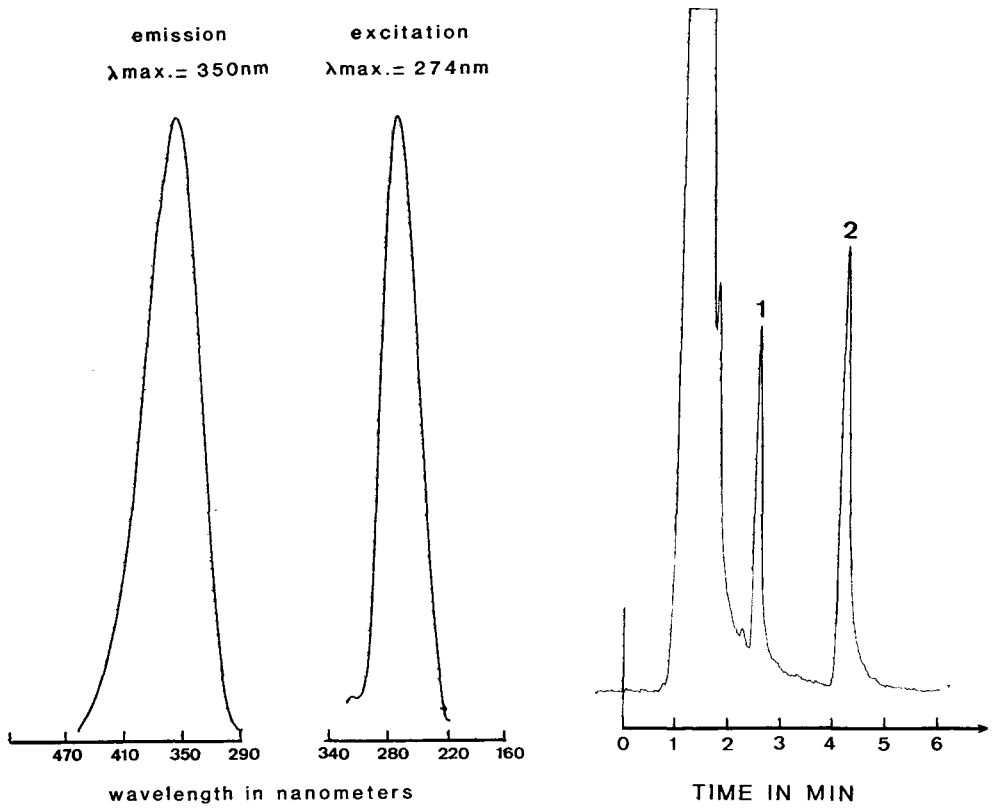

Fig. 3. Excitation and emission spectra of a 20 μg/ml solution of LM 22070 in acetonitrile—water—0.2 *M* phosphate buffer (pH 4.4) (65:15:20).

Fig. 4. Chromatogram of a 0.6-ml urine extract containing LM 22070 (1) 8.9 μg/ml and internal standard (2) 4 μg/ml.

TABLE I

ABSOLUTE ANALYTICAL RECOVERY OF LM 22070

Concentration (μg/ml)	*n*	Recovery (%)
0.5	3	97.5
2	4	96.5
6	4	99

Accuracy varied from 1.2 to 4.6%. It was still good at low concentrations and within the generally accepted limits for drug assays.

The results of calibration curves showed that a good linear fit was obtained in the therapeutic range of concentration (0.2—8 μg/ml) (slope 0.280; intercept 0.0238; $r = 0.999$).

TABLE II

REPEATABILITY AND ACCURACY

LM 22070 added (μg/ml)	*n*	LM 22070 measured (μg/ml)*	Mean error	Relative error (%)	CV** (%)
0.5	10	0.48 (0.022)	0.069	4	4.6
2	5	1.91 (0.051)	0.023	4.5	2.6
6	5	5.72 (0.068)	0.030	4.6	1.2

*Standard deviation in parentheses.
**Coefficient of variation.

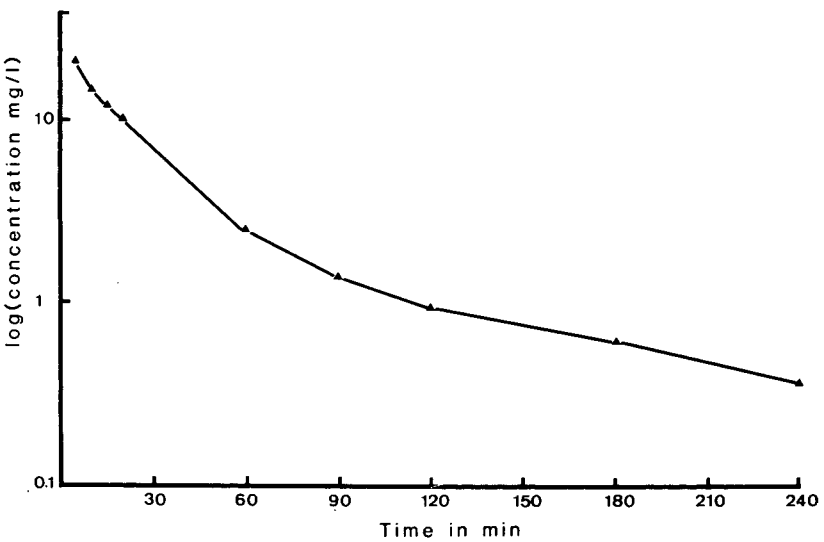

Fig. 5. Plasma concentration—time curve after a 50-mg intravenous injection of LM 22070.

Fig. 5 shows the plasma concentration—time curve after a 50-mg intravenous injection of LM 22070. Plasma levels varied from 21.5 μg/ml 5 min after administration to 0.36 μg/ml 4 h after dosing. The amount of LM 22070 excreted by the urine (free and conjugated) was 14.53 mg per 24 h (29.06%).

The detection limit is about 90 ng/ml in plasma and urine, both measured at a signal-to-noise ratio of 3:1.

The proposed RPLC method for the assay of 1-isobutyl-3,4-diphenylpyrazole-5-acetic acid is simple and sensitive enough to be used in pharmacokinetic and biopharmaceutic studies with this new drug.

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Note

Rapid determination of procetofenic acid in plasma by high-performance liquid chromatography

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(First received May 8th, 1981; revised manuscript received August 5th, 1981)

Procetofenic acid (I, Fig. 1) is the active metabolite of the new drug procetofene, or fenofibrate, which is its corresponding isopropyl ester derivative. This acid is structurally related to the well-known hypolipidaemic agent, clofibrate, in which the *p*-chlorine atom has been replaced by a *p*-chlorobenzoyl group in order to improve its activity and tolerance.

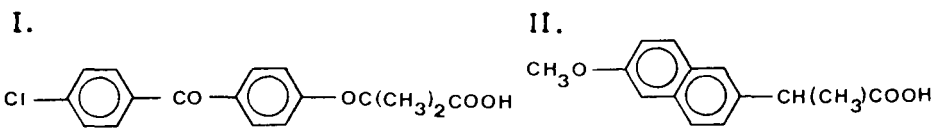


Fig. 1. Chemical structure of procetofenic acid (I) and naproxen (II).

The metabolism and pharmacokinetics of fenofibrate have been recently reported [1, 2]. The analytical methods used for the active metabolite determination in plasma include gas chromatography [3] and gas chromatography—mass spectrometry [4]. The compound was determined as its methyl ester and the parent drug, procetofene, was used as the internal standard. However, under the reported conditions [3] a small interfering peak appears in plasma samples.

The method described in this paper makes use of high-performance liquid chromatography (HPLC), and is faster, more sensitive and more specific than the gas chromatographic method. It is therefore suitable for the routine analysis of large numbers of biological samples.

EXPERIMENTAL

Reagents and materials

Diethyl ether, methanol and potassium dihydrogen phosphate were of reagent grade (E. Merck, Darmstadt, G.F.R.) and used as received. Procetofenic acid and the internal standard, naproxen (II, Fig. 1), were of analytical purity.

Plasma samples were obtained from male Wistar rats of approx. 300 g, orally medicated with doses of 50 mg of procetofenic acid per kg of body weight. Sets of eight rats were used for each determination.

Extraction procedure

A 3-ml aliquot of plasma was pipetted into a glass-stoppered centrifuge tube, acidified with 1.5 ml of 3 *M* hydrochloric acid and mechanically shaken (Vortex mixer) for 15 sec. After standing for 5 min, 1 ml of the internal standard solution of naproxen (150 μg/ml in methanol) and 15 ml of diethyl ether were added to the acidified plasma. The mixture was shaken again for 30 sec and centrifuged at 1500 *g* for 5 min. The upper layer was transferred to another tube and the extraction was repeated once more. The ether extract were carried to dryness under vacuum at 40°C in a rotary evaporator. The resulting residue was dissolved in 1 ml of methanol and 10 μl were injected into the chromatograph.

Apparatus and chromatography

An M 600 pump and a U6K injector (Waters Assoc., Milford, MA, U.S.A.) were coupled to a flexible-walled cartridge packed with 5-μm reversed-phase material (100 × 5 mm, μBondapak C_{18}) fitted in a radial compression module (RCM-100)*. A Waters M440 UV detector, with a fixed wavelength of 254 nm, and a DM730 data processing module were used to measure the concentrations of compounds I and II (Fig. 1).

The mobile phase consisted of a mixture of 70% (v/v) methanol and 30% water containing 0.4% (w/v) phosphate–phosphoric buffer to maintain the pH at 4. A constant flow-rate of 2 ml/min was used with a pressure of 103 bar. Under these conditions, the retention times of naproxen and procetofenic acid were 2.8 and 5.2 min, respectively.

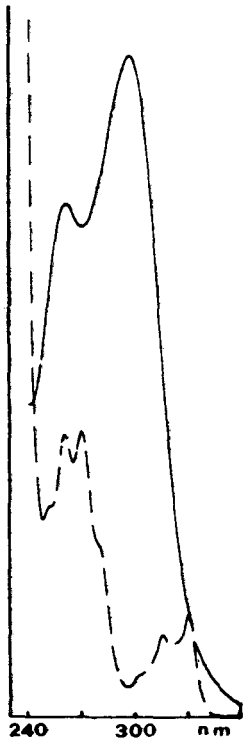
RESULTS AND DISCUSSION

The UV absorption spectra of procetofenic acid (I) and naproxen (II) show maxima at about 260 nm (Fig. 2). Therefore a 254-nm fixed-wavelength detector is suitable.

Fig. 3 shows representative chromatograms obtained and demonstrates the absence of interfering endogenous compounds. The short column length provides a satisfactory retention time, thus improving sensitivity.

A linear calibration curve of peak area ratios of procetofenic acid/naproxen vs. plasma concentrations of procetofenic acid was obtained in the range of 1–100 μg/ml. The correlation coefficient for this linear relationship was

*Trade-mark Waters Assoc.



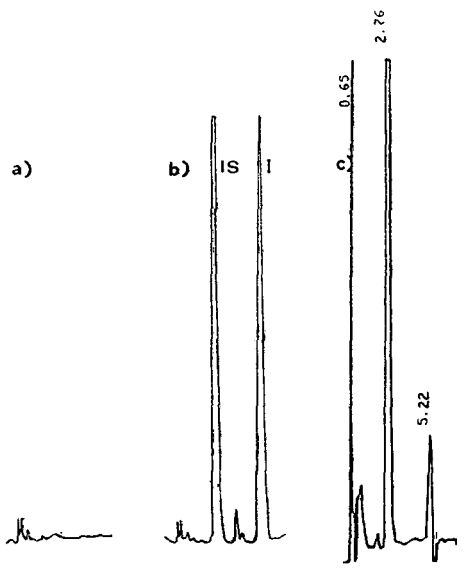


Fig. 2. Ultraviolet absorption spectra of procetofenic acid (——) and naproxen (— — —) in methanol.

Fig. 3. Typical chromatograms of: (a) control plasma; (b) plasma extract from a medicated rat (I = procetofenic acid; IS = internal standard); (c) plasma containing procetofenic acid at a level of 100 ng/ml. Retention times of procetofenic acid (5.22 min) and internal standard (2.76 min) are indicated.

consistently greater than 0.999 (n = 3) and the value of the intercept was not significantly different from zero. The corresponding equation was y = 52.63 x + 0.12, where y represents the concentration of procetofenic acid in plasma in μg/ml and x the indicated peak area ratios, using 150 μg/ml of internal standard.

The reproducibility of six replicate measurements of procetofenic acid added to plasma in the concentrations 100, 50 and 10 μg/ml was found to be 1.0, 1.4 and 3.2%, respectively. The recovery was in the order of 60% in the first extraction and better than 98% with the second one.

The sensitivity of the method as described was about 50 ng/ml, which allowed the study of the pharmacokinetic parameters of this drug in rats. Table I summarizes the results of a bioavailability trial where it is clearly shown that the administration of the acid as a salt derivative does not produce any significant change in its pharmacokinetics compared to the acid itself.

However, if therapeutic levels in biological fluids need to be measured, it is possible to detect as low as 10 ng/ml. Consequently, the sensitivity of

TABLE I

PHARMACOKINETIC PARAMETERS FOR ORAL ABSORPTION OF PROCETOFENIC ACID IN RATS, IN TWO DIFFERENT FORMULATIONS

Procetofenic acid was administered (50 mg/kg) (A) as the acid and (B) as its cinnarizine salt. Values are means ± S.D. of eight animals.

Formulation	Mean peak plasma conc. (μg/ml)	Mean time to peak plasma conc. (h)	Mean area under curve (0—48 h) (μgh/ml)	Mean plasma half-life (h)
A	155 ± 18.1	2	2732	21.73 ± 1.07
B	165 ± 24.4	2	2719	18.8 ± 1.2

this method is higher than that previously described for gas chromatography [3]. In addition, it possesses several advantages for routine analysis; namely, an easier and faster handling procedure of the samples and a higher specificity. Nevertheless, the specificity must be tested when the assay needs to be applied to the plasma of patients receiving multi-drug therapy.

ACKNOWLEDGEMENT

We thank Waters Española, S.A. (A. Torres) for the use of the radial compression module RCM-100.

NOTE ADDED IN PROOF

A method for the determination of procetofenic acid in plasma by gas chromatography with electron-capture detection has been published recently [5]; the limit of detection was 1 μg/ml.

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Note

High-performance liquid chromatographic analysis of miconazole in plasma

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Miconazole is a synthetic imidazole derivative with broad-spectrum antifungal activity [1—3]. It is relatively non-toxic and has been used experimentally in the treatment of various systemic mycoses, including candidiasis, coccidiodomycosis, cryptococcosis and histoplasmosis. Interest prevails in evaluating the bioavailability and distribution of various oral formulations of the drug and comparing them with formulations intended for intravenous (i.v.) administration.

Therefore, an analytical method suitable for specifically measuring parent drug levels in plasma was sought. Although gas chromatography with electron-capture detection has been used for monitoring miconazole [4—6], this technique has disadvantages associated with it. One of the reported procedures [4] requires derivatization of the drug prior to chromatography to avoid poor peak shapes (pronounced tailing). The authors suggest that subsequent to derivatization the drug may be pyrolyzed in the injection port and, therefore, only a fragment of the parent is detected. Under these conditions, the ability of the method to differentiate between parent drug and potential metabolites is questionable. A ^{63}Ni source was used and resulted in limited linearity of detector response. The other method [5,6] employed a Sc^3H detector source to circumvent the limited detector linearity associated with ^{63}Ni. However, because of the restricted detector temperature ($\leqslant 300°C$) with Sc^3H, the detector was not self-cleaning for miconazole and after ca. 50 determinations, the Sc^3H foil had to be removed and cleaned in an ultrasonic bath. These methods reported an overall recovery of 50—75% from biological fluid.

To avoid the problems of (a) thermal decomposition, (b) need for derivatization, (c) limited detector linearity and lifetime, and (d) mediocre recovery of drug, a high-performance liquid chromatographic (HPLC) method for miconazole has been developed with the drug being detected spectrophotometrically.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, Model U6K injector and Model 440 absorbance detector operated at 254 nm. A Waters μBondapak C_{18} (30 cm × 3.9 mm I.D.; 10 μm particle size) column was used throughout.

Materials

Miconazole nitrate was obtained from Prodotti Chimici Farmaceutici (Rome, Italy) and the internal standard, R-18,648 was obtained from Janssen Pharmaceutica (Beerse, Belgium). Both were used without further purification. Miconazole (free base) was obtained by neutralization of an aqueous solution of the nitrate with sodium hydroxide and extraction into chloroform. Evaporation of the chloroform extract yielded a yellow oil which was recrystallized from benzene—hexane (10:90) to yield crystals of the free base, m.p. 79—81°C (elemental analysis calculated for $C_{18}H_{14}N_2OCl_4$, C=51.9; H=3.4; N=6.7; found C=51.98; H=3.50; N=6.60).

Separation of miconazole from dog blood

Blood samples (3 ml) were centrifuged at 1100 *g* for 10 min. A 1-ml aliquot of the resulting plasma was transferred to a 15-ml glass centrifuge tube, 25 μg of internal standard, R-18, 648 (25 μl of a 1 mg/ml stock solution prepared in methanol) and 1 ml of a 0.2 *M* sodium hydroxide solution (final pH ca. 12.6) was added and the mixture agitated. A 5-ml aliquot of *n*-heptane—isoamyl alcohol (98.5:1.5) was then added and the mixture extracted with vortex mixing. The layers were separated by centrifugation and the plasma layer frozen in a dry ice—acetone bath. The organic layer was then transferred by decantation into a second 15-ml glass centrifuge tube. The plasma was extracted a second time as described above and the two organic layers were combined. The extract was evaporated to dryness under a stream of nitrogen in a 60°C water bath, and the residue dissolved in 100 μl of HPLC grade methanol (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Chromatography

A 60-μl aliquot of the reconstituted extract was chromatographed as an octanesulfonate ion-pair on an octadecylsilane bonded phase column thermostated at 50°C with UV absorbance detection of the effluent at its λ_{max}, 254 nm. A ternary mobile phase of methanol—tetrahydrofuran—2.5 mmol/l aqueous acetate buffer, pH 5 (62.5:5:32.5) containing 5 mmol/l octanesulfonate was used for separation. Flow-rate was maintained at 2.0 ml/min.

Quantitative analysis

Miconazole was quantitated by determining the analyte:internal standard (R-18,648) peak height ratio and comparing this value with a standard curve constructed after analysis of plasma samples containing known amounts of drug. The calibration curve was generated by analyzing two samples of plasma

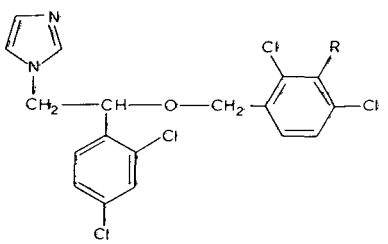
at each of six concentrations of miconazole ranging from 1—50 μg/ml. To determine overall recovery, a second standard curve was constructed by spiking the HPLC mobile phase with miconazole and internal standard at the same concentrations used in the generation of the plasma calibration curve and then subjecting these solutions to direct HPLC analysis.

Animal studies

A beagle dog (weighing 11.5 kg) was administered 250 mg of miconazole as an i.v. bolus (administration time, 1 min) via the jugular vein. Blood samples (3 ml) were drawn from the other jugular vein 5, 10, 15, 30, 60, 120, 180, 240, 300 and 360 min post-dosing. Samples were centrifuged and plasma stored at 4°C until analyzed (within 48 h).

RESULTS AND DISCUSSION

Analysis of miconazole, 1, in plasma is carried out in two stages: initial extraction of the drug from the biological mixture into a water-immiscible solvent and chromatographic separation of the resulting extract on a reversed-phase column with spectrophotometric monitoring of the column eluent.



1 R = H
2 R = Cl

Extraction

Plasma was initially alkalinized to pH 12 with sodium hydroxide to present the amine functions in a totally non-ionized form. Subsequent extraction of the plasma with the moderately polar solvent diethyl ether afforded 20% recovery of drug. Extraction efficiency could be increased to 83 ± 2% by 1:1 volume dilution of the (ether) extractant with hydrocarbon (e.g., *n*-hexane). Taking full advantage of the hydrophobic nature of the free base and extracting the pH-adjusted plasma with *n*-heptane containing 1.5% isoamyl alcohol to avoid emulsification and adsorption of drug onto the glass container surfaces, afforded quantitative recovery (100 ± 2%) of drug. The extract contained minimal contamination by other materials present in the plasma as subsequently determined by HPLC. Extractants containing diethyl ether were much less specific and in addition to being less efficient, gave extracts that subsequently produced chromatograms showing significant contamination.

Depending on the animal species used for the bioavailability studies, either 0.1-ml or 1.0-ml blood samples will be drawn. In this study recovery of miconazole was independent of sample size over the concentration range 1—50 μg of drug per ml of plasma.

Overall recovery from sample-to-sample was determined by the addition of a constant amount of internal standard, R-18,648, 2, directly to the plasma

sample. The internal standard chosen was a halogenated analogue of the drug and also was quantitatively recovered (100 ± 2%) by extraction with heptane (containing 1.5% isoamyl alcohol). In all cases two extractions of plasma with 2.5 volumes of heptane were required for quantitative recovery of 1 and 2.

Chromatography

Extracts were separated on an RP-18 column. Initial attempts at separation involved methanol—water or acetonitrile—water mobile phases with and without sodium octanesulfonate as ion-pairing agent. These systems were incapable of separating the drug from the internal standard or from co-extracted contaminants. Peak shape and resolution could be improved by thermostating the column at an elevated temperature (e.g., 50°C). Under these conditions, capacity factor (k') for all components decreased, a situation that could be balanced by decreasing the volume fraction of organic modifier in the mobile phase. With this system optimum resolution was achieved using methanol—2.5 mmol/l aqueous acetate buffer, pH 5 (75:25) containing 1 mmol/l octanesulfonate mobile phase. For most samples, this eluent offered adequate resolution of 1 and 2 from contaminants with short analysis times (t_R = 5.5±0.5 min). Unfortunately, about 20% of the plasma samples contained a contaminant (inter-animal variation) that co-eluted with the internal standard. By reducing the methanol concentration in the mobile phase and concomitantly adding a small amount of tetrahydrofuran (a more powerful organic modifier), a ternary eluent of methanol—tetrahydrofuran—2.5 mmol/l aqueous acetate buffer, pH 5 (62.5:5:32.5) containing 6 mmol/l octanesulfonate was finally selected which was capable of resolving all peaks of interest. The column was still maintained at 50°C to minimize band broadening. Under these conditions miconazole and internal standard eluted with k' values of 3.7 and 5.5, respectively (Fig. 1). Equal sensitivity was gained by monitoring the column effluent spectrophotometrically at 254 or 280 nm; however, at 254 nm contributions to the chromatogram from plasma contaminants were minimized and this wavelength was therefore chosen for subsequent analysis.

Quantitative analysis

Miconazole was quantitated by comparing peak height ratio (drug:internal standard) for the unknown with a standard curve prepared by adding known amounts of miconazole and a constant amount of internal standard to plasma samples and carrying the resulting solutions through the analysis sequence. A typical curve generated after analysis of six duplicate plasma samples containing 1—50 μg of 1 and 25 μg of 2 per ml is described by the equation, peak height ratio = 6.99·(concentration of 1) + 8.56 (correlation coefficient 0.998) (Fig. 2). To determine overall recovery of 1 from plasma, a second standard curve was constructed after analysis of samples of 1 prepared in mobile phase (at the same concentration employed with the curve generated from plasma samples) and then immediately analyzed by HPLC (without extraction). The resulting line is described by peak height ratio = 6.96·(concentration of 1) — 3.20 (correlation coefficient = 0.999) (Fig. 2). Overall recovery, determined as the ratio of the slopes of these two calibration plots, was 100%.

The injection-to-injection reproducibility of analysis was determined by

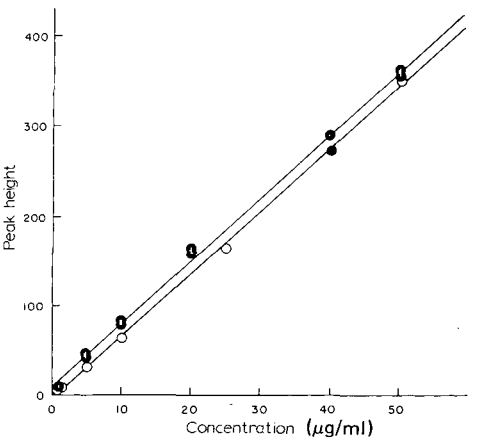


Fig. 1. Chromatogram of miconazole from dog plasma. Miconazole (1 μg/ml of plasma), retention volume, 28 ml; internal standard (25 μg), retention volume, 38 ml. Separation carried out on octadecylsilane bonded phase column with 6 mmol/l sodium octanesulfonate in methanol—tetrahydrofuran—2.5 mmol/l aqueous acetate buffer, pH 5 (62.5:5:32.5) as the mobile phase. Eluent monitored at 254 nm. Flow-rate 2 ml/min.

Fig. 2. Calibration curve for analysis of miconazole from dog plasma (•). Plasma was spiked with known amounts of miconazole and carried through the analysis sequence. Calibration curve generated by HPLC analysis of samples containing known amounts of miconazole prepared directly in mobile phase (○) (without extraction or other pretreatment of samples).

repetitive injection of a single sample containing 10 μg/ml of miconazole. A relative standard deviation of ± 0.4% was obtained from five replicate injections. Sample-to-sample variation was determined by analysis of six plasma samples spiked with 30 μg of miconazole per ml and six containing 1 μg of miconazole per ml, representing high and low concentrations in bioavailability studies. At the high concentration, a relative standard deviation of ± 5% was observed, whereas at the lower level (1 μg/ml), a relative standard deviation of ± 1% was determined. The trend toward lesser variance at lower drug levels was not expected, and cannot be explained at this time. The sensitivity limit for the method determined from analysis of increasingly lower concentrations of drug in plasma was 250 ng/ml at a signal-to-noise ratio of 3:1.

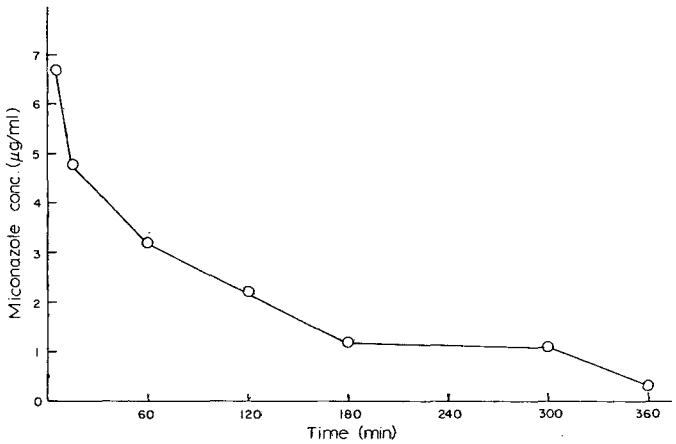


Fig. 3. Plasma levels of miconazole present in a dog administered the drug i.v. (250 mg) over a 1-min period.

Bioavailability studies

To demonstrate the applicability of the method for evaluating distribution of various dosage forms of miconazole for i.v. administration, a standard dose (250 mg) of drug was administered to a beagle dog. The plasma—time profile (Fig. 3) demonstrates peak levels of 7 μg/ml declining to levels of ca. 500 ng/ml at 6 h post-dose. Elimination of the drug from plasma can thus be followed for an adequate period of time to evaluate and describe the distribution pattern.

In conclusion, a simple, rapid HPLC method is described for analysis of miconazole in plasma. Although it lacks the sensitivity of methods previously described based on gas—liquid chromatographic separation with electron-capture detection, it is sufficiently sensitive to monitor the drug after i.v. dosing. In addition, it overcomes the difficulties associated with these gas—liquid chromatographic procedures of (a) thermal decomposition of the drug, (b) need for derivatization, (c) limited detector linearity and/or lifetime and (d) poor overall recovery of drug from plasma.

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Note

Determination of miconazole in human saliva using high-performance liquid chromatography

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Miconazole nitrate [1-(2,4-dichloro-β-((2,4-dichlorobenzyl)oxy)phenethyl)-imidazole nitrate] is a broad-spectrum antifungal drug. It is established as a useful drug for the topical treatment of superficial fungal infections, and it has been used in the treatment of certain systemic fungal infections [1]. Several methods have been described for its determination in serum. These methods have been based on microbiological [2,3] or gas chromatographic [4] procedures and are not suitable for the rapid investigation of large numbers of samples. A sensitive and specific high-performance liquid chromatographic (HPLC) method has been described for the related imidazole antifungal drug econazole [5] using miconazole as the internal standard. This method did not, however, give good results with our chromatograph.

The method described in this paper was devised to permit the investigation of the persistence of miconazole in the mouth after dosing with an oral gel formulation of the drug. Chromatography was performed on de-proteinated saliva samples using a C_8 reversed-phase radial compression column and UV detection.

The method is simple, rapid and sensitive and will measure concentrations of miconazole down to 0.5 mg/l.

MATERIALS AND METHODS

Apparatus

The chromatograph consisted of a Waters Model M6000A pump, a Model U6K injector, a Bondapak C_{18} Corasil guard column, an RCM 100 radial compression module containing a Radial-Pak C8 analytical column (5 mm I.D.), and a Model M450 variable-wavelength detector operating at 230 nm (Waters Assoc., Hartford, Great Britain). A Model M730 data module plotter/integrator (Waters Assoc.) was used to record the chromatograms.

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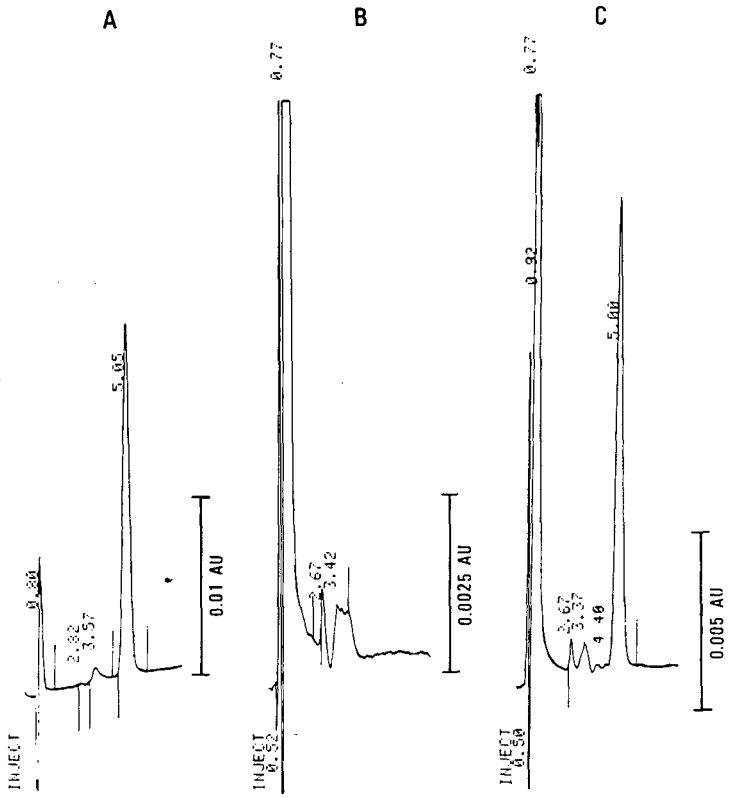

Fig. 1. (A) Chromatogram of miconazole (retention time 5.05 min) standard in water. (B) Chromatogram of predose saliva from subject No. 4. (C) Chromatogram of saliva from subject No. 4, 15 min after dosing with 10 ml miconazole oral gel.

TABLE I

CONCENTRATIONS OF MICONAZOLE IN SALIVA OF HUMAN SUBJECTS AFTER SINGLE DOSES OF 5 OR 10 ml MICONAZOLE ORAL GEL

Time after dosing (min)	Miconazole concentration (mg/l)							
	Subject 1		Subject 2		Subject 3		Subject 4	
	5-ml dose	10-ml dose	5-ml dose	10-ml dose	5-ml dose	10-ml dose	5-ml dose	10-ml dose
0	0	0	0	0	0	0	0	0
15	9.2	45.8	3.5	5.3	12.5	45.8	40.0	51.6
30	1.8	4.7	5.0	1.6	2.3	10.0	5.3	13.9
45	0.7	3.1	2.8	0.9	2.0	6.7	7.6	8.1
60	0.4	2.8	<0.5	0.9	0.8	4.8	1.2	0.8
90	0.6	2.5	<0.5	0.7	1.9	2.3	1.2	5.3
120	<0.5	1.4	<0.5	0.5	1.0	2.6	<0.5	1.2
150	0.7	1.6	<0.5	<0.5	1.5	2.9	1.8	10.9
180	<0.5	1.3	<0.5	0.6	<0.5	1.4	0.6	0.7

Reagents and solvents

Miconazole nitrate was obtained from Janssen Pharmaceutica (Beerse, Belgium). A stock solution was prepared in distilled water (acidified to pH 3 with concentrated phosphoric acid) at a concentration of 1000 mg/l. All chemicals and reagents except *n*-nonylamine were analytical grade.

Chromatographic eluent

The mobile phase was composed of 77% methanol in 0.01 *M* EDTA with 0.005 *M* *n*-nonylamine and was used at a flow-rate of 1.5 ml/min. The eluent was filtered and degassed under reduced pressure before use.

Sample preparation

Saliva samples (150 μl) were mixed with an equal volume of acetonitrile, allowed to stand for 5 min and then centrifuged at 1000 *g* for 2 min. The supernatant was collected and 20 μl injected into the liquid chromatograph. Most saliva samples were analysed on the same day they were prepared. However, no deterioration was found in deproteinated samples stored at −20°C for up to seven days.

Quantitation

Standards were prepared by spiking normal human saliva with small volumes of a concentrated stock solution of miconazole. A standard curve was produced by plotting peak area counts against miconazole concentration.

Saliva samples

The method of analysis was applied to samples obtained from volunteers who were participating in studies of the persistence of miconazole in the mouth. Samples were collected at intervals for 3 h after a 5- or 10-ml dose of miconazole oral gel. A predose sample was also collected from each volunteer on each occasion.

RESULTS AND DISCUSSION

The mean recovery of miconazole from saliva samples was determined to be 101% (S.D. 6.93, n=5), over the range of 10—100 mg/l. This was calculated by comparing peak area counts of standards prepared in saliva to those of standards prepared in acidified water. The calibration curve was linear over the concentration range 0.5—100 mg/l (y=0.1476+0.000017x, correlation coefficient, r=0.9831) with the intercept close to zero.

Fig. 1 shows some representative chromatograms. None of the control or predose samples of saliva showed interfering peaks at the retention time of miconazole.

The results of the assays on the saliva samples from some of the volunteers are shown in Table I. There are considerable variations in the peak concentrations of miconazole detected 15 min after dosing and in the increase in peak concentration obtained in response to doubling the dose. Most subjects had reached the limit of detection (0.5 mg/l) of miconazole at 3 h after dosing.

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CHROMBIO. 1072

Note

Measurement of haloperidol in human plasma using reversed-phase high-performance liquid chromatography

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Dosage and plasma concentrations of the potent antipsychotic drug haloperidol vary over a range of almost two orders of magnitude with its present pattern of usage. Concentrations of 2—5 ng/ml seen after small dosages, push most analytical techniques to the limit of their sensitivity. Despite this, the relatively simple metabolism of haloperidol [1] makes it particularly attractive for studies relating plasma concentrations to various clinical and biochemical measures.

Gas chromatography using electron-capture [2] and nitrogen detectors [3, 4] as well as mass fragmentography [5, 6] have been applied to the measurement of haloperidol in plasma. Radioimmunoassay procedures have been reported [7, 8] and recently, Creese and Snyder [9] have published a radioreceptor binding assay for determing total plasma concentrations of active antipsychotic compounds as a class. We describe a high-performance liquid chromatography (HPLC) procedure for the quantitation of haloperidol concentrations in plasma*.

MATERIALS AND METHODS

Apparatus

We used a Series 2 high-pressure liquid chromatograph equipped with a Model 65T variable-wavelength detector (Perkin-Elmer, Norwalk, CT, U.S.A.),

* After submission of this manuscript, an HPLC procedure for haloperidol was published by Miyazaki et al. [10]. The above referenced procedure required an evaporation step, and as described with 250 nm detection, had a lower sensitivity limit of 5 ng/ml.

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a Model 7120 injector with a 100-μl loop (Rheodyne, Berkeley, CA, U.S.A.) and a 250-mm reversed-phase column (Bio-sil ODS-10 (10 μm), Bio-Rad Labs., Rockville Center, NY, U.S.A.). As alternatives, LiChrosorb RP-8 (10 μm), and Spherisorb C6 (5 μm) (Applied Science, State College, PA, U.S.A.) columns were also evaluated. Chromatograms were recorded on a Linear Instruments (Irvine, CA, U.S.A.) strip chart recorder set to 0.01 a.u.f.s.

Reagents and standards

Haloperidol and chlorohaloperidol were gifts of McNeil Pharmaceutical (Springhouse, PA, U.S.A.). Desipramine was a gift of USV Pharmaceutical Corp. (Scarsdale, NY, U.S.A.), Hexane, acetonitrile, and methanol, all distilled in glass, were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Isoamyl alcohol, reagent grade, was redistilled in glass prior to use. All other reagents were obtained from usual commercial sources.

Stock standard solutions of haloperidol, chlorohaloperidol and desipramine (1 mg/ml calculated as free base) were prepared in acidified methanol (0.1 *M* hydrochloric acid in methanol) and stored at about −15°C.

Plasma standards over a concentration range of 2—30 ng/ml were prepared by appropriately supplementing drug-free plasma with 1:100 dilution of the stock standard in 0.01 *M* hydrochloric acid. Although we prepared the plasma standards freshly on each day of analysis, an aliquoted and frozen plasma control was stable at −15°C for at least six months.

A working internal standard(s) solution was prepared by adding 5 μl of desipramine and 10 μl of chlorohaloperidol stock solutions to 10 ml of 0.1 *M* hydrochloric acid.

Serum or plasma was stored frozen at −15°C until the time of analysis.

Procedure

Two ml of plasma or serum (samples, plasma standards, or controls) and exactly 100 μl of working internal standard solution were added in succession to PTFE-lined screw-top glass test tubes, mixed, and allowed to stand for 5 min. Following addition of 0.2 ml of 2 *M* sodium hydroxide, the sample was extracted for 20 min on a rotator with 10 ml of hexane containing 2% isoamyl alcohol. After centrifugation for 5 min, the organic (upper) phase was transferred to 12-ml PTFE-lined screw-capped conical centrifuge tubes. This can be accomplished with a pasteur pipette or, more efficiently, the tube can be immersed briefly in an acetone—dry ice bath, and the unfrozen supernatant organic phase decanted.

About 150 μl of 0.1 *M* hydrochloric acid were added and after vigorous mixing using a vortex type mixer, and a 5-min centrifugation, most of the organic (upper) layer was aspirated and discarded. Approximately 100 μl of the aqueous (hydrochloric acid) phase were submitted to chromatography.

Chromatography conditions

The mobile phase consisted of 40% (v/v) acetonitrile in phosphate buffer, pH 3.8 (6.5 g KH_2PO_4 in 1000 ml water adjusted to pH 3.8—4.0 with orthophosphoric acid). The optimal proportion of acetonitrile varied with the age and condition of the column. The column oven was maintained at 50°C. The

overall performance of the system was verified periodically by injecting about 20 ng each of a mixture of haloperidol, chlorohaloperidol and desipramine.

RESULTS AND DISCUSSION

Fig. 1 illustrates chromatograms resulting from the analysis of serum from two patients who had been treated with haloperidol. Fig. 2 shows serial steady state concentrations in a patient maintained on a stable dose of haloperidol. Table I illustrates steady state levels in ten patients receiving various doses of haloperidol. Steady state concentrations are known to vary considerably between individuals on the same dosage. [11].

Peak height ratios of haloperidol to each of the internal standards showed a linear relationship to serum haloperidol concentration over a range of at least 2—60 ng/ml. While concentrations as low as 1 ng/ml could be detected, 2 ng/ml

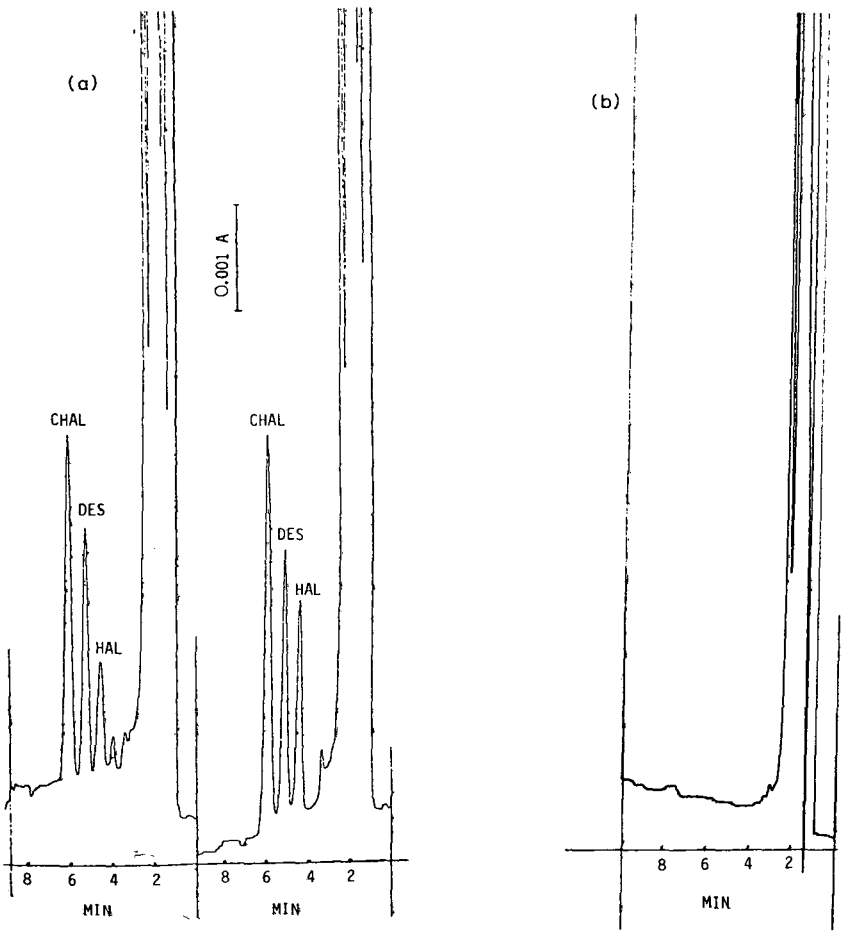

Fig. 1. (a) Chromatogram of extracts from patient sera containing 9.4 ng/ml (left) and 17.1 ng/ml (right) of haloperidol, respectively. Peaks: HAL = haloperidol; DES = desipramine; CHAL = chlorohaloperidol. (b) Chromatogram of extract from a blank (haloperidol-free) serum.

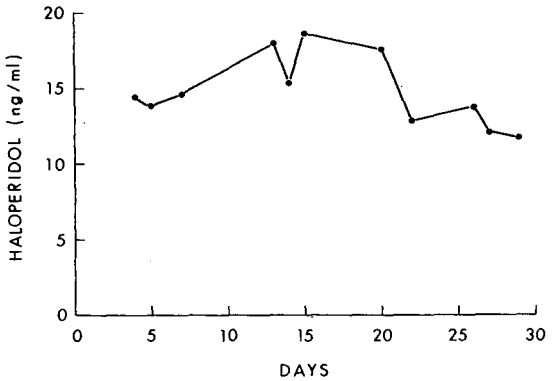

Fig. 2. Steady state serum haloperidol concentrations in a patient receiving 17 mg of drug per day. Values are a.m. trough concentrations, approximately 10 h after last dose.

TABLE I

ILLUSTRATIVE STEADY STATE TROUGH HALOPERIDOL CONCENTRATIONS

Values are trough concentrations collected a.m. before morning dose, approximately 10 h after last dose.

Dose (mg/day)	Haloperidol concentration (mg/ml)
10	3.8
10	7.8
12	7.7
12	5.9
15	17.5
17	17.2
20	6.0
40	32.1
40	32.2
80	105

is probably the lower limit for useful quantitation. For measurement of concentrations below 5 ng/ml, a UV detector capable of maintaining a reasonably stable and smooth baseline at 0.01 a.u.f.s., at 195 nm, is needed. Most modern variable-wavelength detectors can achieve this.

The within-day and between-day coefficients of variation were 5.0 and 7.3%, respectively, at a concentration of 5 ng/ml. Multiple drug-free sera were tested and found to be free of interfering peaks. Absolute uncorrected recovery achieved with the described double extraction procedure was 78%, but was compensated by carrying serum or plasma standards through the extraction procedure.

When measuring very low concentrations of drugs, trace contamination (endogenous or exogenous) can significantly distort the data. We have therefore found it useful to use two internal standards in that consistency in their peak height ratio provides assurance that otherwise undetectable interfering peaks

are not co-eluting with either internal standard. In the absence of interferences, either desipramine or chlorohaloperidol can be used to calculate haloperidol concentrations. Basic drugs tested that did not interfere included caffeine, nicotine, cogentin, codeine, meperidine, normeperidine, propoxyphene, chlordiazepoxide, norchlordiazepoxide, flurazepam, desalkylflurazepam, and various antihistamines. Nortriptyline eluted close to chlorohaloperidol, necessitating that desipramine be used as the internal standard. Concurrent administration of imipramine or desipramine of course necessitates use of chlorohaloperidol for quantitation. While chloropromazine itself did not interfere, the easily recognized cluster of chloropromazine metabolites may obscure the peaks of interest. Concurrent use of two neuroleptics is uncommon in clinical practice, and certainly not likely in clinical research studies.

We found that the ODS column provided the best overall performance in routine use. There are, however, selected instances of polypharmacy when the alternative columns are helpful. Using the ODS column, nordiazepam and diazepam were not adequately resolved from haloperidol and chlorohaloperidol, respectively. The presence of these interferences can be detected by distortion of the ratio between the two internal standards. Should it be necessary to measure haloperidol in patients receiving diazepam, this benzodiazepine and its major metabolite were well separated from haloperidol and chlorohaloperidol on a LiChrosorb RP-8 column. Similarly, diphenhydramine (Benadryl) co-eluted with haloperidol. Although alternative medications to diphenhydramine are readily available, it was separated from haloperidol (using 33% acetonitrile) on the Spherisorb C6 column, although retention times were much longer. Desipramine did not adequately separate from haloperidol on the C6 column or from nordiazepam on the RP-8 column. Therefore, with either alternative column only one internal standard (chlorohaloperidol) is suitable.

Various options are now available for measurement of haloperidol in plasma. Some radioimmunoassays have been reported to yield higher concentrations than gas–liquid chromatography [12] suggesting that the former may cross-react with one or more circulating metabolites. On the other hand, the receptor binding assay has been reported to produce higher concentrations than a radioimmunoassay [13]. The receptor binding assay [8] may be especially attractive for correlating total active drug concentration with various measures of pharmacological activity. On the other hand, a specific physical measurement (e.g., chromatography) is more desirable for any studies which include pharmacokinetic considerations.

Evaluating gas–liquid chromatography using a variety of columns we were troubled by apparent adsorptive losses, and non-linear recovery. We had much greater success with reversed-phase HPLC which was also more efficient in that sample preparation was simpler and no evaporation step was required.

Sufficient data are not yet available to support a major clinical role for the therapeutic monitoring of antipsychotic drugs. However, there are preliminary data to suggest that haloperidol may be a good candidate in this instance [13–16]. Certainly, its apparent lack of major active metabolites suggests that measurement of the parent compound may be useful in clinical correlation studies. The HPLC procedure described is sufficiently sensitive to cover the range of concentrations anticipated after clinical use.

ACKNOWLEDGEMENT

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Note

High-performance liquid chromatographic analysis of pentazocine in blood and plasma

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Pentazocine [1,2,3,4,5,6-hexahydro-*cis*-6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocin-8-ol] is a narcotic analgesic, structurally related to morphine, which has been widely used in man. Various analytical techniques including fluorimetry [1], gas chromatography [2,3], radioimmunoassay [4] and thin-layer chromatography [5], have been developed for pentazocine, although to date no high-performance liquid chromatographic (HPLC) method has been reported.

In the present investigation, an HPLC method for pentazocine has therefore been developed to allow rapid quantification of the drug in biological fluids.

Pentazocine has a relatively low molar extinction coefficient of 1910 (λ_{max} = 278 nm), and per se is not readily detected by its UV absorption. To overcome this sensitivity limitation, a derivative of pentazocine with 2-*p*-chlorosulphophenyl-3-phenylindone (DIS-CL), previously used by Vinson et al. [6] in the thin-layer chromatographic analysis of narcotic analgesics has been used. DIS-CL reacts with the phenolic hydroxyl group of pentazocine to produce a derivative with a λ_{max} at 275 and has a considerably greater UV absorption than pentazocine itself.

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MATERIALS AND METHODS

Extraction of pentazocine

One milliliter of whole blood or whole blood β-glucuronidase—sulphatase hydrolysate was alkalinised by the addition of 0.2 ml 1 *M* sodium hydroxide, 1 ml of levallorphan (internal standard, 0.2 mg/ml in water) was added, and the mixture was extracted by shaking with 2 × 5 ml dichloromethane for 10 min. After centrifugation, the organic phase was removed, combined and evaporated to dryness at 45°C under a stream of dry nitrogen.

The residue was redissolved in 100 μl acetonitrile and 5-μl aliquots of DIS-CL (1 mg/ml in acetonitrile), and 2 *M* sodium carbonate were added. Derivatization was complete after heating at 45°C for 10 min. The reaction mixture was evaporated under a stream of dry nitrogen and the residue redissolved in 100 μl of the HPLC mobile phase.

High-performance liquid chromatography

Analyses were performed using a Waters Assoc. (Milford, MA, U.S.A.) HPLC system Model M6000A pump, Model 440 detector set at 280 nm, U6K injector and C_{18} μBondapak column (30 cm × 4 mm I.D.; 10 μm particle size). A mobile phase of acetonitrile—0.7% ammonium chloride (adjusted to pH 8 with ammonium hydroxide) (80:20) was pumped at a flow-rate of 2 ml/min.

In situ isolated rabbit intestinal loops

The technique used was essentially that described by George et al. [7]. Briefly, using unanaesthetized rabbits, a 10—15 cm section of jejunum was isolated between two catheters and pentazocine solution (10.4 m*M*) was introduced into the gut lumen. Venous blood from the isolated loop was collected in fractions (2—4 min periods for 46 min) by means of a 21-gauge scalp vein catheter placed in the mesenteric vein draining the isolated loop. Aliquots of blood (0.2—0.4 ml), 1 ml of acetate buffer pH 5.0, and 1 ml of β-glucuronidase (E.C. 3.2.1.31)—sulphatase (E.C. 3.1.6.1) mixture (1000 U/ml) were incubated at 37°C for 48 h to achieve total hydrolysis of conjugated pentazocine.

Materials and reagents

Dichloromethane (nanograde, Mallinckrodt, St. Louis, MO, U.S.A.), acetonitrile (HPLC grade, Waters Assoc.), levallorphan·HCl (Roche Products, Sydney, Australia), pentazocine (Winthrop Labs., Sydney, Australia), 2-*p*-chlorosulphophenyl-3-phenylindone (DIS-CL; Polysciences, Warrington, PA, U.S.A.). β-Glucuronidase—sulphatase was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical reagent grade.

RESULTS AND DISCUSSION

Under the conditions used for the HPLC separation, pentazocine and levallorphan have retention times of approximately 5.7 min and 7.8 min respectively (Fig. 1).

Other structurally similar narcotics had very similar retention times (codeine 5.5 min, morphine 5.9 min, ethylmorphine 6.2 min, and methadone 5.8 min).

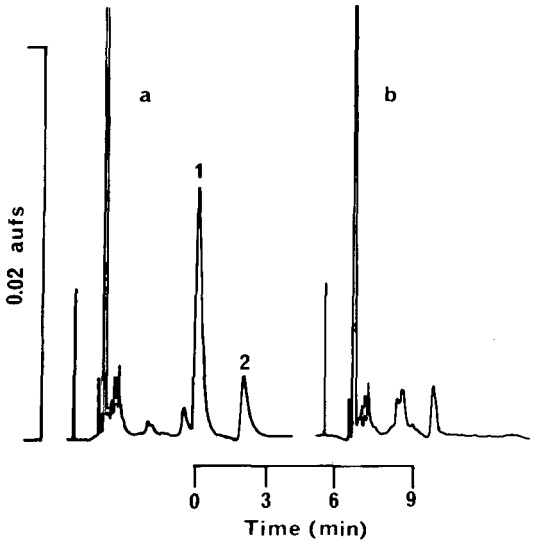

Fig. 1. Chromatograms of an extract of whole blood (pentazocine added) showing (a) separation of pentazocine (1) 5 μg/ml and the internal standard levallorphan (2) 2 μg/ml, and (b) an extract of drug-free blood.

TABLE I

COMPARISON OF ANALYTICAL METHODS FOR PENTAZOCINE

Method	Sensitivity	Comments	Reference
Thin-layer chromatography	1 μg/ml		Reid and Gerbeck [5]
Fluorimetry	50 ng/ml	Selectivity?	Berkowitz et al. [9]
Gas chromatography (flame ionisation detector)	not stated	Large volume of plasma required	Vaughn and Beckett [2]
Gas chromatography (electron-capture detector)	100 ng/ml	Extensive clean-up needed in sample preparation	Pittman and Davison [10]
Gas chromatography (electron-capture detector)	1 ng/ml		Brötell et al. [11]
Gas chromatography (electron-capture detector)	1 ng/ml		Swezey et al. [3]
Radioimmunoassay	1 ng/ml		Peterson et al. [4]

Results were quantified from a plot of peak height ratio pentazocine to internal standard versus pentazocine concentration, which was found to be linear over the range 20 ng—10 μg pentazocine base per ml blood.

Recovery of pentazocine through the extraction procedure, assessed by measuring peak height of the pentazocine derivative prepared from a blood extract (1 μg/ml) with that from a non-extracted standard, was found to be 78%. The relative standard deviations of the method at 200 ng, 1 μg and 10 μg pentazocine per ml blood were 4% (n = 6), 3.8% (n = 4) and 3.5% (n = 4), respectively. Blood to plasma ratios (rabbit blood) of 2.05 ± 0.2 (mean ± S.D.; n = 6) were obtained over the concentration range 1—10 μg/ml. Ehrnebo et al. [8] obtained a mean value of 1.06 for human blood at 200 ng pentazocine per ml.

This difference may be a reflection of the drug concentration range used, or of inherent differences in blood chemistry between the two species.

The HPLC method described above can be compared to previously reported methods by reference to Table I. It has a detection limit of approximately 10 ng/ml, which makes it 5—10 times more sensitive than fluorimetric or some gas chromatographic methods, but is some 10 times less sensitive than electron-capture gas chromatography or radioimmunoassay. A major advantage of the HPLC method is that it requires minimal sample preparation by comparison to the extensive clean-up procedures needed for electron-capture gas chromatography.

Fig. 2 illustrates the application of the HPLC method to the measurement of pentazocine in venous blood from an isolated in situ rabbit intestinal loop preparation. Both free (unmetabolised) and total (after hydrolysis with β-glucuronidase—sulphatase) blood pentazocine were measured. The difference between total and free drug gives a measure of the extent of conjugation of pentazocine. The results indicate that peak concentration of pentazocine occurs at about 4 min, and that there is substantial metabolism of the drug during intestinal absorption.

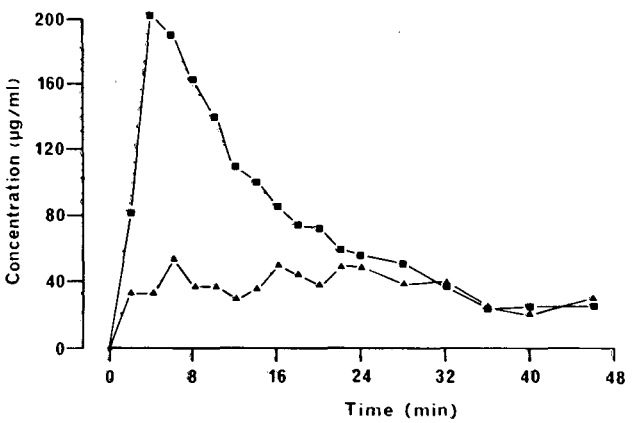

Fig. 2. Blood concentration—time curve for unmetabolized pentazocine (■) and conjugated pentazocine (▲) in mesenteric venous blood from an isolated in situ rabbit intestinal loop containing 10.4 m*M* pentazocine solution.

In summary, we have developed a rapid, simple, sensitive analytical technique for pentazocine in blood and have demonstrated its application in studying the intestinal absorption of this drug. Use of a fluorimetric detector would further increase sensitivity. Since steady-state concentrations of pentazocine in blood following administration of the usual analgesic dose to man are in the range 10—50 ng/ml [2] this method should also be applicable to the monitoring of pentazocine in the clinical situation.

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Note

Micro determination of gentamicin in serum by high-performance liquid chromatography

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Gentamicin is a widely used aminoglycoside antibiotic for treatment of serious infections with gram-negative bacilli. It has, however, a narrow therapeutic range and exerts adverse side-effects of nephrotoxicity and ototoxicity. Therefore, monitoring of gentamicin levels in serum is necessary for the best therapeutic effects.

Recently, several methods using high-performance liquid chromatography (HPLC) have been developed for determination of the three major components of gentamicin in serum or plasma [1—11]. Some of these methods have used fluorescence detection with pre-column or post-column derivatization of gentamicin [1—8]. Others have employed ultraviolet absorption detection with pre-column derivatization of gentamicin [8—10]. All these methods require time-consuming sample pretreatment and a large volume of sample for the determination of gentamicin in serum or plasma.

This report describes a simple micro-scale method for the analysis of gentamicin in serum.

EXPERIMENTAL

Reagents

Gentamicin sulfate (manifested potency, 552 μg/mg) was obtained from Shionogi (Osaka, Japan), *o*-phthalaldehyde was from Nakarai Chemicals

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(Kyoto, Japan), Brij-35 from Wako Pure Chemicals (Osaka, Japan), sodium octanesulfonate from Kanto Chemicals (Tokyo, Japan) and disodium 1,2-ethanedisulfonate from Tokyo Kasei Kogyo (Tokyo, Japan). De-ionized distilled water was used throughout this investigation. Acetonitrile and methanol were of liquid chromatographic grade. All other chemicals were of reagent grade.

Counter-ion reagent was prepared to contain 0.2 *M* disodium 1,2-ethanedisulfonate and 0.01 *M* sodium octanesulfonate in water adjusted to about pH 2.5 with acetic acid.

Mobile phase was prepared to contain 0.1 *M* disodium 1,2-ethanedisulfonate and 0.005 *M* sodium octanesulfonate in a water—acetonitrile mixture (85 : 15, v/v) adjusted to about pH 3.5 with acetic acid.

o-Phthalaldehyde (OPA) reagent was prepared according to the method of Anhalt and Brown [5].

Apparatus and chromatographic conditions

Fig. 1 shows the flow diagram of the chromatographic system. A Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.) was used to deliver the mobile phase (1.5 ml/min) and a Model U6K universal injector (Waters Assoc.) was used for injection of samples. A μBondapak C_{18} column (particle size 10 μm, 30 cm × 3.9 mm I.D., Waters Assoc.) was used for the analyses. The OPA reagent was delivered at a flow-rate of 0.8 ml/min to the column effluent via a mixing T-piece with a Model 3000 pump (Waters Assoc.). A reaction coil consisting of a Teflon tube (5 m × 0.25 mm I.D.) was placed in a 50°C water-bath between the mixing T-piece and a detector. A Model S-FL-330 fluorometer (Soma Optics, Tokyo, Japan) equipped with a FL4BLB lamp (energy maximum at 365 nm, excitation), a 440-nm cut-off filter (emission) and a 70-μl quartz flow-cell was used to detect the fluorescent product. Detector signals were recorded with a Model VP6621A recorder (Matsushita Communication Industrial, Osaka, Japan).

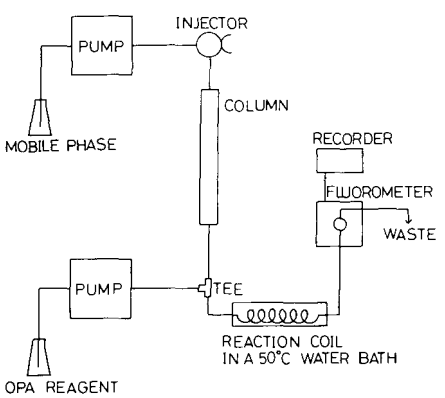

Fig. 1. Flow diagram of the chromatographic system used for separation and fluorescence detection.

Procedure

A 20-μl serum sample in a 1.5-ml tapered polypropylene centrifuge tube was vortex-mixed with 100 μl of methanol for a few seconds, then centrifuged at about 7800 *g* in a Model H-25FI centrifuge (Kokusan, Tokyo, Japan) for 1 min. Then 200 μl of the counter-ion reagent were directly added to the supernatant, which was vortex-mixed again for a few seconds and centrifuged at about 7800 *g* for 1 min. A 240-μl aliquot of the supernatant containing the counter-ion reagent was injected into the chromatograph.

Standard serum samples supplemented with various concentrations of gentamicin (potency 2.7—16.5 μg/ml) were prepared for chromatographic analysis. Peak area measurements were used to construct the standard curve.

Every sample was analysed in duplicate. The sum of the areas of gentamicin components was measured and averaged.

RESULTS

It was observed frequently that gentamicin-containing sera after deproteinization gave inconsistent chromatograms. To avoid this problem, a counter-ion reagent was added to the protein-free supernatant prior to injection of samples into the chromatograph. It was found that the chromatograms obtained after this treatment were virtually identical with those obtained with standard gentamicin dissolved in mobile phase.

Typical chromatograms of gentamicin components from a patient's serum and of serum blanks with and without antibiotic supplementation are shown in Fig. 2. Individual components were identified by their retention times. The serum blanks so far examined showed no interfering peaks. A small peak appearing between gentamicin C_2 and C_1 was observed with standard gentamicin samples as well as with extracts of patient's sera. The peak presumably corresponds to a minor component of a gentamicin C complex.

Linear regression analysis of the standard curve from 2.7 to 16.5 μg/ml yielded the following equation: $y = 0.792x - 0.126$ ($r = 0.999$). A good linear relationship was obtained between peak areas and gentamicin concentrations in

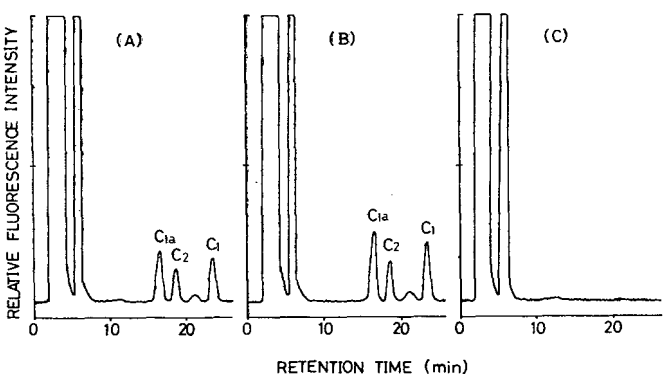

Fig. 2. Chromatograms of gentamicin components: (A) a patient serum; (B) a serum blank supplemented with 10.0 μg of gentamicin per ml; (C) a serum blank. C_{1a} = gentamicin C_{1a}, C_2 = gentamicin C_2, C_1 = gentamicin C_1.

serum in the range studied, even though no internal standard was used in the method. The limit of sensitivity of this method is about 0.5 μg/ml.

Analytical recovery of gentamicin added to serum was determined by comparing the peak areas from a serum sample containing 5.5 μg of gentamicin per ml with the results obtained from an aqueous standard of the same concentration. The recovery was between 97 and 103%.

The precision of this method is summarized in Table I. Within-run precision was estimated by analyzing ten aliquots of each of two serum pools containing gentamicin in concentrations of 5.5 and 11.0 μg/ml, respectively. Day-to-day precision was estimated by analyzing an aliquot of each pool for ten days. The within-run precision was less than 2.5% and day-to-day precision was less than 3.2%.

The results obtained by the proposed method were compared with those obtained using a conventional microbiological method (Fig. 3). The correlation coefficient was 0.934.

TABLE I

PRECISION STUDIES

Gentamicin concentration in serum (μg/ml)	Coefficient of variation (%)	
	Within-run (n = 10)	Day-to-day (n = 10)
5.5	2.5	3.2
11.0	2.0	2.3

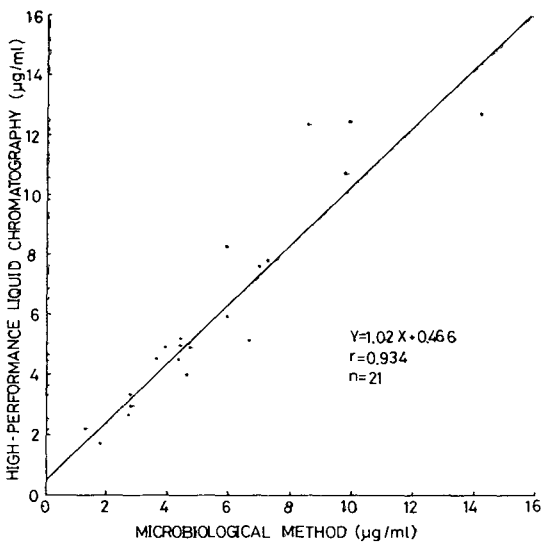

Fig. 3. Comparison with the microbiological method.

DISCUSSION

As reported previously [12], at a serum/methanol ratio of 1:5 (v/v), deproteinization was essentially complete. The counter-ion reagent could be

added directly to the supernatant without disturbing the precipitate of serum proteins denatured with methanol which adhered tightly to the bottom of the tapered polypropylene centrifuge tubes after centrifugation. No serum proteins were detected by the Lowry method in the supernatant containing the counter-ion reagent.

The method described above simplifies sample pretreatment greatly by avoiding tedious steps such as solvent extraction [1, 4, 6—11] and column chromatography [2, 3, 5] and reduces the analysis time significantly.

Ion-pair chromatography was effective in eliminating the interference caused by serum components such as amino acids, small peptides and amines, which form fluorescent products with the OPA reagent during gentamicin determination. In the presence of sodium octanesulfonate the retention time of gentamicin with five amino groups was longer than of those with fewer amino groups. The addition of disodium 1,2-ethanedisulfonate increased the resolution of the three major components of gentamicin with subtle molecular differences. The attempts to use sodium sulfate [7], potassium methanesulfonate or sodium methylsulfate instead of disodium 1,2-ethanedisulfonate resulted in the appearance of an interfering peak and poor resolution of the gentamicin components.

This procedure for the determination of gentamicin in serum appears to be potentially applicable to the determination of other aminoglycosides used in clinical treatment such as kanamycin, tobramycin and amikacin. Serum volumes of as little as 20 μl provide a further advantage for monitoring pediatric patients.

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Note

Quantitative determination of pertechnetate by high-performance liquid chromatography with UV detection

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In the practice of diagnostic nuclear medicine, some chemical form of a gamma-ray emitting isotope is administered to a patient with the goal of having the isotope localize in a specific organ. Subsequent scanning of the organ with a gamma-ray camera provides valuable diagnostic and prognostic data by an essentially noninvasive technique [1,2].

Technetium-99m is the isotope of choice for diagnostic nuclear medicine because of its optimal nuclear properties, its diverse chemistry, and its ready availability by means of the $^{99}Mo/^{99m}Tc$ generator [3—5]. This generator is based upon the nuclear decay scheme

$$^{99}Mo \xrightarrow[66\ h]{\beta^-} {}^{99m}Tc \xrightarrow[6\ h]{\gamma} {}^{99}Tc$$

and the chemical fact that the highest oxidation states of Mo and Tc have different ionic charges (MoO_4^{2-} and TcO_4^-, molybdate and pertechnetate, respectively in neutral, aqueous solution). The manufacturer loads $^{99}MoO_4^{2-}$ onto a shielded alumina column and then ships this generator to the point of use. In the hospital the column is eluted with normal saline (0.15 *M* sodium chloride) once every working day; upon elution, the —1-charged pertechnetate (both $^{99m}TcO_4^-$ and $^{99}TcO_4^-$) is eluted whereas the —2-charged molybdate is retained. This eluate can be used directly to obtain a ^{99m}Tc pertechnetate scan. But more often the eluate is subjected to one of a variety of chemical reactions in which the technetium is reduced to a lower oxidation state and simultaneously chelated by a ligand to generate a reduced ^{99m}Tc radiopharmaceutical with specific biological properties [1—6].

The concentration of ^{99m}Tc in the generator eluate is of prime importance in nuclear medicine applications since it is the gamma-ray emission from this

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isotope which provides the diagnostic image. However, the eluate also contains significant amounts of ^{99}Tc because of the characteristics of the ^{99}Mo—^{99m}Tc—^{99}Tc decay scheme, and this concentration is important for two reasons. First, it is the total chemical concentration of pertechnetate (both $^{99m}TcO_4^-$ and $^{99}TcO_4^-$) in the eluate that determines the chemistry and kinetics of the conversion of pertechnetate into radiopharmaceuticals containing technetium in a reduced oxidation state [3]. Secondly, the amount of ^{99}Tc, which is a long-lived ($t_{1/2} = 2 \cdot 10^5$ year) beta-emitter, injected into a patient must be taken into account when calculating the total radiation dose received by the patient.

The total amount of technetium, as both ^{99}Tc and ^{99m}Tc, in generator eluates varies with the age and history of the generator, and, of course, with the time elapsed since the last elution of the generator. The amounts of ^{99m}Tc and ^{99}Tc in any given generator eluate can be calculated from the ^{99}Mo—^{99m}Tc—^{99}Tc decay scheme, given certain assumptions about the history of the generator [7,8]. However, these assumptions are difficult to verify and, to date, the calculations have not been experimentally validated. Determination of ^{99m}Tc in the eluate presents no problems since this isotope can be accurately monitored by means of its gamma-emission. Determination of ^{99}Tc by radiochemical means is very difficult because of its low-energy beta-emission (0.29 MeV), the low concentration of this isotope in the eluate, and the presence of other long-lived radioactive impurities in most generator eluates [9]. We have therefore undertaken this study to determine the total amount of chemical pertechnetate (as both $^{99m}TcO_4^-$ and $^{99}TcO_4^-$) in generator eluates by chemical means. This non-radiochemical procedure employs high-performance liquid chromatography (HPLC) to separate pertechnetate from other generator constituents and impurities, and UV detection to quantitatively monitor the concentration of pertechnetate.

MATERIALS AND METHODS

Chemicals

Technetium in the form of TcO_4^- in 0.9% sodium chloride was obtained from the Radioisotope Laboratory at Cincinnati General Hospital as the first elution of a $^{99}Mo/^{99m}Tc$ generator (7.5 Ci, Union Carbide, Tuxedo, NY, U.S.A.).

Crystalline $NH_4{}^{99}TcO_4$ (Oak Ridge National Laboratories, Oak Ridge, TN, U.S.A.) was converted to $K^{99}TcO_4$ by metathesis with potassium hydroxide. This material was recrystallized and dried before being used for the preparation of standard solutions.

Standard solutions

Stock solutions of $6.00 \cdot 10^{-4}$ *M* and $4.35 \cdot 10^{-4}$ *M* $K^{99}TcO_4$ were prepared by weight in 0.10 *M* and 0.03 *M* acetate, pH 4.5, respectively. Appropriate dilutions of these with the corresponding elution buffers yielded a series of standard solutions.

Reagents

Elution buffers, 0.10 *M* and 0.03 *M* in total acetate concentration, pH 4.5, were freshly prepared from glacial acetic acid, A.R., sodium hydroxide, A.R., and triply distilled, charcoal filtered water. Prior to use, these were filtered through 0.22-μm GS membranes (Millipore, Befored, MA, U.S.A.) and deaerated by sonication under vacuum.

Chromatographic apparatus

The chromatographic equipment included a Waters Assoc. Model M45 solvent delivery system, a Rheodyne Model 7125 injection valve fitted with a 100-μl sample loop and a PD-2 12-in. pulse dampener, all obtained from Bioanalytical Systems (West Lafayette, IN, U.S.A.).

A LiChrosorb 10-μm amino bonded phase 5-cm cartridge guard column (Brownlee Labs., Santa Clara, CA, U.S.A.) was installed between the injection valve and the analytical column.

The analytical column was a 250 mm × 4.6 mm stainless-steel Knauer column (Unimetrics, Anaheim, CA, U.S.A.) slurry packed with Spherisorb amino bonded phase, 5 μm (Phase Separations, Hauppauge, NY, U.S.A.). Isocratic elution of these columns with the aqueous acetate buffers at 1.5 ml/min resulted in typical operating pressures around 12.4 MPa (1800 p.s.i.) at ambient temperature.

UV detection

The absorbance of the eluent was monitored at 254 nm with a Beckman Model 153 UV detector equipped with an 8-μl flow-cell of 1 cm pathlength. The detector output was recorded with an Omniscribe B-5000 dual-channel strip chart recorder (Houston Instruments, Austin, TX, U.S.A.).

Radiometric detection

Chromatographic detection of the gamma activity of eluting $^{99m}TcO_4^-$ was accomplished with a scintillation spectrometry system (Harshaw Chemical Co., Solon, OH, U.S.A.). This consisted of a NA-23 stabilized amplifier/single channel analyzer, a NR-22 linear ratemeter, and a NV-32A high-voltage power supply all mounted in an AP-2H nuclear instrument module (Berkeley Nucleonics, Berkeley, CA, U.S.A.). The detector was a shielded 5.1 × 5.1 cm cylindrical NaI(Tl) crystal with a 1.4 cm I.D. hole optically coupled to a 5.1-cm Amperex PM 2202 photomultiplier tube. The ratemeter signal was fed to the second channel of the recorder permitting simultaneous monitoring of gamma activity and optical absorbance. Finally, absolute amounts of ^{99m}Tc activity in collected fractions were determined with a CRC-6A Radioisotope Calibrator (E.R. Squibb and Sons, Princeton, NJ, U.S.A.).

Quantitative analysis

A standard curve (Fig. 1A) under conditions of minimum retention and maximum detectability was generated by sampling eighteen different concentrations of $KTcO_4$, ranging from $6.00 \cdot 10^{-8}$ *M* to $6.00 \cdot 10^{-4}$ *M* in 0.10 *M* acetate buffer, pH 4.5. A second standard curve (Fig. 1B) under conditions more suited to the analysis of $^{99}Mo/^{99m}Tc$ generators was obtained by sampling

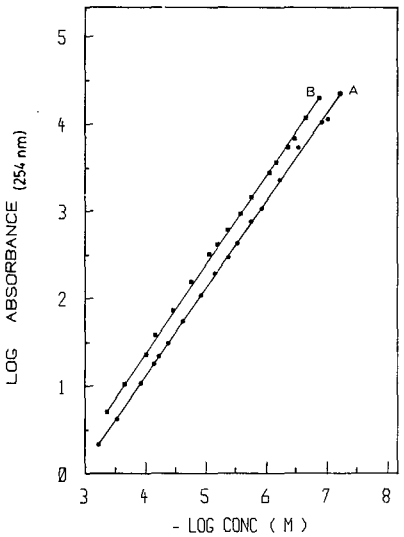

Fig. 1. Calibration graphs of $K^{99}TcO_4$ with mobile phases of (A) 0.10 *M* acetate, pH 4.5 (t_R = 4.3 min) and (B) 0.03 *M* acetate, pH 4.5 (t_R = 7.3 min). Conditions: flow-rate 1.5 ml/min; detection 254 nm; temperature ambient.

seventeen different concentrations of $KTcO_4$, ranging from $1.30 \cdot 10^{-7}$ *M* to $4.35 \cdot 10^{-4}$ *M* in 0.03 *M* acetate buffer, pH 4.5. For each concentration of $K^{99}TcO_4$, six replicate chromatograms were obtained; the peak heights were corrected for noise and measured manually. The six peak heights were then averaged, and the standard deviation of the mean (σ_m) was calculated. Logarithmic calibration curves were constructed by plotting average peak height [in absorbance units, weighted as $1/(\sigma_m)^2$] vs. $K^{99}TcO_4$ concentration, and were then analyzed by a linear least squares treatment (Table I).

RESULTS AND DISCUSSION

Initial experiments designed to optimize conditions for pertechnetate analysis resulted in a detection limit of $6.00 \cdot 10^{-8}$ *M* TcO_4^- in 0.10 *M* acetate, pH 4.5, the retention time of pertechnetate being 4.3 min. However, it was found that analysis of pertechnetate eluted from a $^{99}Mo/^{99m}Tc$ generator was not possible under these conditions due to refractive index changes and other unknown chromatographic interferences occurring at or near this retention time. The mobile phase was therefore adjusted to increase the retention time of pertechnetate. With 0.03 *M* acetate, pH 4.5, the retention time of pertechnetate is 7.3 min and the detection limit for $K^{99}TcO_4$ is $1.30 \cdot 10^{-7}$ *M*. A typical chromatogram obtained under these conditions is shown in Fig. 2A.

Fig. 1 shows that the calibration curves under both sets of conditions are linear over four orders of magnitude of pertechnetate concentration. This linearity extends at least two orders of magnitude beyond the pertechnetate concentrations expected in $^{99}Mo/^{99m}Tc$ generator eluates. Statistical data describing each calibration curve are summarized in Table I.

Analysis of the first eluate from 7.5 Ci Union Carbide $^{99}Mo/^{99m}Tc$ generator

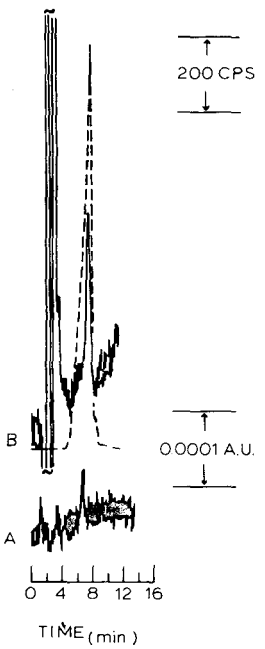

Fig. 2. Typical chromatograms of (A) $1.30 \cdot 10^{-7}$ *M* $K^{99}TcO_4$ and (B) $^{99}Mo/^{99m}Tc$ generator first eluent (37 μCi/ml). Conditions: eluent 0.03 *M* acetate, pH 4.5; flow-rate 1.5 ml/min; detection 254 nm (——), γ (— — —); temperature ambient.

TABLE I

WEIGHTED LINEAR LEAST SQUARES ANALYSIS FOR $K^{99}TcO_4$ STANDARD CURVES (LOG VS. LOG)

	t_R (A) (4.3 min)	t_R (B) (7.3 min)
Slope	1.011 ± 0.004	1.043 ± 0.006
Y-Intercept	−2.926 ± 0.015	−2.80 ± 0.03
Correlation coefficient	0.9998	0.9997

(detection at 254 nm) yields the chromatogram shown in Fig. 2B (solid line), with a single component eluting at a retention time of 7.3 min. Gamma detection (Fig. 2B, broken line) also shows a single major component with a retention time of 7.3 min, confirming that the component is indeed pertechnetate. From the slope and intercept parameters for standard curve B (Table I) the concentration of pertechnetate in the $^{99}Mo/^{99m}Tc$ generator eluate is calculated to be $(8.0 \pm 1.6) \cdot 10^{-7}$ *M*, five times greater than the detection limit. In this experiment, 100 ± 7% of the initially injected gamma activity is recovered from the HPLC column within the peak at a retention time of 7.3 min. Therefore, this particular generator eluate does not contain significant amounts of reduced forms of ^{99m}Tc, and the HPLC procedure itself does not induce significant reduction of pertechnetate. Upon increasing the gamma sensitivity, two additional radioactive components eluting prior to TcO_4^- are observed

(Fig. 3). These extremely minor components each comprise approximately 0.01% of the total activity applied and have been verified by multichannel pulse height analysis to contain only ^{99m}Tc (possibly as reduced hydrolyzed species).

Fig. 3. Chromatogram of $^{99}Mo/^{99m}Tc$ generator eluent (≈60 mCi/ml) with gamma-detection. Conditions: eluent 0.1 *M* acetate, pH 4.5; flow-rate 1.5 ml/min; temperature ambient.

Attempts at detecting MoO_4^{2-} (a breakthrough product of $^{99}Mo/^{99m}Tc$ generators) were performed by chromatographing standard Na_2MoO_4 solutions under similar conditions of generator analysis. Since no peaks were observed after 5.5 h of elution (1.5 ml/min), it is concluded that MoO_4^{2-} is either totally retained on the column or has been sufficiently diluted so as to be undetectable, and it thus poses no serious interference in the analysis of TcO_4^-.

From these results it is clear that HPLC with UV detection has the sensitivity and selectivity necessary to monitor total chemical pertechnetate in $^{99}Mo/^{99m}Tc$ generator eluates. The applicability of this technique has been substantiated by our current investigation involving the measurement of total TcO_4^- in the eluates of several $^{99}Mo/^{99m}Tc$ generators over their entire useful clinical lifetime [10]. We have noticed that the absolute sensitivity varies slightly, however, due to both the age of the column and variability in column-to-column efficiencies.

Although some column deterioration (loss of retention) was observed over the course of the study, this analysis should be of considerable utility in monitoring the function of clinical generators, as well as in the routine analysis of reduced ^{99m}Tc radiopharmaceuticals for the presence of undesired pertechnetate.

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Note

Screening method for the detection of aflatoxin and metabolites in human urine: aflatoxins B_1, G_1, M_1, B_2a, G_2a, aflatoxicols I and II

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Ingestion of aflatoxins has been associated with the occurrence of hepatocellular carcinoma (HCC) [1], and the identity of the eventual carcinogenic agent has been closely linked to the way in which aflatoxin is metabolised in the liver [2]. It has been shown that the sensitivity of animal species to the carcinogenic effects of aflatoxin B_1 can be directly correlated with hepatic activity and its transformation into aflatoxicol [3]. In man, hepatic metabolism and detoxification of aflatoxins may be related to a number of environmental influences [1]. Among these, the presence of acute or chronic liver pathology (cirrhosis, HCC, hepatitis) might influence the metabolism of aflatoxin in the liver, and this effect may be manifested in the type and concentration of metabolites excreted. Identification and quantification of aflatoxin metabolites may yield information concerning the mechanisms of aflatoxin metabolism in man.

This paper describes the successful extension of a simple technique for the extraction and quantification of aflatoxin M_1 from human urine [4] to aflatoxin B_1 and the metabolites aflatoxicol I and II, aflatoxin B_2a, G_2a and the derivative tetrahydrodeoxyaflatoxin B_1.

EXPERIMENTAL

The method used for aflatoxin extraction was based on the technique described by Messripour and Nesheim [4]. Aflatoxin M_1, aflatoxicols I and II, aflatoxins B_2a, G_2a and tetrahydrodeoxyaflatoxin B_1 were supplied by Makor Chemicals (Jerusalem, Israel). Aflatoxins B_1 and G_1 were supplied by Aldrich (Milwaukee, WI, U.S.A.). The recovery experiments used human urine spiked with a known amount of standard aflatoxin in the range 0.2—1.0 ng/ml for aflatoxins B_1, G_1, and 2.0—4.0 ng/ml for the metabolites. Celite 545 filter

aid (10 g) (Johns Manville, Denver, CO, U.S.A.) was added to 100 ml urine. The mixture was stirred continuously for 10 min with a magnetic stirrer whilst 200 ml of distilled acetone were added slowly, then the mixture was filtered into a 600-ml beaker.

The filtrate was stirred and 100 ml of distilled water added, followed by 10 ml of a 20% lead acetate solution. The mixture was allowed to stand for 5—10 min, until the precipitate coagulated, and 5 ml of saturated sodium chloride slowly added with stirring, followed by 5 g of Celite. Stirring was continued for a further 1—2 min before the mixture was filtered into a 600-ml beaker. The filtrate was stirred and a freshly prepared ferric hydroxide slurry (30 ml 6.7% $FeCl_3$ and 170 ml 0.2 *M* sodium hydroxide) added, followed by 5 g Celite 545. The mixture was transferred into two 500-ml separating funnels and 50 ml of 0.1% sulphuric acid added to each. The filtrate in each flask was extracted with two portions of distilled chloroform (15 ml and 10 ml) and the two extracts combined and washed by shaking with 100 ml of 5% sodium chloride solution. The washed extract was evaporated to dryness over a water bath under a gentle stream of nitrogen. It was then quantitatively transferred to a small sample bottle and again evaporated to dryness. The final mixture was redissolved in 0.6 ml AR chloroform (Merck, Darmstadt, G.F.R.).

The extract was analysed by thin-layer chromatography (TLC). The 0.5 mm plates 20 cm × 20 cm, were prepared from silica gel G-HR (Macherey, Nagel and Co., Düren, G.F.R.), activated for 1 h at 105°C and stored in a desiccator. The chromatography tank was equilibrated overnight with acetone (distilled)—chloroform (distilled)—propan-2-ol (AR) (10:85:5). The chloroform extract (30 μl) was spotted in parallel with the standard aflatoxin metabolite at different concentrations. The original concentration of aflatoxin in the urine which would be expected from 30 μl of a 0.6-ml extract was calculated and different percentages of this used. To allow for the background fluorescence of other urine constituents, an aflatoxin-free urine extract was prepared as a control, and this was spotted on the same spot as the standards, so that they ran together. All plates were developed up to 15 cm from the bottom edge. The temperature was in the range 23—29°C, and the relative humidity in the range 40—80%. The concentration of aflatoxin metabolite was estimated by visual comparison of the fluorescence with the intensity of the standard spots plus control urine under 365 nm UV light (Hanovia Fluorescence 16).

The Messripour and Nesheim [4] method includes a detection column, however our experience showed that some interfering fluorescent substances were contained in the charcoal and acid alumina. These could be removed by washing with chloroform—methanol (97:3, v/v), however after this treatment some of the aflatoxin M_1 was retained by the first part of the column, which consisted of a mixture of acid-treated charcoal, magnesia, Celite 545 and glasswool in the ratio 2:4:8:1. In view of this and considering the large volume of extract required (0.5 ml out of a total of 0.6-ml sample) the column detection technique was not used in these studies.

LE I

OVERY OF AFLATOXIN METABOLITES EXTRACTED FROM HUMAN URINE

bolite	Limit of detection on TLC plate (ng)*	Recovery (%)			Mean value ± S.D. (%)	R_F**	Lowest detectabl concentration in urine*** (ng per
		Run 1	Run 2	Run 3			
oxin B_1	0.3	60	75	75	70 ± 9	0.73 ± 0.08	9
oxin G_1	0.5	75	78	75	76 ± 2	0.59 ± 0.06	13
oxin M_1	1.0	80	85	60	75 ± 15	0.44 ± 0.08	27
oxin B_2a	1.0	25	10	12	16 ± 6	0.39 ± 0.08	125
oxin G_2a	2.0	60	55	50	55 ± 5	0.29 ± 0.07	73
oxicol I atural isomer)	1.0	25	30	50	35 ± 13	0.69 ± 0.07	57
oxicol II nnatural isomer)	1.0	45	48	50	48 ± 3	0.62 ± 0.06	42
hydrodeoxyaflatoxin B_1	1.0	60	60	60	60 ± 0	0.79 ± 0.06	33

mated in presence of urine with a high fluorescent background.
e R_F values are quoted to 1 S.D. of all estimations carried out in this study using standard metabolites.
or a 30-μl application from a 0.6-ml concentrate per 100 ml urine.

RESULTS

The percentage yield obtained of each aflatoxin metabolite is presented in Table I. A representative thin-layer chromatogram is shown in Fig. 1, demonstrating the clear separation of metabolites. The limit of visible detection of aflatoxin on the TLC plate was measured in the presence of urine extract. These data, together with the average recovery obtained for each metabolite were used to calculate the lowest aflatoxin concentration detectable by this method. If the sample is concentrated to 0.2 ml rather than 0.6 ml sensitivity is increased, but this increases the amount of background fluorescence from the urine and reduces the volume available for confirmatory tests.

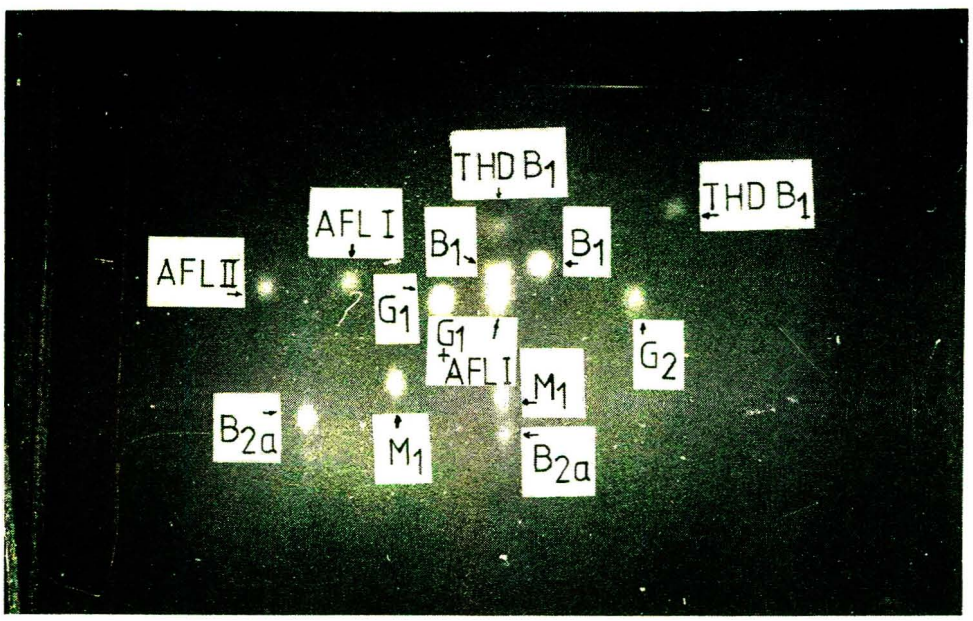

Fig. 1. A representative thin-layer chromatogram showing separation of aflatoxins B_1, G_1, M_1, B_2a, G_2a, aflatoxicols I (AFL I) and II (AFL II) and tetrahydrodeoxyaflatoxin B_1 (THD B_1); 0.5-mm silica gel G-HR plate equilibrated overnight, and run in acetone—chloroform—propan-2-ol (10:85:5).

Derivatisation with trifluoroacetic acid on the TLC plates was attempted for all the compounds in Table I [5]. Apart from aflatoxins B_1, G_1 and M_1 only the tetrahydrodeoxyaflatoxin B_1 responded to a slight degree.

This method has been used in this laboratory to analyse 200 samples of human urine in duplicate of which six have been shown to contain aflatoxin M_1 at greater than 30 ng per 100 ml [6]. This was confirmed by formation of the derivative with trifluoroacetic acid, seen on two-dimensional chromatography. No other suspected metabolite was observed. The urine samples were collected from hospital patients just after admission, in Lusaka.

DISCUSSION

A number of extraction and detection methods has been used to examine foodstuffs, animal tissue, human and animal milk, excreta and urines. In many reports, total recovery rates for the whole extraction and detection procedures are not stated, however the following values have been noted: 79—84% [7], 90—100% [8], 85% [9] and 80—83% [10] for aflatoxin B_1; 90—100% [8] and 100% [10] for aflatoxins G_1, G_2 and 87—88% [11], 92% [12] and 65% [9] for aflatoxin M_1. These compare reasonably with the data presented in this paper. This method uses simple instrumentation and a technique which is suitable for countries where aflatoxin is a problem and screening would be advocated.

However, it is necessary to assess the suitability of this method in relation to the local aflatoxin levels suspected. A previous study of plate-food samples collected in the dry season in the Eastern Province of Zambia gave the mean level of contamination of positive samples equal to 10 μg aflatoxin B per kg food [13]. Assuming an intake of 1 kg of food daily and a urine output of 1 l daily, and using the data available that 3.8% of aflatoxin is detectable as metabolites in vitro [3], the expected concentration of aflatoxin metabolites will be 38 ng per 100-ml urine sample. If 75% recovery is assumed approximately 1.5 ng would be present in the urine extract on the TLC plate for 30 μl of an 0.6-ml concentrate. Table I demonstrates that this metabolite concentration is at the lower levels of detection of this technique.

Using this method, urine samples obtained both from patients newly admitted to hospital with acute or chronic liver disease and from control patients without evidence of liver pathology, are being examined for aflatoxin metabolites. This is to assess the frequency of exposure to aflatoxins and the possible influence of liver disease on metabolite excretion patterns.

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Note

Isotachophoretic determination of adriamycin and adriamycinol in human plasma

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Adriamycin (doxorubicin) is one of the potent anticancer drugs and used for the treatment of neoplasms such as malignant lymphoma, leukemia and lung cancer. However, since it shows severe side-effects when administered in excess, pharmacokinetic studies are necessary for therapeutic designs.

Several analytical methods have been reported for the determination of adriamycin in human plasma and urine. These methods are based on either fluorescence measurement [1, 2] or radioimmunoassay [3], but they have disadvantages due to the lack of specificity. The use of fluorescence scanning on thin-layer chromatograms [4] and of reversed-phase liquid column chromatography [5—7] made a considerable improvement in the specificity of assay.

In the present paper we report a method for the specific determination of adriamycin and its active metabolite (adriamycinol) in human plasma by means of isotachophoresis.

EXPERIMENTAL

Human plasma (1 ml) was diluted four times with water, and mixed vigorously with 4 ml of *n*-butanol for 5 min. The mixture was then centrifuged at 2000 *g* for 10 min and the resultant butanol layer was concentrated to dryness in a flask under vacuum at 40°C. The wall of the flask was washed twice with 0.5 ml of methanol and the solvent was transferred to a pyrex tube and evaporated under nitrogen gas. The dried residue was dissolved in 50—100 μl of 90% methanol and was subjected to isotachophoresis. The recovery of adriamycin extraction from plasma was 89.1 ± 0.94% (mean ± S.E.M.).

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Isotachophoretic separations were performed in a 23 cm × 0.5 mm I.D. capillary using the LKB 2127 Tachophor. One or 10 μl of the sample were injected. The separations were done at a constant current of 100 μA. The light transmission at 254 nm was recorded at a chart speed of 6 cm/min. The leading electrolyte was 10 m*M* sodium acetate—acetic acid buffer in 60% methanol (pH 6.0) and the terminator was 10 m*M* β-alanine in 60% methanol.

Adriamycin was obtained from Kyowa Hakko Co., Tokyo, Japan. Adriamycinol (13-hydroxydoxorubicin) and adriamycin aglycones were purified by thin-layer chromatography from the incubation mixture of adriamycin with the supernatant fraction (105,000 *g*, 60 min) of mouse kidney homogenate by the method of Bachur et al. [8]. Ampholine (pH 3.5—10) was purchased from LKB, Bromma, Sweden.

RESULTS AND DISCUSSION

Fig. 1 shows the isotachophoretic pattern of adriamycin. A typical isotachophoretic run of the butanol extract of plasma containing no adriamycin

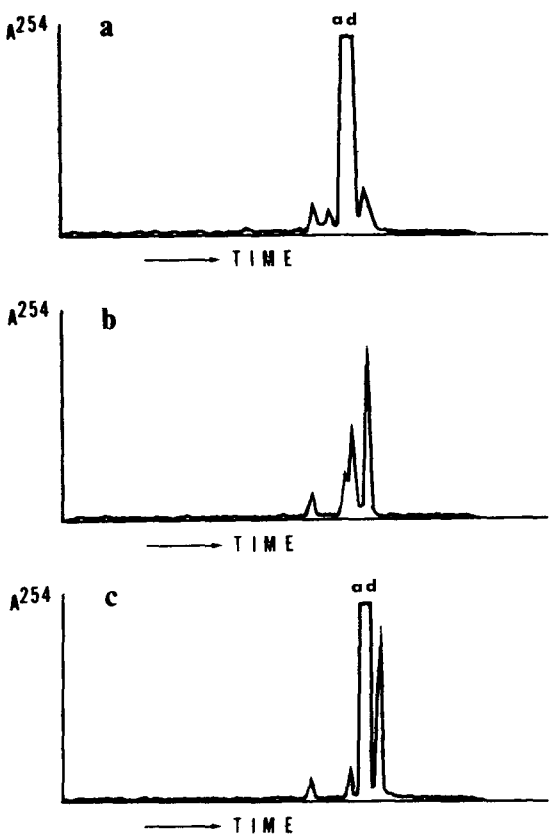

Fig. 1. Isotachopherograms of adriamycin. Ten microliters of the sample were injected. (a) Adriamycin (200 μg/ml of saline); (b) *n*-butanol extract of plasma; (c) *n*-butanol extract of plasma containing 200 μg/ml adriamycin. ad = adriamycin.

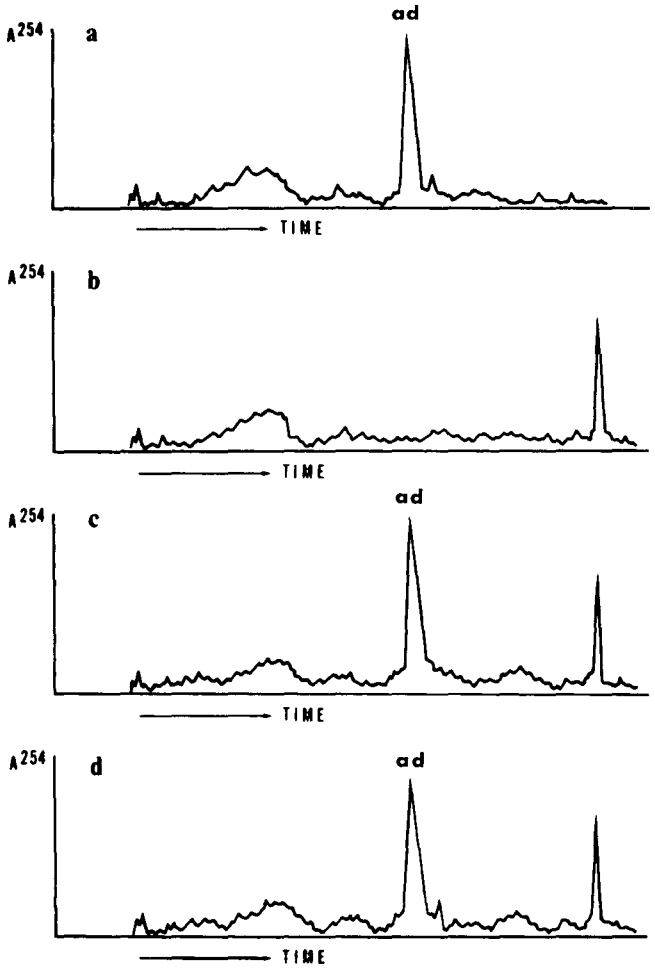

Fig. 2. Isotachopherogram of adriamycin using Ampholine as a spacer. Ten microliters of sample were mixed with 1 μl of 1% Ampholine (pH 3.5—10) and injected. (a) Adriamycin (10 μg/ml of saline); (b) *n*-butanol extract of plasma; (c) *n*-butanol extract of plasma to which adriamycin (10 μg/ml) was added before injection; (d) *n*-butanol extract of plasma containing 10 μg/ml adriamycin. ad = adriamycin.

(Fig. 1b) shows two UV-negative zones and three UV-positive spikes. When adriamycin was added to the extract, the UV-positive zone of adriamycin was positioned just behind the second UV-positive spike (Fig. 1c). Since the UV-positive spikes disturbed the detection of small amounts of adriamycin, Ampholine (pH 3.5—10, final concentration 0.1%) was added to the extract and electrophoresed. As shown in Fig. 2, the peak corresponding to adriamycin was well separated from the distinct UV-positive spike in blank plasma. Adriamycinol, a major metabolite with antineoplastic activity [9], co-migrated with adriamycin (data not shown). A linear relationship [y (mm^2) = 11.2 x (ng) — 0.3; r = 0.999] was obtained between the integrated peak area (y) and the injected amount of adriamycin (x) within the range 2.5—100 ng. Since adriamycinol has a molar extinction coefficient at 254 nm essentially

identical to that of adriamycin [8], this concentration range almost covers combined levels of the drug and its active metabolite in plasma in ordinary therapeutic designs [10].

Eksborg et al. [7] and Pierce and Jatlow [6] have studied the use of reversed-phase liquid chromatography for the determination of adriamycin and adriamycinol. The detection sensitivity of our method was not better than but as good as that of their procedures. The recovery of the drug and its hydroxyl metabolite was higher than that reported by Pierce and Jatlow and was almost the same as that found by Eksborg et al.

Aglycones of adriamycin, which are pharmacologically inactive metabolites [9], did not move into the capillary and stayed at the origin. The present method, therefore, gives the levels of the active compounds in plasma, avoiding the contamination by the inactive aglycones which in the usual fluorescence method interferes with the determination of the active compounds due to their similar fluorescence spectra.

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Vol. 227, No. 1

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

3rd INTERNATIONAL SYMPOSIUM ON ISOTACHOPHORESIS

The 3rd International Symposium on Isotachophoresis will be held in Goslar (near Hannover), G.F.R., on June 1–4, 1982. The areas covered by the Symposium will include all aspects of isotachophoresis, from theoretical treatments of phenomena associated with this method, to instrumental developments, and all types of applications in the field of analytical chemistry and biochemistry, the uses of isotachophoresis in experimental biology and medicine, as well as industrial applications. Both applications on an analytical and on a preparative scale are relevant, either in free-flow systems, in gels or in other media.

The deadline for registering a paper or poster for consideration by the scientific committee is January 1st, 1982. The deadline for the submission of abstracts is March 1st, 1982.

For further information contact: Dr. C.J. Holloway, Organising Chairman, ITP 82, Abteilung für Klinische Biochemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, G.F.R. Phone: (0511) 532 2838/2834.

XXXth ANNUAL COLLOQUIUM "PROTIDES OF BIOLOGICAL FLUIDS"

The XXXth Annual Colloquium "Protides of Biological Fluids" will be held on May 3–6, 1982, at the Sheraton Hotel, Rogiersplein 3, in Brussels, Belgium. Three main topics will be covered. They are: Nervous System Specific Proteins, Monoclonal Proteins as a Reagent, and New Separation Techniques for Polypeptides & Proteins.

Summaries of posters should be submitted before January 1st, 1982. The deadline for registration is April 1st, 1982.

Immediately preceeding the Colloquium will be a two-day workshop, "Fast Liquid Chromatography of Proteins", on May 1–2, 1982. The workshop is sponsored by Pharmacia Fine Chemicals and will be held at the Institute for Medical Biology in Brussels.

For further information contact: Colloquium "Protides of Biological Fluids", Secretariat, Institute for Medical Biology, Alsembergsesteenweg 196 Chaussée d'Alsemberg, 1180 Brussels, Belgium. Tel.: (02) 344·19·50 (inside Belgium), 32·2·344·19·50 (outside Belgium); Telex CDHBRU 26501.

CALENDAR OF FORTHCOMING EVENTS

Jan. 19–20, 1982 Amsterdam, The Netherlands	**Symposium on "Detection in High-Performance Liquid Chromatography"** Contact: Mrs. Peschier, Hewlett-Packard Nederland B.V., Analytical Department, van Heuven Goedhartlaan 121, 1181 KK Amstelveen, The Netherlands (Tel.: 020-47 20 21). (Further details published in Vol. 212, No. 2)
March 8–12, 1982 Atlantic City, NJ, U.S.A.	**1982 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Linda Briggs, Program Secretary, Pittsburgh Conference, Department J-057, 437 Donald Road, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 212, No. 2)
March 28–April 2, 1982 Las Vegas, NV, U.S.A.	**183rd American Chemical Society National Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 5–8, 1982 Las Vegas, NV, U.S.A.	**17th 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. (Complete program published in Vol. 219, 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	**18th 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. (Complete program published in Vol. 234, No. 1.)
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 3–6, 1982 Brussels, Belgium	**XXXth Annual Colloquium "Protides of Biological Fluids"** Contact: Colloquium "Protides of Biological Fluids", Secretariate, Institute for Medical Biology, Alsembergsesteenweg 196 Chaussée d'Alsemberg, 1180 Brussels, Belgium. Tel.: (02)344 19 50; (Int.) 32.2.344 19 50. Telex: CDHBRU 26501.

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. (Further details published in Vol. 226, No. 2.)
May 16–18, 1982 Indiannapolis, IN, U.S.A.	**1982 LCEC Symposium: Biomedical Applications of LCEC and Voltammetry** Contact: LCEC Symposium, P.O. Box 2206, West Lafayette, IN 47906, U.S.A. Tel.: (317) 463-2505; Telex: 276 141. (Further details published in Vol. 226, No. 1.)
June 1–4, 1982 Goslar (near Hannover), G.F.R.	**3rd International Symposium on Isotachophoresis** Contact: Dr. C.J. Holloway, ITP 82, Abteilung für klinische Biochemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, G.F.R. (Further details published in Vol. 219, No. 3.)
June 6–11, 1982 Kansas City, MO, U.S.A.	**International Symposium on the Synthesis and Application of Isotopically Labeled Compounds** Contact: Dr. Alexander Susán, Scientific Secretary of the Symposium, c/o Midwest Research Institute, 425 Volker Boulevard, Kansas City, MO 64110, U.S.A. Tel: (816) 753-7600, extension 268. (Further details published in Vol. 225, No. 1.)
June 7–11, 1982 Philadelphia, PA, U.S.A.	**VI International Symposium on Column Liquid Chromatography** Contact: R.A. Barford, ERRC – SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A. (Further details published in Vol. 211, No. 3).
June 18–21, 1982 Lund, Sweden	**Flow Analysis II** Contact: Flow Analysis II, c/o The Swedish Chemical Society, Upplandsgatan 6A, 1 tr., S-111 23 Stockholm, Sweden. (Further details published in Vol. 216.)
June 20–23, 1982 Bordighera (near San Remo), Italy	**International Symposium on Chromatography and Mass Spectrometry in Biomedical Sciences** Contact: Dr. Alberto Frigerio, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62 – 20517 Milan, Italy. Tel.: 35.54.546; Telex: 331268 NEGRI I. (Further details published in Vol. 225, No. 1.)
June 20–24, 1982 Toronto, Canada	**North American Medicinal Chemistry Symposium** Contact: Symposium Secretariat, North American Medicinal Chemistry Symposium, c/o Ayerst Laboratories, P.O. Box 6115, Montreal, Quebec H3C 3J1, Canada.
July 11–16, 1982 Washington, DC, U.S.A.	**6th International Conference on Computers in Chemical Research and Education (ICCCRE)** Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.
Aug. 15–21, 1982 Perth, Australia	**The 12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia

Aug. 22–28, 1982 Vancouver, Canada	**The XIth International Carbohydrate Symposium** Contact: Mr. K. Charbonneau, Executive Secretary, XIth International Carbohydrate Symposium, c/o National Research Council of Canada, Ottawa, Ontaria, Canada K1A 0R6. Tel.: (613) 993-9009; Telex: 053-3145.
Aug. 30–Sept. 3, 1982 Vienna, Austria	**9th International Mass Spectrometry Conference** Contact: Interconvention, P.O. Box 105, A-1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.
Sept. 5–9, 1982 Liège, Belgium	**8th European Workshop on Drug Metabolism** Contact: Professor Jacques E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, Université de Liège, Bâtiment B 23, B-4000 Sart Tilman par Liège 1, Belgium. Tel.: (32-41)-56.24.80/81. (Further details published in Vol. 225, No. 2.)
Sept. 6–9, 1982 Bath, Great Britain	**4th European Symposium on Chemical Structure – Biological Activity: Quantitative Approaches** Contact: Dr. J.C. Dearden, School of Pharmacy, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, Great Britain.
Sept. 6–9, 1982 Hradec Králové, Czechoslovakia	**8th International Symposium on Biomedical Applications of Chromatography** Contact: Dr. K. Macek, Institute of Physiology, Czechoslovakian Academy of Sciences, Vídeňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia. (Further details published in Vol. 225, No. 2.)
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)
Sept. 19–24, 1982 Singapore, Republic of Singapore	**2nd Asian-Pacific Congress on Clinical Biochemistry** Contact: 2nd Asian-Pacific Congress on Clinical Biochemistry, Singapore Professional Centre, 129B Block 23, Outram Park, Singapore 0316, Republic of Singapore.
Oct. 12–14, 1982 Salzburg, Austria	**DIOXIN 82, 3rd International Symposium – Workshop on Chlorinated Dioxins and Related Compounds** Contact: Dr. E. Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland. (Further details published in Vol. 219, No. 3.)
May 30–June 3, 1983 Melbourne, Australia	**International Conference on Chromatographic Detectors** Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria 3052, Australia. (Further details published in Vol. 216.)
July 17–23, 1983 Edinburgh, Scotland, Great Britain	**SAC '83, International Conference and Exhibition on Analytical Chemistry** Contact: The Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel.: 01-734-9971. (Further details published in Vol. 214, No. 2.)
Aug. 28–Sept. 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

Affinity chromatography and related techniques – Theoretical aspects/Industrial and biomedical applications *(Proc. 4th Int. Symp., Veldhoven, June 22–26, 1981)* (Analytical Chemistry Symposia Series, Vol. 9), edited by T.C.J. Gribnau, J. Visser and R.J.F. Nivard, Elsevier, Amsterdam, Oxford, New York, 1982, XVIII + 584 pp., price Dfl. 195.00, US$83.00, ISBN 0-444-42031-2.

Recent developments in mass spectrometry in biochemistry, medicine and environmental research, 7 *(Proc. 7th Int. Symp., Milan, June 16–18, 1980)* (Analytical Chemistry Symposia Series, Vol. 7), edited by A. Frigerio, Elsevier, Amsterdam, Oxford, New York, 1981, IX + 360 pp., price Dfl. 170.00, US$2.25, ISBN 0-444-42029-0.

Advances in steroid analysis *(Proc. Symp., Eger, May 20–22, 1981)* (Analytical Chemistry Symposia Series, Vol. 10), edited by S. Görög, Elsevier, Amsterdam, Oxford, New York, 1982, 464 pp., price Dfl. 225.00, US$95.75, ISBN 0-444-99711-3.

Biochemical and clinical aspects of coenzyme Q, Vol. 3 *(Proc. 3rd Int. Symp., Austin, TX, January 18–21, 1981)*, edited by K. Folkers, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1981, XIV + 414 pp., price Dfl. 151.00, US$64.25, ISBN 0-444-80319-X.

Clinical pharmacology in psychiatry, edited by E. Usdin, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1981, 384 pp., price Dfl. 135.00 (outside U.S.A. and Canada), US$ 59.00 (in U.S.A. and Canada), ISBN 0-444-00556-0.

MANUFACTURERS' LITERATURE

N-1644

BROCHURE ON ELECTROPHORESIS CHAMBER

A brochure describing Shandon Southern's Model 600 electrophoresis chamber is now available from the manufacturer. The brochure shows how this instrument can be combined with custom-designed accessories and application systems to perform specific electrophoretic techniques. These techniques include isoelectric focusing, immunoelectrophoresis, cellulose acetate electrophoresis and thin-layer electrophoresis.

NEW PRODUCTS

N-1645

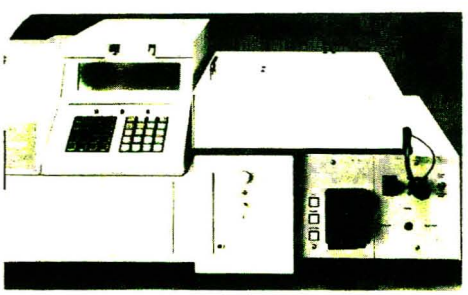
NEW GLC COLUMNS

Whatman's Chemical Separation Division recently expanded its line of μPartisorb GLC columns with the addition of eleven new configurations and one new liquid phase. According to the manufacturer, the new PEG-BASIC low-load liquid phase columns exhibit as much as 2200 plates per ft. efficiency and are particularly useful for the analysis of basic compounds such as free amines. All μPartisorb columns are 6 ft. × 2 mm glass columns.

N-1643

SLAB GEL SCANNING SYSTEM

Beckman Instruments has introduced a slab gel scanning system as an accessory to the DU-8 UV-VIS spectrophotometer. This accessory permits the analysis of slab gels with the same spectrophotometer used for kinetic rate determinations and DNA denaturation and renaturation experiments. The accessory can be used with every DU-8 spectrophotometer. It is permanently installed in the instrument and does not interfere with the other DU-8 accessories. The DU-8 slab gel scanning system accommodates 20 × 20 cm slab gels or autoradiograms without gel cutting, and calculates and tabulates peaks, peak areas and percentage of total area and determines molecular weight.



For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH		J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography		234/1 234/2 235/1 235/2	236/1 236/2	The publication schedule for further issues will be published later.									
Chromatographic Reviews													
Biomedical Applications		227/1	227/2										

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.

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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".

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Electron Capture – Theory and Practice in Chromatography

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

JOURNAL OF CHROMATOGRAPHY LIBRARY – Volume 20

Sept. 1981 xii + 418 pages
Price: US $76.50/ Dfl. 180.00
ISBN 0-444-41954-3

This book provides the first comprehensive coverage of all aspects of the theory, design, operation and applications of the electron capture detector (ECD) from the chromatographer's point of view. In addition, an up-to-date look at the ancillary techniques of selective electron-capture sensitization, atmospheric pressure ionization and plasma chromatography has been included. ECD users will find the solutions to instrumental and technical problems which arise during practice particularly valuable. These have been derived from the experiences of the internationally distinguished team of authors.

Each chapter has been prepared by experts in their field and provides an in-depth coverage of its topic. The basic theory of the mechanisms of electron capture detection is included. Practical sections form the bulk of the book and are devoted to such topics as the construction and operating principles of the detector, including the establishment of instrument design criteria, and the different methods of derivatization. A more personal touch is provided by the inventor of the ECD, J.E. Lovelock, in his review of the development of the technique. Other chapters illustrate the importance of ECD in trace analysis in environmental and biomedical research. A unique feature is the extensive tabulation of all the pertinent data concerning the use of ECD in gas and liquid chromatography.

For those analytical chemists who use chromatography in their research, this book should become a standard text.

CONTENTS: Chapter 1. The electron-capture detector – A personal odyssey *(J.E. Lovelock)*. 2. The design and operation of the electron-capture detector *(C.F. Poole and A. Zlatkis)*. 3. Theory of electron capture *(W.E. Wentworth and E.C.M. Chen)*. 4. Selective electron-capture sensitization *(F.C. Fehsenfeld, P.D. Goldan, M.P. Phillips and R.E. Sievers)*. 5. Oxygen-doping of the carrier gas in electron-capture detection *(E.P. Grimsrud)*. 6. Wide-range calibration of electron-capture detectors *(R.E. Kaiser and R.I. Rieder)*. 7. Response of the electron-capture detector to compounds with natural electrophores *(J. Vessman)*. 8. Sensitive derivatives for the determination of organic compounds by electron-capture gas chromatography *(C.F. Poole and A. Zlatkis)*. 9. The detection of inorganic and organometallic compounds by electron-capture gas chromatography *(C.F. Poole and A. Zlatkis)*. 10. Environmental applications of the electron-capture detector – pesticides *(W.P. Cochrane and R.B. Maybury)*. 11. Environmental applications of the electron-capture detector – dioxins *(F. Bruner)*. 12. The electron-capture detector as a monitor of halocarbons in the atmosphere *(P.G. Simmonds)*. 13. Biomedical applications of the electron-capture detector *(J. Vessman)*. 14. Negative ion atmospheric pressure ionization mass spectrometry and the electron-capture detector *(E.C. Horning, D.I. Carroll, I. Dzidic and R.N. Stillwell)*. 15. Electron-capture process and ion mobility spectra in plasma chromatography *(F.W. Karasek and G.E. Spangler)*. 16. The electron-capture detector as a detector in liquid chromatography *(U.A. Th. Brinkman)*. Subject index.

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