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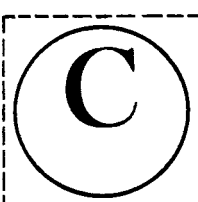

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GAS CHROMATOGRAPHIC DETERMINATION OF FREE AMINO ACIDS AND AMINO ACIDS ATTACHED TO TRANSFER RNA IN BIOLOGICAL SAMPLES

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(First received April 13th, 1982; revised manuscript received June 4th, 1982)

SUMMARY

A method was developed to analyze quantitatively free amino acids and amino acids attached to transfer RNA (tRNA) in tissue samples by gas chromatography. Free amino acids were purified by ion-exchange chromatography after deproteinization. Total cellular aminoacyl-tRNA was extracted from rabbit reticulocytes and liver by a modified phenol extraction method under conditions which were designed to prevent deacylation of the attached amino acids. After deacylation and separation from tRNA by pressure ultrafiltration, eighteen amino acids were determined by gas chromatography as their N-heptafluorobutyryl isobutyl derivatives.

INTRODUCTION

Alterations in the levels of specific transfer RNA (tRNA) species have been observed in association with changes in the rate of specific protein synthesis, e.g. in silk gland of silkworms [1], in avian liver during yolk protein synthesis [2], and in rabbit reticulocytes specialized for hemoglobin synthesis [3, 4]. The method generally used to determine the abundance of specific tRNA species in a purified sample of tRNA is that of acylation of radioactive amino acids, one at a time, to the tRNA in vitro. There are several potential sources of error, however, when this method is used. They include variation in the activity and purity of the synthetase, purity of the tRNA sample, nature and composition of the acylation buffer, salt and substrate concentration, etc. It is thus possible that the maximal level of acylation is not achieved in vitro for a given amino acid. On the other hand, the level of aminoacylation achieved in vivo may be lower than what is found in vitro, depending on the physiological state of the cell.

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We have been interested in developing a direct method for determination of amino acids attached to tRNA in vivo. The gas chromatographic method used in this study is sensitive enough to allow analysis of endogenous amino acids attached to a tRNA preparation derived from a single tissue sample (e.g. rabbit liver). Butler et al. [5] have reported results from a similar study. They determined twelve amino acids attached to rabbit liver tRNA as their trifluoroacetyl methyl derivatives. We have analyzed all twenty amino acids (glutamine is determined with glutamic acid and asparagine with aspartic acid) attached to rabbit reticulocyte and liver tRNA as their N-heptafluorobutyryl isobutyl esters using both packed and capillary columns.

MATERIALS AND METHODS

Reagents and standards

Cycloheximide was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), sodium heparin from Medica (Helsinki, Finland) and sodium pentobarbital from Abbot S.A. (Ottignies, Belgium). Triton X-100 was from BDH Chemicals (Poole, Great Britain), and phenylhydrazine, chloroform, phenol and most of the commonly used chemicals were from Merck (Darmstadt, G.F.R.). Sephadex G-100 was from Pharmacia Fine Chemicals (Uppsala, Sweden) and Diaflo ultrafiltration membranes, Type UM 10, from Amicon Co. (Lexington, MA, U.S.A.). Dowex-1 (1-X8-200 Cl^-) and Dowex-50W (50-X8-200, H^+) were purchased from Sigma.

An amino acid calibration mixture (type 1) was obtained from Beckman (Spinco Division, Palo Alto, CA, U.S.A.). It contained 2.500 ± 0.004 m*M* alanine, arginine, aspartic acid, phenylalanine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, proline, serine, threonine, tyrosine and valine, and 1.250 ± 0.004 m*M* cysteine. Norleucine and ornithine were purchased from Fluka (Buchs, Switzerland), and hydroxyproline, tryptophan and cystine were from Merck.

Isobutanol—HCl was prepared by bubbling anhydrous HCl through three traps containing concentrated sulphuric acid and then into isobutanol at 0—4°C. The amount of HCl (3 *M*) was determined by titration. Isobutanol (Merck) was dried and distilled before use. Methylene chloride and ethyl acetate were obtained from Merck and purified by fractional distillation. Heptafluorobutyric acid anhydride was from Fluka.

Gas chromatography

Gas chromatography was carried out with either a Varian Aerograph Model 1400 or a Varian Series 3700 gas chromatograph. Both were fitted with flame ionization detectors and temperature programmer units. The glass column (183 cm × 2 mm I.D.) was packed with Chromosorb W AW (80—100 mesh; Johns-Manville Celite Division, Denver, CO, U.S.A.) coated with 3% (w/w) SE-30 stationary phase (Varian Aerograph, Walnut Creek, CA, U.S.A.). The glass capillary column (25 m × 0.39 mm I.D.) SE-30 (WCOT) was purchased from Varian. Nitrogen was used as a carrier gas. The flow-rate was 20 ml/min in the packed column and 1 ml/min in the capillary column. The temperature program was from 80°C (4°C/min). The pre time was changed from 0 to 8 min.

Attenuation was 32×10^{-11} with the packed column and 32×10^{-12} with the capillary column. With the capillary column, a splitless injection with a delay of 30 sec was used. Direct manual measurement of peak heights was used as the basis for all quantitative calculations. A Servogor 210 potentiometric strip chart recorder was used (Goerz Electro, Vienna, Austria).

tRNA preparation

The rabbits were injected subcutaneously with 2.5% phenylhydrazine solution (pH 7.0) according to a standard schedule [6]. On the seventh day, the rabbits were anesthesized by injection of 60 mg/kg of sodium pentobarbital intraperitoneally. An intraperitoneal injection of cycloheximide (40 mg/kg in saline) was given and after about 5 min the rabbits were bled via direct cardiac puncture. The blood was collected with a heparinized needle and syringe and immediately frozen in liquid nitrogen. All subsequent steps were carried out at 0—4° C.

The frozen blood was weighed, pulverized in a cooled mortar and mixed with one volume of a buffer containing 0.1 *M* potassium acetate, pH 5.0, 25 m*M* KCl, 5 m*M* $MgCl_2$, 0.2 mg/ml sodium heparin, 2% (w/v) Triton X-100 and 1 m*M* cycloheximide. After homogenization in a glass—Teflon homogenizer with ten strokes using a tight pestle, an equal volume of cold, water-saturated phenol was added, and the mixture was shaken in a mechanical shaker at 4° C for 60 min. The phases were separated by centrifugation for 10 min at 27,000 *g*, and the phenol phase re-extracted with one volume of 5 m*M* sodium acetate, 5 m*M* $MgCl_2$, pH 5.0, and one volume of chloroform. The resulting aqueous phase was combined with the aqueous phase from the first extraction and one additional phenol extraction was performed for 30 min at 4° C. The nucleic acids were precipitated from the aqueous phase by addition of 0.1 volume of 20% (w/v) potassium acetate, pH 4.9, and 2.5 volumes of cold ethanol. After mixing, the nucleic acids were allowed to precipitate overnight at —20° C. Livers from untreated, anesthesized rabbits were exposed, cut out and immediately frozen in liquid nitrogen. Extraction of the nucleic acids from frozen tissue was as explained above for frozen blood.

tRNA was purified by passing the RNA sample through a column of Sephadex G-100 (2.5 × 100 cm) in a buffer containing 0.5 *M* NaCl, 10 m*M* $MgCl_2$ and 5 m*M* sodium acetate, pH 5.0. The absorbancy at 254 nm was monitored and the major A_{254}-absorbing peak corresponding to tRNA was precipitated with ethanol, redissolved in 5 m*M* formic acid, pH 5.0, and the absorbance units measured at 260 nm. One A_{260} unit of tRNA is defined as that quantity which, when dissolved in 1 ml of distilled water, gives a solution having an absorbance of 1 at a path length of 1 cm and a wavelength of 260 nm.

Deacylation of aminoacyl-tRNA

The tRNA sample was dialyzed overnight against 5 m*M* formic acid, pH 5.0. After dialysis, the endogenous amino acids were released from the tRNA by adjusting the pH to 9—10 with dilute ammonium hydroxide. The mixture was incubated at 37° C for 120 min and the released amino acids were separated from the tRNA by pressure ultrafiltration using a UM 10 Diaflo membrane.

Free amino acids

Ten grams of frozen rabbit liver were pulverized in a cooled mortar, mixed with 25 ml of 20% trichloroacetic acid and homogenized with a glass—Teflon homogenizer. After centrifugation for 30 min at 32,000 *g*, the supernatant and 2.5 μmol of the internal standard, norleucine, were evaporated with a rotary evaporator and dried with a stream of nitrogen. The residue was dissolved in 0.1 *M* HCl and passed through a cation-exchange resin (Dowex-50W). The resin was eluted with 3 *M* ammonium hydroxide and the eluate dried and further purified by passing through an anion-exchange resin (Dowex-1). The resin was eluted with 3 *M* acetic acid and the eluate evaporated with a rotary evaporator and dried with a stream of nitrogen. The free amino acids were determined either by an automatic amino acid analyzer (Kontron, Zürich, Switzerland) or by gas chromatography.

Derivatization of amino acids

Derivatization of amino acids for gas chromatography was performed essentially as described by MacKenzie and Tenaschuk [7, 8]. Ten nmol of the internal standard, norleucine, was added and the amino acid sample was evaporated at 50° C with a stream of nitrogen. The standard amino acid mixture contained 100 nmol of each amino acid plus norleucine with the exception of cysteine (50 nmol). Isobutanol—HCl (0.25 ml) was added and the solution was heated at 120° C for 30 min in an oven. After cooling to room temperature, excess reagent was evaporated with a stream of nitrogen; 50—75 μl of heptafluorobutyric anhydride were added and the tubes were closed tightly and placed at 150° C for 10 min. After cooling, the contents of the tube were evaporated to dryness with a stream of nitrogen. The amino acid derivatives were dissolved in ethyl acetate and immediately analyzed by gas chromatography. Methylene chloride was used in each evaporating step to dry the sample completely. Co-injection of the sample and acetic anhydride for the on-column acylation of histidine [7, 8] was tested and it resulted in increased yields of histidine in some columns. However, in the analyses presented in this study, the yields of histidine were similar with and without co-injection of acetic anhydride.

RESULTS

tRNA isolation

Cycloheximide treatment of the rabbits prior to liver removal or reticulocyte collection, rapid freezing of the tissue sample in liquid nitrogen, and handling of the tRNA throughout the purification scheme in ice-cold solutions buffered to pH 5.0 were designed to inhibit elongation reactions on ribosomes during tissue collection and also non-enzymatic deacylation of tRNA during purification. The phenol-extracted tRNA eluted as a symmetric peak on Sephadex G-100 gel filtration (not shown). RNA samples were chromatographed in two or three portions to ensure that RNA species of higher molecular weight were not mixed with the tRNA peak during gel filtration. The average yield of tRNA obtained from 100 ml of blood was 3.1 ± 0.5 mg (n = 7) and from 100 g of

rabbit liver 12.3 ± 2.3 mg (n = 6). The latter figure compares well with the value of 11.6 mg reported by Butler et al. [5] for rabbit liver.

Gas chromatography of standard amino acids

Cystine, hydroxyproline, norleucine, ornithine and tryptophan were added to the standard amino acid mixture containing seventeen amino acids (Beckman Amino Acid Calibration Mixture). A representative elution profile on a glass capillary column is shown in Fig. 1. Each peak represents about 50 pmol of injected amino acid with the exception of cysteine (25 pmol). The relative molecular responses (RMR) for individual amino acids were calculated on the basis of peak heights. Results from samples containing unknown quantities of amino acids were calculated using the molecular responses relative to norleucine internal standard. Retention times and elution profiles from the standard amino acid mixture were used as a reference. In uncertain cases,

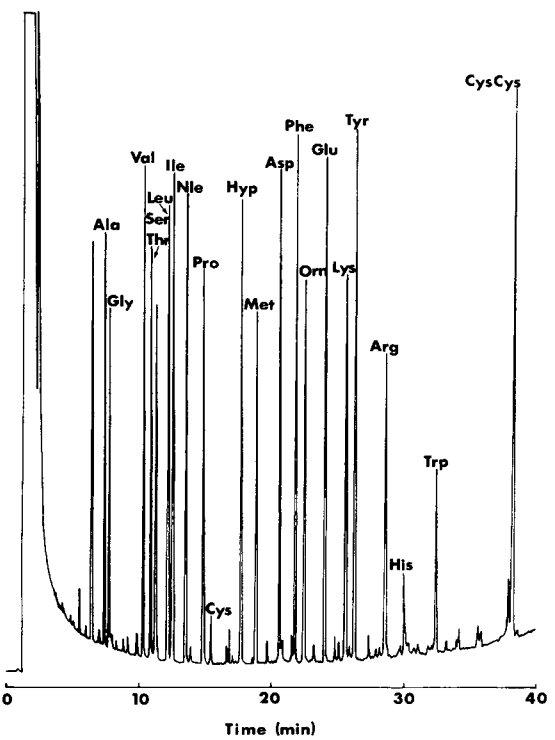


Fig. 1. Gas chromatographic separation on a glass capillary column of N-heptafluorobutyryl isobutyl esters of standard amino acids. Temperature program rate was 4°C/min. Other chromatographic conditions are given in the text.

spiking with amino acid standard injected with the sample was used to identify an unknown peak. All amino acids except glutamine and asparagine were well separated and could be quantitated. Glutamine and asparagine eluted with glutamic acid and aspartic acid, respectively. N-Heptafluorobutyryl n-propyl derivatives were also tested but the isobutyl derivatives gave better yield for

methionine and better separation of ornithine and glutamic acid (results not shown).

Amino acids attached to reticulocyte and liver tRNA

Representative elution profiles of amino acid derivatives from reticulocyte and liver aminoacyl-tRNA are shown in Figs. 2—4. There were a number of unknown peaks when biological samples containing amino acids from both types of tRNA were analyzed. The impurities made an accurate quantification with packed column difficult (Fig. 3). With the capillary column, separation of the individual peaks was much better and quantification of sixteen amino acids and Asn + Asp and Gln + Glu was possible (Table I). The correlation between determinations on packed and capillary columns was good (r = 0.947). The results obtained with amino acids derived from reticulocyte and liver tRNA were qualitatively similar. However, with respect to quantities of individual amino acids, certain differences were observed. The relative scarcity of leucine and isoleucine in aminoacyl-tRNA derived from reticulocytes was especially noteworthy (Figs. 2 and 4).

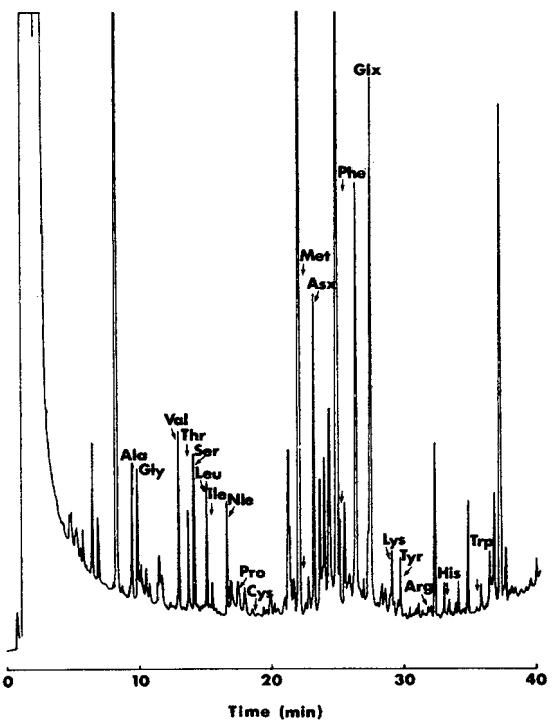
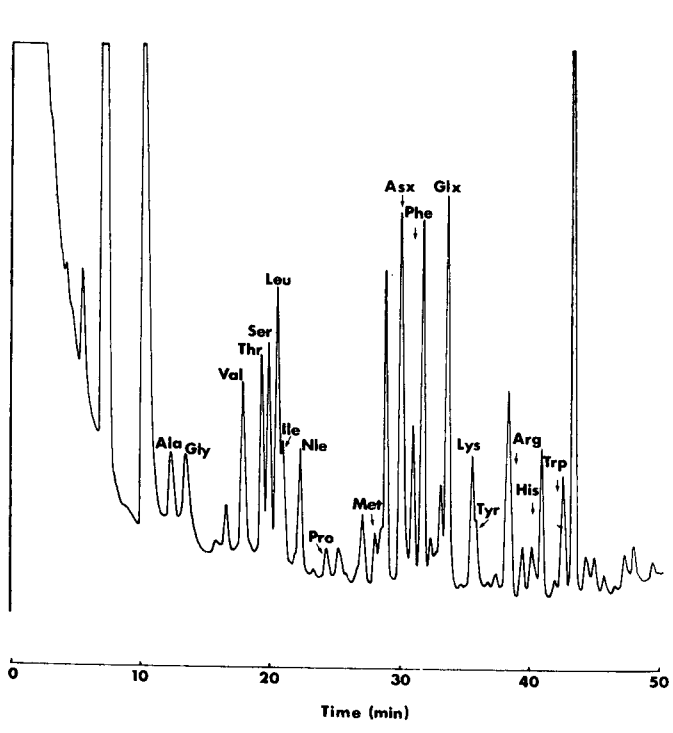


Fig. 2. Gas chromatographic separation on a glass capillary column of N-heptafluorobutyryl isobutyl esters of amino acids derived from rabbit reticulocyte tRNA. Conditions for chromatography are as in Fig. 1.



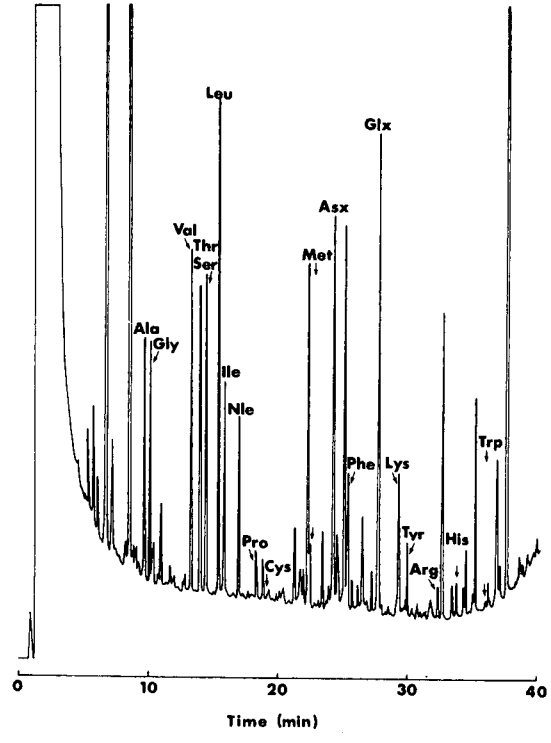


Fig. 3. Gas chromatographic separation on a packed column of N-heptafluorobutyryl isobutyl esters of amino acids derived from rabbit liver tRNA. Conditions of chromatography are given in the text.

Fig. 4. Gas chromatographic separation on a glass capillary column of N-heptafluorobutyryl isobutyl esters of amino acids derived from rabbit liver tRNA. Conditions of chromatography are as in Fig. 1.

TABLE I

AMINO ACIDS ATTACHED TO RABBIT LIVER tRNA IN VIVO

Comparison of results from gas chromatography of a tRNA sample on packed and glass capillary columns (pmol/nmol tRNA, mean ± S.E.M., n = 3). The detection limit for the method was 3 pmol/nmol tRNA.

Amino acid	Packed column	Glass capillary column
Ala	31.9 ± 6.5	45.9 ± 2.4
Arg	—*	6.0 ± 1.4
Asn + Asp	47.8 ± 1.8	39.8 ± 1.9
Cys	—*	—*
Gln + Glu	49.0 ± 3.5	48.4 ± 2.0
Gly	33.6 ± 5.5	49.5 ± 2.7
His	23.5 ± 3.9	23.9 ± 1.4
Ile	20.8 ± 0.8	22.7 ± 0.7
Leu	48.3 ± 2.9	53.5 ± 0.8
Lys	18.7 ± 2.1	15.8 ± 1.0
Met	11.3 ± 1.0	12.3 ± 1.0
Phe	16.6 ± 0.3	12.2 ± 0.8
Pro	5.8 ± 1.0	6.3 ± 0.4
Ser	44.3 ± 1.4	43.6 ± 1.3
Thr	37.9 ± 1.3	38.0 ± 0.5
Trp	6.6 ± 2.4	3.5 ± 0.2
Tyr	8.1 ± 0.7	7.1 ± 0.6
Val	36.5 ± 3.3	44.3 ± 1.4
Total	440.7	472.8

*Value below the detection limit.

Free amino acids

A representative elution profile of free amino acid derivatives from rabbit liver is shown in Fig. 5. Separation and quantification with glass capillary column was relatively simple due to the preliminary purification with ion-exchange resin and the abundance of free amino acids in the liver. Determination of the same samples was also made with a conventional amino acid analyzer (Table II). The results correlated well (r = 0.979). No significant correlation was observed with quantities of free amino acids in liver and amino acids attached to tRNA (r = 0.450) (Tables I and II).

DISCUSSION

There are only a few reports in the literature of a direct determination of amino acids attached to tRNA in vivo. Yegian et al. [9] determined sixteen amino acids attached to *Escherichia coli* tRNA with an automatic amino acid analyzer. They also estimated the acceptor capacity of the tRNA with and without periodate oxidation of tRNA. Smith and McNamara [3, 4] determined the extent of aminoacylation of rabbit reticulocyte tRNA in vivo indirectly by using periodate treatment and enzymatic acylation in vitro with

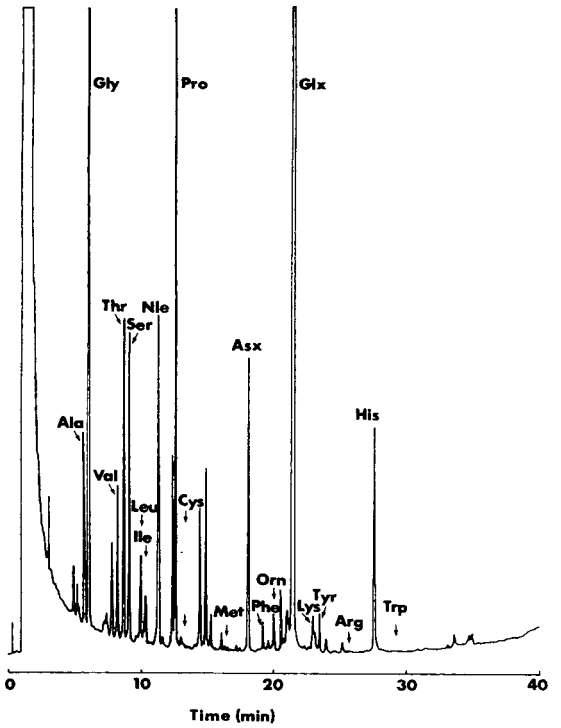


Fig. 5. Gas chromatographic separation on a glass capillary column of N-heptafluorobutyryl isobutyl esters of free amino acids from rabbit liver. Conditions for chromatography are as in Fig. 1.

radioactive amino acids. Butler et al. [5] reported results from a study where twelve amino acids attached to rabbit liver tRNA were determined directly as their trifluoroacetyl methyl derivatives using gas chromatography on packed columns. We have shown here that eighteen amino acids (Gln is determined with Glu, and Asn with Asp) attached to rabbit reticulocyte and liver tRNA can be separated and quantified as their N-heptafluorobutyryl isobutyl esters using gas chromatography on capillary columns. The method also allows analysis of free amino acids in tissue samples.

Determination of amino acids attached to tRNA in vivo involves a number of problems. One is preservation of the aminoacyl-tRNA ester bond during isolation and purification of tRNA. Another is separation and quantification of small amounts of amino acids obtained from tRNA by deacylation. A third factor is interpretation of the results in terms of different forms of tRNA present in the cell. Cycloheximide was given to rabbits prior to collection of reticulocytes or removal of liver to "freeze" the elongation process on ribosomes and thus to maximize the proportion of tRNA that would be in aminoacylated form. Rapid freezing of tissue samples in liquid nitrogen and maintenance of low pH until deacylation in vitro were further efforts to protect the aminoacyl-tRNA ester bond.

Transfer RNA is cycled in the cell through several stages during translation.

TABLE II

FREE AMINO ACIDS IN RABBIT LIVER

Comparison of results from determination of an amino acid sample with gas chromatography on a glass capillary column or with an amino acid analyzer (μmol/g liver, mean ± S.E.M., n = 3).

Amino acid	Gas chromatography	Amino acid analyzer
Ala	0.19 ± 0.007	0.18 ± 0.009
Arg	—*	—*
Asn + Asp	0.21 ± 0.003	0.32 ± 0.02
Cys	—*	—*
Gln + Glu	3.55 ± 0.16	3.20 ± 1.60
Gly	4.21 ± 0.11	5.31 ± 0.75
His	0.60 ± 0.06	0.39 ± 0.03
Ile	0.03 ± 0.001	0.04 ± 0.003
Leu	0.07 ± 0.002	0.07 ± 0.003
Lys	0.03 ± 0.001	0.02 ± 0.001
Met	0.01 ± 0.001	0.01 ± 0.002
Orn	0.03 ± 0.002	0.04 ± 0.003
Phe	0.02 ± 0.001	0.10 ± 0.003
Pro	0.83 ± 0.01	0.32 ± 0.07
Ser	0.29 ± 0.003	0.38 ± 0.007
Thr	0.28 ± 0.001	0.33 ± 0.01
Trp	—*	—*
Tyr	0.02 ± 0.002	0.03 ± 0.003
Val	0.12 ± 0.003	0.14 ± 0.007
Total	10.49	10.88

*Value below the detection limit.

These include free tRNA, tRNA attached to its specific aminoacyl-tRNA synthetase, free aminoacyl-tRNA, aminoacyl-tRNA attached to its specific codon on the ribosome and finally peptidyl-tRNA. Smith and McNamara [3, 4] have estimated by in vitro acylation with and without periodate oxidation that the level of tRNA aminoacylation in rabbit reticulocytes ranges from 71% for tRNA accepting leucine and phenylalanine to most values being close to 90%. The remaining 10% consists of free tRNA and peptidyl-tRNA. The proportion of peptidyl-tRNA probably varies according to the rate of protein synthesis and the amount of ribosomes in the cell. There may also be situations where tRNA aminoacylation is not maximal due to a limited supply of amino acids or energy although high levels of aminoacylation have been found in rat liver during starvation [10]. Some potential sources of error have probably been avoided in the present study by using only well-fed animals and the precautions outlined above during collection of the cells and handling of the aminoacyl-tRNA during preparation. Determination of amino acids attached to tRNA by gas chromatography on capillary columns under these conditions gives an estimate also of the abundance of amino acid specific tRNA species in the cell although a small source of error is included in the form of free and peptidyl-tRNA which are not measured by this method.

Smith and McNamara [3, 4] found that only about half of the material absorbing at 260 nm and eluted as a single symmetrical peak from the Sephadex G-100 column accepts amino acids in vitro. This was confirmed in the present study, since only about 47% of liver tRNA contained attached amino acids (Table I). The nature of the "inactive" tRNA is not known, but it may consist of incomplete tRNA molecules lacking the CCA end or being partly degraded, tRNAs with altered modifications, or RNA molecules other than tRNA.

Taken together, separation and quantification of free amino acids and amino acids attached to tRNA in vivo from a single tissue sample is shown by a direct gas chromatographic method. The method is rapid and sensitive and yields data for all amino acids in a single chromatographic run. Further, it gives information on tRNA which may not be possible to obtain from in vitro experiments. Other uses of the method include studies of in vivo acylation of tRNA in situations where the supply of amino acids or energy may become limiting for protein synthesis.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF METHYLATED PHOSPHOLIPIDS

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SUMMARY

A rapid high-performance liquid chromatographic method for the separation of methylated phospholipids is described. The separation is accomplished on an amine column using acetonitrile—methanol—water as the eluting solvent and UV detection at 203 nm. The choice between gradient and isocratic elution for the separation depends upon the condition of column. The method is suitable for the isolation of phosphatidylcholine, sphingomyelin, lysophosphatidylcholine, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine and lysophosphatidylethanolamine from tissues. It is applicable to the study of reaction products in phosphatide methyltransferase assay mixtures. Choline and ethanolamine plasmalogens can be determined indirectly by converting them into lysophosphatidylcholine and lysophosphatidylethanolamine with exposure to hydrochloric acid fumes.

INTRODUCTION

Phosphatidylcholine (PC), a major constituent of mammalian cell membranes, can be synthesized by two pathways: by stepwise methylation of phosphatidylethanolamine (PE), and by the CDP-choline pathway [1]. The methylation pathway utilizes S-adenosyl-L-methionine (SAM) as the methyl donor, and is catalyzed by two methyltransferases with phosphatidyl-N-monomethylethanolamine (PMME) and phosphatidyl-N,N-dimethylethanolamine (PDME) as intermediates [2]. Recently Hirata and Axelrod [3] have demonstrated in many cell types an important role of phospholipid methylation in the transduction of receptor-mediated signals through the cell membranes. Phospholipid methyla-

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tion is coupled to calcium influx and the release of arachidonic acid. A simple, rapid and quantitative analytical method for the isolation of PE, PMME, PDME and PC will be very useful for studying the function and regulation of phospholipid methylation. The isolation of the different methylated phospholipids by thin-layer chromatography (TLC) [4] is tedious and time consuming. Gas—liquid chromatographic analysis of the bases after acid hydrolysis of the lipids is also a laborious procedure [5]. Axelrod and co-workers [6, 7] recently reported a high-performance liquid chromatographic (HPLC) method for separation and characterization of the major phospholipids formed by methyltransferases in the rat liver. Using a silica gel column, a solvent system of chloroform—methanol—water—ethanolamine (77.8 : 20 : 2 : 0.2, v/v), a fraction collector and liquid scintillation counting, the method is suitable for the analysis of radiolabelled phospholipids. However, since the solvent system absorbs in the 200-nm range, direct monitoring of lipid separation with UV detection is not possible. In this report we describe a rapid and simple HPLC method which is suitable for the isolation of methylated phospholipids from tissues.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine, sphingomyelin (SPH), lysophosphatidylcholine (LPC), phosphatidylethanolamine and lysophosphatidylethanolamine (LPE) were obtained from Sigma (St. Louis, MO, U.S.A.). Egg yolk PMME and PDME were purchased from Gibco (Grand Island, NY, U.S.A.). They were derivatives of egg yolk PC by the exchange of bases in the presence of phospholipase D. Dipalmitoyl PMME and PDME were synthetic products of Calbiochem-Behring (La Jolla, CA, U.S.A.). Acetonitrile and methanol were of HPLC grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). S-Adenosyl-L-[methyl-^{14}C]methionine (59 Ci/mol), and [dipalmitoyl-1-^{14}C]phosphatidylcholine (100 mCi/mmol) were purchased 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. A 1-g amount of rat tissue from cerebrum or right lobe of liver 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. [8]. An aliquot of the lower phase was injected into the chromatograph for analysis. Lipid phosphorus in tissue extracts was measured by the Bartlett procedure [9].

Hydrolysis of choline and ethanolamine plasmalogens

For the analysis of choline and ethanolamine plasmalogens present in tissues, an aliquot of the lipid extract was dried under nitrogen. The open vial was then inverted and held over an open bottle of concentrated hydrochloric acid for 10 min. After flushing the vial with nitrogen, chloroform was added in an amount identical with that of the original sample. An aliquot was injected into the chromatograph for analysis.

Chromatographic conditions

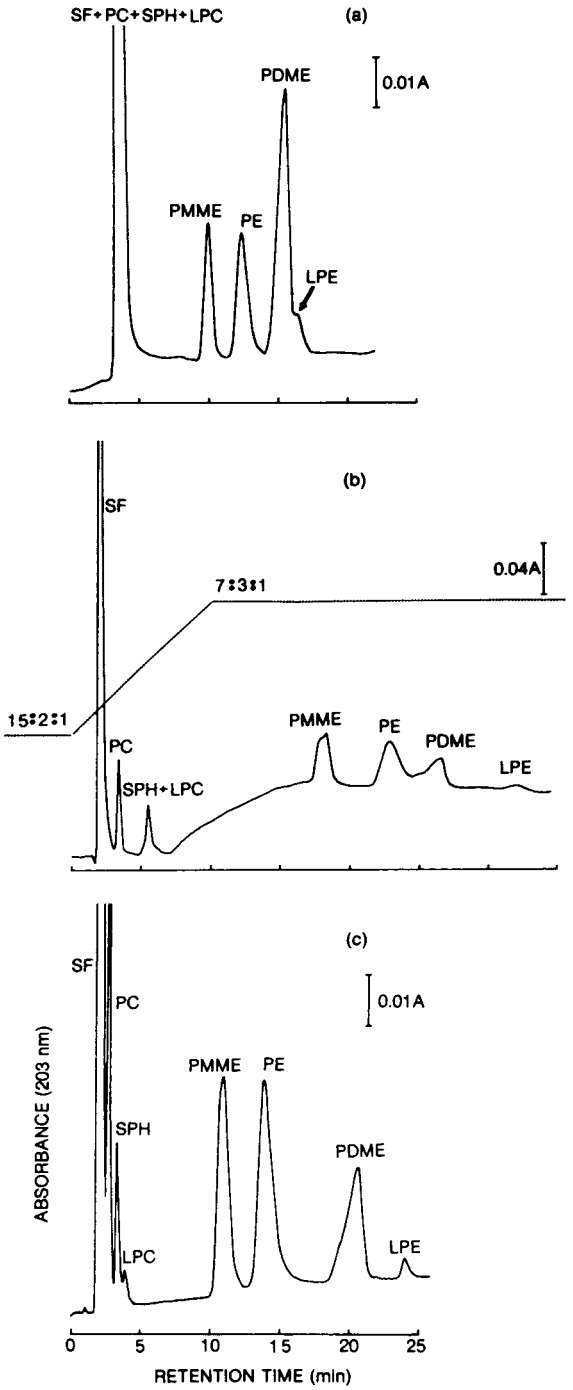
We used a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatographic system consisting of a Model 6000 solvent delivery system, a Model 660 solvent programmer, a Model U6K injector, a Model 450 variable-wavelength detector and a strip chart recorder. The column was a 30 cm × 3.9 mm I.D. prepacked stainless-steel column which contained μBondapak NH_2, particle size 10 μm (Cat. No. 84040). The packing material has an amino group chemically bonded to silica at 9% (w/w) (—Si—R—NH_2). Solvent composition, flow-rate, sample size and recorder response are indicated in the legends to the figures. The temperature was approximately 21°C. The detection was at 203 nm. The reference cell contained air. 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.

Analysis of reaction products in methyltransferase assay

The rat liver was minced and homogenized at 4°C in 3 volumes of 0.25 *M* sucrose with a Polytron (Brinkmann Instruments, Westbury, NY, U.S.A.) for 1 min. The homogenate was centrifuged for 20 min at 10,000 *g* to sediment cell debris, nuclei and mitochondria. Aliquots of the postmitochondria supernatant were used as enzyme suspensions in the phosphatide methyltransferase assays. The enzyme activity was assayed by measuring the incorporation of methyl group from [methyl-^{14}C]SAM into phospholipids as described by Tanaka et al. [10]. The assay mixture contained 5.9 μ*M* [methyl-^{14}C]SAM (0.1 μCi), 10 m*M* L-cysteine, 10% glycerol, 0.1 *M* Tris · HCl buffer (pH 8.8) and enzyme suspension (2.5 mg protein) in a total volume of 0.5 ml. The reaction was initiated by the addition of radioactive SAM. The mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 8 ml of chloroform—methanol (2 : 1, v/v). Lipids were extracted according to the Folch procedure [8]. After washing once with the pure solvent upper phase [8], the lower chloroform layer was dried down under nitrogen and redissolved in 30 μl of chloroform. An aliquot (20 μl) was injected into the chromatograph for the analysis of reaction products. We attempted to confirm that the reaction products were methylated phospholipids by comparing the retention times of ^{14}C-radioactivity peaks with those of phospholipids derived from various sources. The chromatogram of ^{14}C-labelled substances was obtained by collecting the effluent for 40 min into 80 scintillation vials, i.e. a 30-sec collection per fraction. The eluate was evaporated to dryness, dissolved in 0.5 ml of methanol and counted for β-emission in 15 ml of Liquifluor (New England Nuclear).

Quantitative analysis of phospholipids in the rat liver and brain extract

Lipid extracts were injected into the chromatograph for analysis. Phospholipid classes were quantitated by collecting the effluent under the peaks and measuring the lipid phosphorus with malachite green [11]. The effluent of PMME peak was also quantified by a Dns derivatization procedure which we previously described [12]. Briefly, the lipid reacted with 1-dimethylaminonaphthalene-5-sulfonyl chloride (Dns-Cl) to form a fluorescent derivative. The derivatization mixture was analyzed by a second high-performance liquid chromatograph using a silica gel column and gradient elution. Dns-PMME was measured by fluorescence detection.


SF+PC+SPH+LPC
(a)
PDME
0.01A
PMME
PE
LPE
(b)
SF
0.04A
7:3:1
15:2:1
PMME
PE
PDME
PC
LPE
SPH+LPC
(c)
SF
PC
0.01A
PMME
PE
SPH
PDME
LPC
LPE
ABSORBANCE (203 nm)
0
5
10
15
20
25
RETENTION TIME (min)

RESULTS

HPLC of phospholipid standards

Aliquots of phospholipid solution, containing egg yolk PC, SPH, LPC, PE, PMME, PDME and LPE were injected onto an amine column for analysis, and acetonitrile—methanol—water mixtures were used as eluting solvents. The selection of solvent composition and the choice between gradient and isocratic elution for the separation depended upon the condition of the column. When the column was new, PE, PMME, PDME and LPE were strongly retained. Only eluting solvents with relatively high methanol and water contents could elute these lipids within a short time. On the other hand, the separation of PC, SPH and LPC required a solvent system of very low polarity. Fig. 1a shows that with an acetonitrile—methanol—water (3:2:1, v/v/v) solvent mixture, PE, PMME and PDME were readily separated. PC, SPH and LPC were eluted with the solvent front (SF), while LPE co-eluted with PDME. To separate all of the methylated phospholipids in a single run, it is necessary to use gradient elution. As shown in Fig. 1b, choline-containing phospholipids were eluted first, while the others were not eluted until the polarity of the solvent was raised. However, after the column was used repeatedly for the analysis of tissue extracts, the ability of stationary phase to retain ethanolamine-containing phospholipids decreased. The polarity of eluting solvent that was required for a rapid separation of these lipids became lower and lower with increasing use of the column. In contrast, the retention times of choline-containing phospholipids tended to increase. In time the use of a solvent system of relatively low polarity (acetonitrile—methanol—water, 13:7:1, v/v/v) in isocratic elution could separate all of the methylated phospholipids rapidly (Fig. 1c). The number of theoretical plates of the column was approximately 1500 in the beginning and decreased to 900 at the time the isocratic elution was employed. Despite the decrease in column efficiency, good resolution was achieved by adjusting the proportion of acetonitrile, methanol and water in the eluting solvent. These results were reproducible in all the three columns that we utilized in this present study. Recoveries of phospholipids applied to the column were determined by measuring the amount of phosphorus in the eluted phosphorus peaks. They were consistently greater than 95%, except for the recovery of PE which was 85%. The quantitative recovery of PC was also confirmed by counting the radioactivity in the eluted PC peak after injecting [dipalmitoyl-1-^{14}C] PC into the column.

Fig. 1. Chromatograms of egg yolk phospholipids. Chromatographic conditions: (a) new column, isocratic elution with acetonitrile—methanol—water (3:2:1, v/v/v), flow-rate, 1.0 ml/min, recorder response, 0.1 a.u.f.s.; (b) new column, gradient elution with acetonitrile—methanol—water mixtures from 15:2:1 to 7:3:1 (v/v/v) linearly in 10 min, flow-rate, 1.5 ml/min, recorder response, 0.4 a.u.f.s.; (c) a column in daily use for 1 month; isocratic elution with acetonitrile—methanol—water (13:7:1, v/v/v), flow-rate, 1.6 ml/min, recorder response 0.1 a.u.f.s. The amount injected was approximately 1 μg of each lipid in (a), 6 μg of each lipid in (b), and 2 μg of each lipid in (c). Peaks: SF = solvent front: PC = phosphatidylcholine; SPH = sphingomyelin; LPC = lysophosphatidylcholine; PE = phosphatidylethanolamine; PMME = phosphatidylmonomethylethanolamine; PDME = phosphatidyldimethylethanolamine; LPE = lysophosphatidylethanolamine.

HPLC of tissue extracts

Aliquots of lipid extracts from rat brain and liver were injected into the chromatograph, and the isocratic technique was used to elute the lipids. Despite the use of crude Folch extracts [8], chromatograms in Fig. 2a and Fig. 3a reveal peaks of PC, SPH and PE free of interferences by other materials. Neutral lipids were eluted in the solvent front. Phosphatidylinositol (PI) and phosphatidylserine (PS) could not be eluted. These chromatographic patterns were consistent with the published results on the relative abundance of phospholipids in various organs of rats [13]. Peaks of PMME, PDME and LPE were detectable only when a larger aliquot was injected and when the sensitivity of the detector was increased (Fig. 3b).

Analysis of plasmalogens

The chromatographic condition described here did not separate choline plasmalogen from PC nor ethanolamine plasmalogen from PE. Plasmalogens

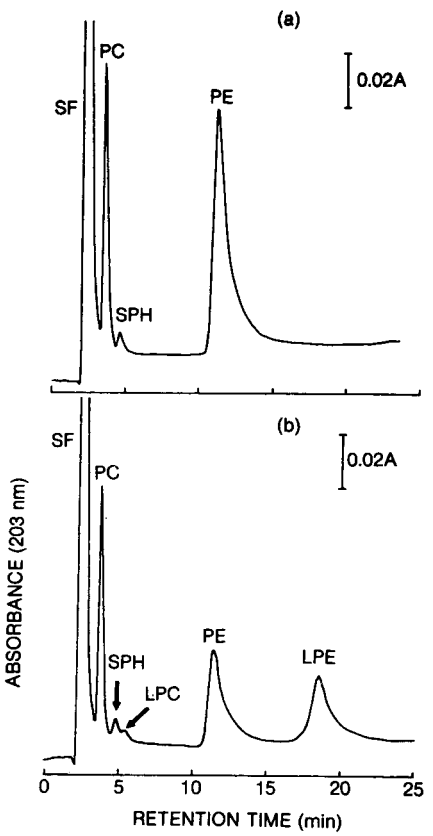


Fig. 2. HPLC analysis of the lipid extract of rat brain before (a) and after (b) exposure to hydrogen chloride fumes. An aliquot of the Folch lipid extract [8], containing 15 μg of total phospholipids, was injected directly into the chromatograph. Another aliquot, also containing 15 μg, was dried, exposed to hydrogen chloride fumes, redissolved in chloroform and then injected. Chromatographic conditions: isocratic elution with acetonitrile—methanol—water (13 : 7 : 1, v/v/v); flow-rate, 1.6 ml/min; and recorder response, 0.2 a.u.f.s.

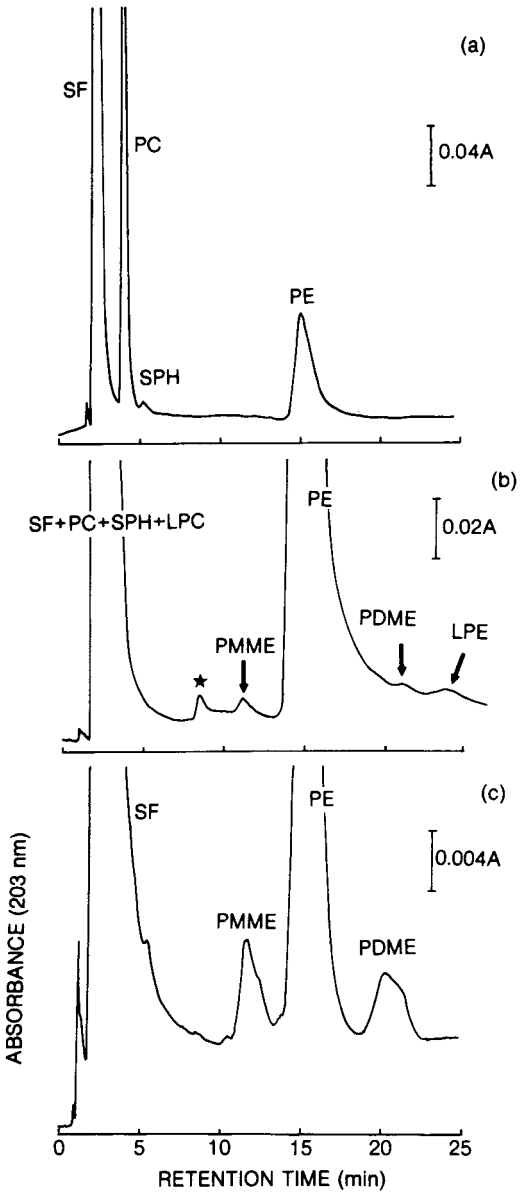


Fig. 3. HPLC analysis of the lipid extract of rat liver. Chromatographic conditions: isocratic elution with acetonitrile—methanol—water (13:7:1, v/v/v); and flow-rate, 1.6 ml/min. (a) An aliquot of the Folch lipid extract [8] containing 15 μg of total phospholipids; recorder response, 0.4 a.u.f.s.; (b) an aliquot containing 360 μg of total phospholipids in 25 μl of chloroform, recorder response, 0.2 a.u.f.s.; (c) isolation of PMME and PDME from rat liver. PMME and PDME peaks were collected and reinjected into the chromatograph for the removal of PE contaminant, see text for explanation. ⋆ = An unidentified peak.

could be analyzed by comparing the chromatogram of the original lipid extract (Fig. 2a) with that of the lipid extract that had been exposed to hydrogen chloride fumes before the HPLC analysis (Fig. 2b). This is based on previous observations that hydrogen chloride fumes quantitatively hydrolyze the alk-1-enyl group from phospholipids and neutral lipids [14]. The chromatogram of the original lipid extract reveals no detectable amounts of lyso-PC and lyso-PE in rat brain. The exposure to hydrogen chloride fumes converted choline plasmalogen into lyso-PC and ethanolamine plasmalogen into lyso-PE, since Fig. 2b shows that the peaks corresponding to PC and PE decreased while the peaks corresponding to lyso-PC and lyso-PE appeared. Table I shows the results of analysis of ethanolamine plasmalogens in the rat brain extract. They are compared with the results obtained by other HPLC and TLC methods. Good agreement between these results is evident.

TABLE I

ANALYSIS OF ETHANOLAMINE PLASMALOGENS IN RAT BRAIN

The values in the present analysis, percentages of the total lipid phosphorus, are mean ± S.D. obtained from three rats. Aliquots of lipid extracts containing 32 μg of total phospholipids were injected into the chromatograph as described in Fig. 2. PE and lyso-PE peaks were collected from the effluent and quantitated by measuring the lipid phosphorus. Ethanolamine plasmalogens were determined by the decrease in PE fraction and the increase in lyso-PE after exposure of the lipid extract to hydrogen chloride fumes.

	Present analysis	Dns derivatization and HPLC by Chen et al. [12]	HPLC by Jungalwala et al. [15]	TLC by Clarke and Dawson [16]
Before exposure to hydrogen chloride				
PE	40.0 ± 2.8	43.6 ± 1.2	41.6 ± 2.6	38.2
Lyso-PE	None detected			
After exposure to hydrogen chloride				
PE	19.2 ± 2.0			15.4
Lyso-PE (derived from plasmalogens)	19.9 ± 0.9	18.9 ± 0.6	22.9 ± 1.3	21.3

Isolation of PMME and PDME from rat liver

The isolation of PMME and PDME from tissues was complicated by the fact that the content of these lipids in the crude lipid extract is extremely small [5, 17]. For PMME and PDME peaks to be detectable with UV detection, a relatively large amount of lipid extract had to be injected into the chromatograph. Because the content of PE in the rat liver is about 1000-fold more than those of PMME and PDME [17], the resolution of ethanolamine-containing phospholipids is sacrificed when the sample size is increased. We used the following procedure to isolate PMME and PDME from the rat liver. In order to avoid excessive contamination by PE, in each injection the sample size of the lipid extract was limited to 360 μg of total phospholipids. PMME and PDME peaks were collected from the effluent. Collections from several injections were combined, evaporated to dryness under nitrogen, and redissolved in 30 μl of chloroform. It was injected for the second time into the chromatograph for the

TABLE II

ANALYSIS OF PMME AND PDME IN THE RAT LIVER AND BRAIN EXTRACTS

The values are the amount of each lipid per mg of total phospholipids in lipid extracts. They are mean ± S.D. obtained from three rats. The method of analysis was described in the text and Fig. 3. PE, PMME and PDME peaks were collected from the effluent and quantitated by measuring the lipid phosphorus with malachite green [11].

	Liver	Brain
PE	0.28 ± 0.01 mg	0.40 ± 0.03 mg
PMME	0.60 ± 0.04 μg 0.76 ± 0.17 μg*	0.50 ± 0.08 μg*
PDME	1.00 ± 0.16 μg	1.94 ± 0.20 μg

*Lipid quantitated by Dns derivatization [12].

removal of PE contamination (Fig. 3c). Identities of the isolated materials, PMME and PDME, were confirmed by comparing their retention times with those of the ^{14}C-labelled phospholipids of the reaction products in the phosphatide methyltransferase assay as described in the next experiment. Table II shows the results of analysis of PE, PMME and PDME in the rat liver and brain extracts. The liver results are in general agreement with those obtained by TLC method as reported by Katyal and Lombardi [17]. We have previously described an HPLC procedure for the analysis of amino group-containing phospholipids in tissues [12]. It involves Dns derivatization of phospholipids followed by HPLC separation of fluorescent derivatives. The usefulness of this procedure for the analysis of methylated phospholipids is limited, because the gradient elution does not separate Dns-PMME from Dns-PE, and because PDME cannot be derivatized with Dns. However, after PMME has been isolated from the tissue extract and PE contamination removed, dansylation can be used for the quantitation of PMME. The major advantage is that because of its sensitivity the effluent collected from only one single injection of the lipid extract is sufficient for quantitation. Table II shows that PMME values obtained by measuring lipid phosphorus with malachite green and by the derivatization procedure were in agreement.

Analysis of reaction products in methyltransferase assay

Experiments were performed in order to determine the usefulness of HPLC in the assay of phosphatide methyltransferases. Intermediates and the product of the methylation pathway in the biosynthesis of PC from PE were labelled by [methyl-^{14}C]SAM using liver homogenates. Previous studies [4, 6, 7] using TLC and HPLC revealed that the labelled reaction products in the assay mixture were PMME, PDME and PC. PE was not significantly labelled. With the method described here the chromatogram of lipid extract (Fig. 4d) shows four peaks of radioactivity. Under the same chromatographic condition, we also injected into the chromatograph separately mixtures of PE, PMME and PDME derived from various sources (Fig. 4a, b and c). The comparison of these chromatograms shows that radioactivity peaks A, B, C and D corresponded to SF, PC, PMME and PDME, respectively. Phospholipids from different sources show-

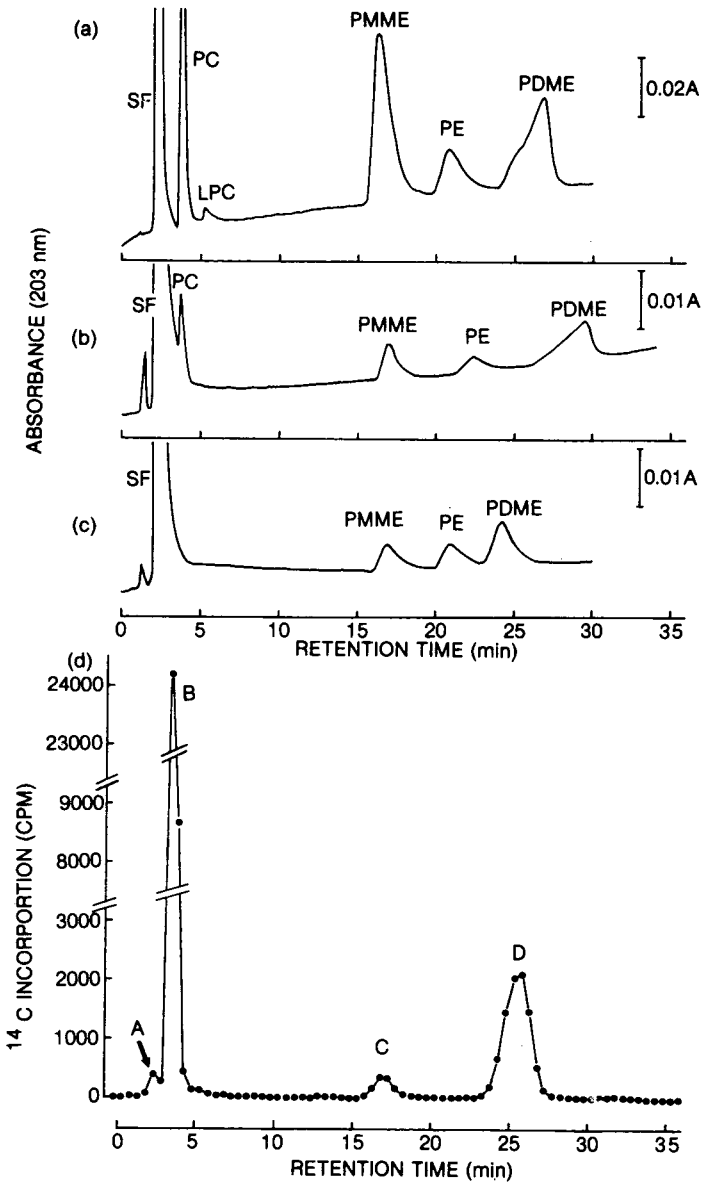


Fig. 4. HPLC analysis of the ^{14}C-labelled lipids of reaction products in the methyltransferase assay mixture, and its comparison with the chromatographic patterns of methylated phospholipids from various sources. Chromatographic conditions: isocratic elution with acetonitrile—methanol—water (110:65:3, v/v/v); flow-rate, 1.5 ml/min. (a) Egg yolk phospholipid mixture, containing approximately 3 μg of PE and 8 μg each of the others; (b) synthetic phospholipid mixture, containing dipalmitoyl PC (15 μg), dipalmitoyl PE (15 μg), dipalmitoyl PMME (30 μg) and dipalmitoyl PDME (30 μg); (c) rat liver PE, PMME and PDME, approximately 1 μg each; (d) reaction products in the stepwise methylation of PE to PC in the rat liver were labelled by [methyl-^{14}C]SAM in vitro as described under Materials and methods. The lipid extract of the assay mixture was injected into the chromatograph. The radioactivity of the effluent was measured. Each dot represents cpm in a 30-sec collection of effluent.

ed variations in the retention time and the peak shape. This is evident particularly in the elution of PDME. This most likely reflected the influence of fatty acid composition on the retention time.

DISCUSSION

The packing material, μBondapak NH_2, contains amino groups chemically bonded to silicas (—Si—R—NH_2). It is a weak anion exchanger (pK_b 6.5) when the pH of the mobile phase is 5.0 or below, but in this present study it was used in a partition mode to separate phospholipid components. The choice of this stationary phase was based on the rule, like-attracts-like. Indeed, ethanolamine-containing phospholipids were eluted from the column much later than choline-containing phospholipids. When the column was new, the differing retention by the stationary phase between choline-containing and ethanolamine-containing phospholipids was so great that gradient elution was required for a rapid separation of all of the methylated phospholipids in a single run. However, with increasing use of the column, the retention of ethanolamine-containing phospholipids decreased gradually. In contrast, the retention of choline-containing phospholipids was increased. It became feasible to use a low-polarity solvent to elute all of the methylated phospholipids isocratically. This most likely was due to a decrease in the number of amino group functionality on the stationary phase resulted from either deactivation of the amino function by peroxides, ketones and aldehydes or the slow accumulation of organic compound contaminants.

The reduction in amino group functionality on the stationary phase enables lipid components to be analyzed by chromatography using isocratic elution without needlessly long separation times. Compared with the analysis using gradient elution, isocratic technique not only is faster, but also avoids a steeply sloped baseline. UV detection with a straight baseline is a prerequisite for operating the chromatograph at maximum sensitivity. A brand new μBondapak NH_2 column was unsuitable for the simultaneous analysis of choline- and ethanolamine-containing phospholipids in tissue extracts, because gradient elution precluded the use of high-sensitivity detection (Fig. 1b). In this present study it was necessary to operate the chromatograph at high sensitivity, because contents of PMME and PDME were very low. For this reason, we did not consider the deactivation and the reduction of amino group functionality in the early stage of the column life to be undesirable. However, after a column has been deactivated to the extent that isocratic elution technique becomes applicable, precautions must be taken to avoid contamination and prevent further reduction in amino group functionality. To minimize the change with time in separation patterns, sample purification prior to HPLC analysis with Sep-Pak silica gel cartridges (Waters Assoc.) and cleaning of the column after use by washing with organic solvents (see Materials and methods) are effective and good practices. The use of a guard column which is changed regularly prolongs the column life, provided that it is packed with suitable material. We have observed that the packing material for guard column recommended by the vendor (Waters Assoc.), Bondapak AX/Corasil, broadens the PE peak. This may be related to the fact that Bondapak AX/Corasil, a strong anion exchanger,

ionizes in a pH range from 1 to 13. This is incompatible with an analytic column which operates in a partition mode.

Because of the different fatty acid composition, phospholipid component derived from various sources may show variations in retention time. This is evident particularly in the elution of PDME and lyso-PE. The tentative identification of chromatographic peaks based on retention times, the elution order and relative abundance of phospholipids in tissues should be confirmed by other independent methods. In this present study the identity of peaks was confirmed by the following methods: (a) the exposure of lipid extracts to hydrogen chloride fumes caused the peaks corresponding to PC and PE to decrease, while peaks corresponding to lyso-PC and lyso-PE appeared (Fig. 2); (b) peaks corresponding to PE, PMME and lyso-PE were collected and confirmed by Dns derivatization and a second HPLC with fluorescence detection [12]; and (c) putative PC, PMME and PDME peaks in the chromatogram of rat liver were confirmed by comparing their retention times with those of the radiolabelled phospholipids of the reaction products in the phosphatide methyltransferase assay (Fig. 4). Previous TLC and HPLC studies [4, 6, 7] have shown that only these three phospholipids became radiolabelled in the assay.

Kiuchi et al. [18] previously used flame ionization detection, a μBondapak NH_2 column and an isocratic solvent system of chloroform—methanol—water to separate lipid mixtures. Using a solvent proportion of 75 : 25 : 4 (v/v/v), the order of lipids eluted were triglycerides, phosphatidic acid, PC, phosphatidylglycerol, PE, PS and PI. Hanson et al. [19] previously performed HPLC of egg yolk lipids with a similar amine column, Ultrasil-NH_2 (Altex, Berkeley, CA, U.S.A.). Using gradient elution of hexane—isopropanol—water mixtures they separated the lipid extract into neutral lipids, PC, SPH, lyso-PC and PE fractions. PS and PI were not eluted. Comparisons of these previous methods which also employed amine columns with the method described here reveal similarity in the order of phospholipid elution.

In the biosynthesis of PC from PE, PMME is the product of methyltransferase I, while PDME and PC are the products of methyltransferase II. HPLC offers an easier and more accurate method for analyzing the reaction products in phosphatide methyltransferase assays than TLC. With the method described here the dynamics of phospholipid methylation in cellular activation can be more readily studied. The method is also well suited for the quantitative analysis of methylated phospholipids and plasmalogens in tissue extracts. For the quantitation of phospholipid components previous investigators [20] suggested that specific peaks are collected and quantified by the analysis of phosphorus. Being sensitive and non-destructive, UV detection is ideal for monitoring the separation of lipids by HPLC. Approximately 1 μg of phospholipid can be detected. However, it is complicated to use UV response directly for lipid quantitation. UV absorption by lipids at the 200-nm region is due largely to the presence of double bonds [20]. The absorption by other functional groups, such as ester carbonyl and amino, also occurs, but it is small in extent. PS and PI possibly were retained by the stationary phase and could not be eluted with an amine column and the solvent systems described in this report. With a differ-

ent solvent, chloroform—methanol—water (75:25:4, v/v/v), PS and PI were eluted behind PE as reported by Kiuchi et al. [18]. They also can be analyzed by HPLC using the methods that we reported previously [12, 21].

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CHROMBIO. 1402

DEVELOPMENT AND UTILIZATION OF A PROCEDURE FOR MEASURING URINARY PORPHYRINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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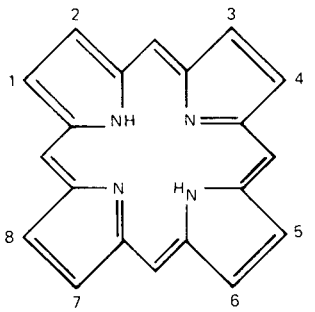
SUMMARY

We describe a procedure for determining the five principal urinary porphyrins — uroporphyrin, heptacarboxylporphyrin, hexacarboxylporphyrin, pentacarboxylporphyrin, and coproporphyrin — by using high-performance liquid chromatography (HPLC). The method involves a 12- to 15-min isocratic separation on a Bondapak® Phenyl column. Studies have indicated that urine samples must be preserved to maintain porphyrin stability for extended periods. We have found that the pH of preserved samples must be adjusted into the acidic range before the samples can be accurately analyzed by this HPLC procedure. Our studies demonstrate that good reproducibility and recovery are achieved with this method. Urinary porphyrin values for normal and porphyric individuals are reported.

INTRODUCTION

The recognition of characteristic urinary porphyrin patterns is important in the diagnosis of porphyria, a disease characterized by excessive porphyrins in body fluids and excreta. Five porphyrins may be found in urine (Fig. 1). Type I porphyrins are unused byproducts of the heme biosynthetic pathway and are usually the principal porphyrin constituents in normal urine. Type III porphyrins are precursors to heme and are not observed in normal urine except in trace amounts. Most normal urine samples contain only coproporphyrin and perhaps a small amount of uroporphyrin. The heptacarboxyl, hexacarboxyl, and pentacarboxyl porphyrins are found in normal urine only in trace amounts, and their detection depends upon the sensitivity of the analytical procedure. The individual measurement of these five porphyrins provides a relative distribution pattern which, in the case of chronic hepatic porphyria, is so characteristic that a diagnosis can be made on the basis of a single examination of a small urine sample [1].

Porphyria is either hereditary (of genetic origin) or acquired from exposure



	Type I	Type III
uroporphyrin (octacarboxylic)	1,3,5,7=A 2,4,6,8=P	1,3,5,8=A 2,4,6,7=P
heptacarboxylic acid	1=M 3,5,7=A 2,4,6,8=P	1=M 3,5,8=A* 2,4,6,7=P
hexacarboxylic acid	1,3=M 5,7=A* 2,4,6,8=P	1,3=M 5,8=A* 2,4,6,7=P
pentacarboxylic acid	1,3,5=M 7=A 2,4,6,8=P	1,5,8=M 3=A* 2,4,6,7=P
coproporphyrin (tetracarboxylic)	1,3,5,7=M 2,4,6,8=P	1,3,5,8=M 2,4,6,7=P
$A=CH_2CO_2H$	$P=CH_2CH_2CO_2H$	$M=CH_3$

* = more than this isomer possible

Fig. 1. Porphyrin structures.

to chemicals. Our particular interest is in chemically induced porphyria. Among the chemicals which cause or precipitate porphyria are halogenated hydrocarbons [2—6], steroid hormones [7], ethanol [8], lead [9], barbiturates, and other drugs [10]. Many of our case studies involve low-level or chronic poisoning or exposure to environmental chemicals, and we are interested in analyses which identify chemically induced disease or injury at early stages before clinical symptoms occur. Doss [11] reported that some definitive early (subclinical) stages of chronic hepatic prophyria can be readily recognized by analyzing the urinary porphyrin pattern.

Classical clinical laboratory methodology for porphyrin analysis involves the separation and spectrometric or fluorescent measurement of porphyrins as two fractions — the uroporphyrins and coproporphyrins — which, for non-porphyric patients, contain principally uroporphyrin and coproporphyrin, respectively. However, for porphyric patients, each fraction actually contains several different porphyrins so that, for example, the uroporphyrin fraction consists of uroporphyrin, heptacarboxylporphyrin, and hexacarboxylporphyrin, and similarly the coproporphyrin fraction contains coproporphyrin, pentacarboxylporphyrin, and hexacarboxylporphyrin. The classical methodology, therefore, is unsuitable for diagnosing porphyria on the basis of porphyrin pattern. Procedures involving thin-layer chromatography have been used for porphyrin separations but, although specific, these analyses are time-consuming and tedious. Recent reports on the use of high-performance liquid chromatography (HPLC) for the analysis of free urinary porphyrin acids have been promising [12—17]. Many of these procedures involve gradient separations [13—15, 17], which are more time-consuming and require more elaborate equipment than isocratic separations [12, 16]. Bonnett [12] reported an isocratic separation for the porphyrin acids, but difficulties with this separation have been reported [14—17]. In 1980, we published a preliminary report of a rapid isocratic HPLC procedure for determining urinary porphyrins [16]. In this present paper, we provide supporting data and experimental details in the development and use of this procedure.

MATERIALS AND METHODS

Porphyrins

Uroporphyrin I, heptacarboxylporphyrin I, hexacarboxylporphyrin I, pentacarboxylporphyrin I, and coproporphyrin I were purchased from Porphyrin Products, Inc., Logan, UT, U.S.A., as separate standards or in mixed standards, known as porphyrin acid-marker kits. These marker kits, which contained 10 nmol of each of the five Type I porphyrins, were used to prepare standard solutions. A stock solution was prepared from a marker kit vial by dissolving the vial contents in 1 ml of 60% methanol—water solution containing 10 mmol/l tetrasodium EDTA (Sigma, St. Louis, MO, U.S.A.). This was either allowed to stand overnight or was put into an ultrasonic bath to effect complete solution of the porphyrin acids. Standards were prepared by diluting various volumes (50—400 μl) of tetrasodium EDTA (0.4—1.5 ml). All porphyrin standards were made by using this solvent system. Concentrations ranged from 4 μg/ml to 0.2 μg/ml. Aliquots of 5 μl each were injected into the HPLC system to produce standard curves. The heptacarboxyl, hexacarboxyl, and pentacarboxylporphyrins were available individually only as the methyl ester and had to be hydrolyzed to free acid forms. The esters (1 mg) were hydrolyzed with 0.5 ml of Ultrex® hydrochloric acid (J.T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.) and 0.5 ml of water for at least 2 h in a water bath at 60—65°C. The solution was evaporated to dryness at 40—45°C, and was appropriately diluted in 60% methanol—water containing 10 mmol/l tetrasodium EDTA.

Reagents

UV-grade methanol was purchased from Burdick and Jackson Labs., Muskegon, MI, U.S.A., or from Fisher Scientific, Fairlawn, NJ, U.S.A. Water was doubly deionized with a Milli-Q-Water Purification System from Millipore, Bedford, MA, U.S.A. The HPLC mobile phase consisted of 15.6 mmol/l sodium 1-pentanesulfonate (Eastman Kodak, Rochester, NY, U.S.A.) and 0.10 mmol/l tetrasodium EDTA in 60—64% methanol in water solution adjusted to pH 2.1 with sulfuric acid (J.T. Baker). The exact concentration of methanol depends upon the retention characteristics of the particular column being used. The 60—64% methanol composition was generally the range used for several columns. The preservative for collected urine specimens was prepared by mixing 14.4 g of sodium carbonate with 19.3 g of tetrasodium EDTA. A 0.3-g portion of this mixture was added to each 250-ml collection bottle.

Equipment

The chromatographic system consisted of a Waters U6K injector (Waters Assoc., Milford, MA, U.S.A.), a Waters M6000A pump, and a Schoeffel FS970 L.C. Fluorometer (Kratos/Schoeffel Instruments, Westwood, NJ, U.S.A.). The detector was set at an excitation wavelength of 403 nm with a 7-59 transmission prefilter and a 600-nm emission cutoff filter. A Waters 10-μm Bondapak® Phenyl column (300 × 3.9 mm) was used with a MPLC® RP-18 guard column (Brownlee Labs., Santa Clara, CA, U.S.A.).

PROCEDURE

Allow collected urine to stand at room temperature for 24 h before analysis to allow oxidation of porphyrinogens to porphyrins. Five to 10 min before analysis, adjust the pH of the urine sample to between 2 and 6 with 6 *N* sulfuric acid. Set the solvent flow-rate at 1.5 ml/min. Inject a 50-μl aliquot of the pH-adjusted urine. Smaller aliquots or dilution of urine may be necessary if peak heights are used for measurement. Compare peak heights or peak areas with a standard curve or linear regression line generated from external standard solutions. Urinary porphyrin levels are reported in micrograms per liter (μg/l) unless the urine analyzed was a 24-h collection, in which case the level may be reported in milligrams per 24-h period.

RESULTS AND DISCUSSION

Our efforts were initially directed toward the use of a previously reported isocratic procedure [12]; however, problems related to reproducibility and sample carryover required a new approach. We obtained good separation for standards with all five urinary porphyrins by using 73% methanol—water with 5.2 mmol/l pentasulfonic acid acidified with phosphoric acid to pH 2.5, with a C_{18} reversed-phase column. When we analyzed the urine samples, however, we found that an unidentified compound had coeluted with the uroporphyrin. This interference was largest in specimens from persons taking vitamin supplements, and we soon recognized the interference as vitamin B_2 or riboflavin — a common, almost ubiquitous component of urine. With a C_{18} column we could resolve the riboflavin and uroporphyrin peaks only by gradient elution, an unsatisfactory method for our purposes since the time of analysis and regeneration to original conditions was at least 30 min. Our goal was to develop a rapid isocratic procedure (a 15-min separation) so that one analyst could analyze at least 15—20 samples with standards and controls in an 8-h work period using simple HPLC equipment (injector, pump, detector, and recorder/integrator).

We found that an isocratic separation could be achieved by using a Bondapak Phenyl® column which resolved riboflavin and uroporphyrin and still allowed elution of the coproporphyrin within 12—15 min (Fig. 2). Optimum conditions for separation on this column were achieved by adjusting the pH and the counter-ion concentration in the mobile phase. Decreasing the pH produced sharper peaks, particularly the coproporphyrin peak. Pentanesulfonic acid and octanesulfonic acid were evaluated as ion pairing agents. The octanesulfonic acid extended the retention time by 20—30% more than the pentanesulfonic acid, but since it did not improve the separation, pentanesulfonic acid was selected as the ion pair reagent. Both sulfuric acid and phosphoric acid were tested as sources of acidification for the mobile phase. Both gave almost identical separations, but the mobile phase with sulfuric acid produced a slightly faster eluting coproporphyrin peak; therefore, we adopted sulfuric acid for acidification.

Tetrasodium EDTA was added to prevent the formation of trace metal complexes and the precipitation of calcium salts. In early experiments, we found

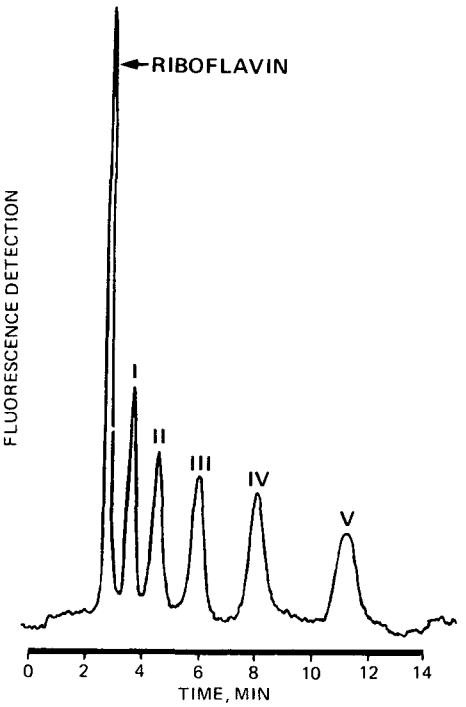


Fig. 2. Chromatogram of porphyrin standard and riboflavin. I = Uroporphyrin (26 ng), II = heptacarboxylporphyrin (25 ng), III = hexacarboxylporphyrin (23 ng), IV = pentacarboxylporphyrin (22 ng), V = coproporphyrin (20 ng).

that porphyrin standards prepared by hydrolysis with ordinary hydrochloric acid apparently formed trace metal complexes with the porphyrins, and these complexes had different retention times and reduced fluorescence. Esters hydrolyzed with Ultrex® hydrochloric acid and diluted with methanol—water containing EDTA were found to be free of these trace metal porphyrin complexes. Whether EDTA is a necessity in the mobile phase is not clear; however, we routinely use EDTA in the mobile phase and have always obtained good results.

We found that the addition of Na_2CO_3 and EDTA as a preservative in the urine samples was necessary to maintain stable samples for extended periods. Unpreserved refrigerated samples showed discernible losses of uroporphyrin and heptacarboxylporphyrin after one week and demonstrated significant losses in the second week (Fig. 3). Although most clinical laboratories would rarely keep samples this long, we must store hundreds of samples for extended periods before analysis. Our usual procedure is to add preservative and then freeze the specimens. Freezing specimens without preservative may also maintain samples for extended periods, but this has not been investigated.

The preservative often changes the pH of the urine sample into the basic range (pH $>$ 7), and we found that urine samples must be adjusted into an acidic range (between pH 2 and 6) just before analysis. In Fig. 4A is shown the chromatogram of a urine specimen with preservative in which the pH

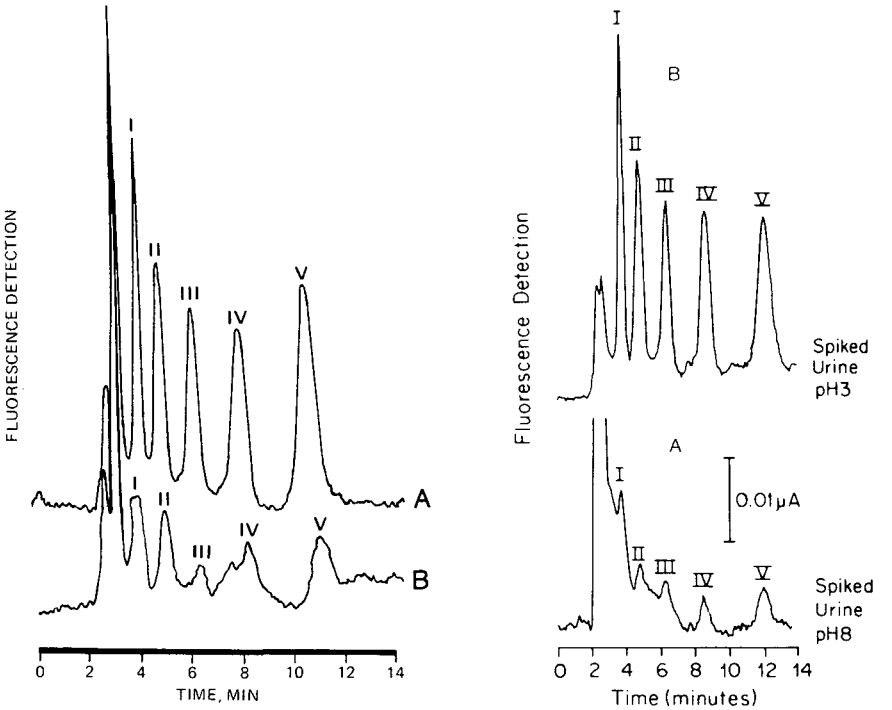


Fig. 3. Chromatograms of spiked urine samples after 2 weeks of refrigerated storage (A) with preservative, (B) without preservative. Peaks as in Fig. 2.

Fig. 4. Chromatogram of spiked urine sample with preservative (A) at pH 8 without acidification, (B) at pH 3 after acidification.

(pH 8) was not adjusted into the acidic range; the chromatogram in Fig. 4B is the same specimen with the pH adjusted to 3.

We attempted unsuccessfully to find a compound which could be used as an internal standard. Our requirements were rather strict, i.e. a compound whose retention time fell just beyond the coproporphyrin peak, was fluorescent under our conditions, and showed similar behavior at differing pH values. All 30 compounds examined either coeluted with the five porphyrins or eluted too late to be considered.

The recovery and reproducibility of the procedure was determined by preparing urine pools spiked at various levels with uroporphyrin, heptacarboxyl, and coproporphyrin. Three aliquots were taken from each of these pools on each of six analytical days covering an 18-day period. Each aliquot was analyzed for the three analytes, and the results are shown in Table I.

The detection limits for each of the porphyrins were estimated to be approximately 5—10 μg/l, or 0.2—0.5 ng per injection. Standards over the range of 5—104 ng/injection produced linear responses with a pooled correlation coefficient for all five porphyrins of 0.9954 ± 0.0065 (X±S.D.) for six successive days of analysis. When refrigerated, standards were found to maintain their stability for at least four weeks.

TABLE I

REPRODUCIBILITY AND RECOVERY IN PORPHYRIN ANALYSIS

Three samples were analyzed for each analyte on each of six days over an 18-day period.

Porphyrin	Overall mean concentration (μg/l)	Relative standard deviation (C.V., %)		Mean recovery (%)
		Within-run	Total*	
Uro-	75	5.7	9.0	104
	150	4.4	11.6	107
	560	6.9	12.7	90
Hepta-	65	5.0	12.1	90
	230	3.8	12.5	99
	500	6.9	12.3	89
Copro-	36	17.6	24.3	110
	180	5.0	6.2	106
	290	6.3	11.7	91

*Combines the within-run and the between-run components of variance.

To establish normal values for this HPLC procedure, we obtained random, fist-morning, and 24-h urine samples from volunteers for analysis. Although we usually collect random urine specimens in our field studies, we have also reported limited data on first-morning and 24-h urine samples (Table II). All samples contained coproporphyrin, and most also had detectable levels of uroporphyrin. We have also analyzed several cases of clinically diagnosed porphyria, and values for these are summarized in Table III. Typical chromatograms of a normal urine sample and some porphyric samples are shown in Fig. 5. Our analytical results confirm earlier reports of characteristic patterns

TABLE II

URINARY PORPHYRIN VALUES FOR NORMAL VOLUNTEERS

	Porphyrin concentration (μg/l)			
	Uro-	Hepta-	Copro-	Total
Random samples (n*=59)				
Mean	6	1	52	58
S.D.	6	3	27	33
Range	0—24	0—9	12—130	12—150
Percentage detectable values	71	10	100	100
1st morning samples (n=36)				
Mean	11	0.4	56	68
S.D.	10	2	35	39
Range	0—37	0—8	5—160	10—170
Percentage detectable values	86	8	100	100
24-h samples (n=10)				
Mean	10 (12)**	0	60 (81)**	70 (92)**
S.D.	11 (12)	0	31 (23)	39 (29)
Range	0—31 (2—44)	0	26—120 (32—120)	33—150 (40—130)
Percentage detectable values	80	0	100	100

*n = number of individuals.
**In parentheses, mg of porphyrin per 24-h period.

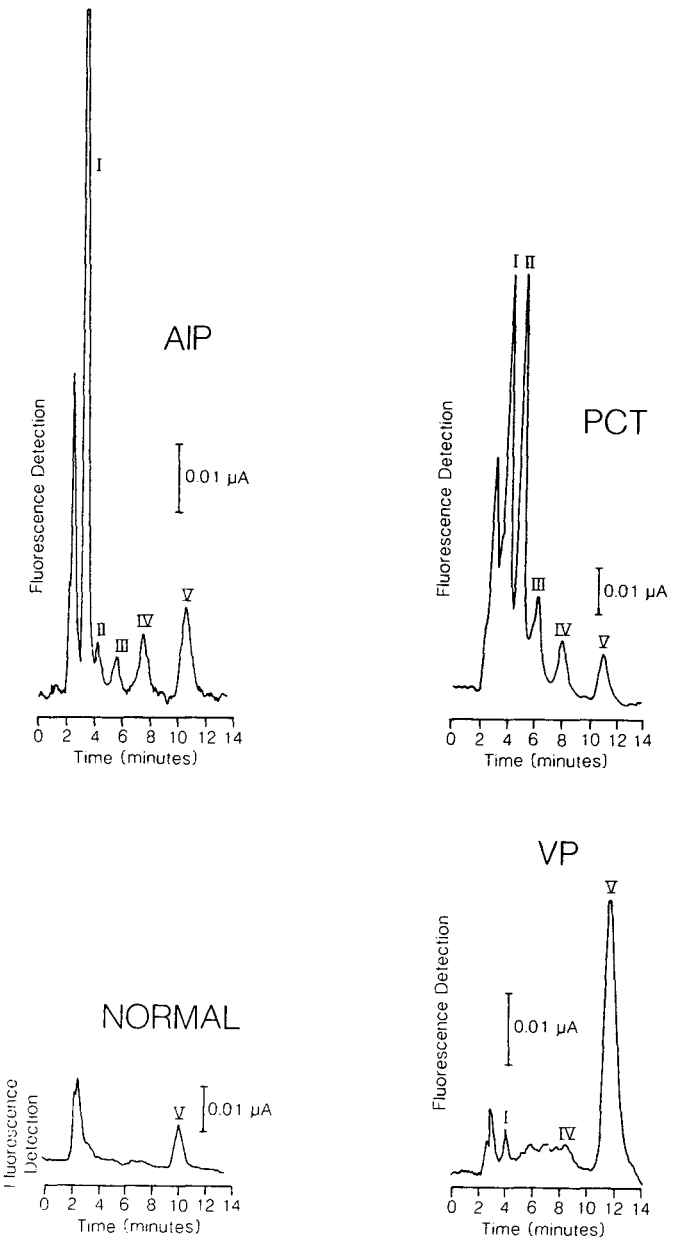


Fig. 5. Chromatograms of urine samples showing patterns for normal individual, acute intermittent porphyria (AIP), porphyria cutanea tarda (PCT), and varigate porphyria (VP).

for various porphyrias. Porphyria cutanea tarda (PCT) is characterized by high levels of uroporphyrin and heptacarboxylporphyrin, the latter being particularly specific for this porphyria [1, 3, 13—15, 17—23]. In PCT, the uroporphyrin/coproporphyrin and heptacarboxylporphyrin/coproporphyrin ratios are greater than one, and — according to some authors — these ratios

TABLE III

URINARY PORPHYRINS IN CLINICALLY CONFIRMED PORPHYRIA

Patient	*n*	Concentrations (μg/l)					Porphyria diagnosis*
		Uro-	Hepta-	Hexa-	Penta-	Copro-	
A	1	1600	400	61	120	95	PCT
B	1	1400	680	29	120	130	PCT
C	5	530 ± 240	140 ± 120	24 ± 30	80 ± 41	53 ± 28	PCT
D	1	670	280	35	83	44	PCT
E	1	1300	1100	45	78	97	PCT
F	1	360	390	78	94	80	PCT
G	4	820 ± 260	540 ± 130	36 ± 28	93 ± 18	80 ± 12	PCT
H	2	1900 ± 410	1200 ± 130	1200 ± 220	670 ± 120	320 ± 36	PCT
I	1	260	290	20	33	86	PCT
J	1	2700	890	110	340	360	PCT
K	1	500	140	23	180	98	PCT
L	1	1000	26	30	54	56	AIP
M	1	1100	110	20	110	2200	VP
N	1	170	20	ND	130	2100	VP

*PCT = porphyria cutanea tarda; VP = varigate porphyria; AIP = acute intermittent porphyria.

can be used to recognize various subclinical states of hepatic porphyria [1, 2]. The single urine specimen that was from a patient with acute intermittent porphyria also conformed to previously reported patterns, showing highly elevated uroporphyrin, but not heptacarboxylporphyrin [9, 15, 18, 23–25]. Specimens from two patients with varigate porphyria (VP) both contained highly elevated coproporphyrin as the dominant component in the porphyrin pattern. Whether this latter pattern is characteristic for VP is less clear. These patterns appear similar to those of patients with cutaneous VP [21]. As better methodology is used for porphyrin analysis, clearer characteristic patterns may emerge.

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

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CHROMBIO. 1393

DETERMINATION OF NIACIN METABOLITES 1-METHYL-5-CARBOXYLAMIDE-2-PYRIDONE AND N-1-METHYLNICOTINAMIDE IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A test suitable for detecting latent niacin deficiency was developed. It measures the 24-h urinary output of the two major metabolites of niacin, 1-methyl-5-carboxylamide-2-pyridone and N-1-methylnicotinamide. The two metabolites were isolated from urine using separate ion-exchange extractions. They and their two internal standards were quantitated simultaneously by high-performance liquid chromatography using a reversed-phase ion pairing separation. Detection was by absorbance at 254 nm.

INTRODUCTION

Niacin or nicotinic acid is one of the B vitamins. Its active form, nicotinamide, serves as a precursor in the biosynthesis of the two coenzymes, NAD and NADP, which are involved in a myriad of enzymatic reactions and metabolic pathways. The deficiency of niacin manifests itself as the disease known as pellagra. Pellagra, while once common in corn-eating areas of the world two centuries ago, is now rarely seen except in occasional cases of alcoholism.

The evaluation of niacin status involves the determination of two major urinary metabolites, 1-methyl-5-carboxylamide-2-pyridone (2-PY) and N-1-methylnicotinamide (N1MN). The weight ratio of 2-PY to N1MN is regarded as an important index of niacin status. De Lange and Joubert [1] defined a 2-PY to N1MN ratio of 1.3 to 4.0 as normal and of less than 1.0 as indicative of latent niacin deficiency.

Until the present method, it has been necessary to use two different tests to quantitate both niacin metabolites and thus calculate the metabolite ratio.

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N1MN in urine has been measured by fluorometric methods [2—5] as well as by high-performance liquid chromatography (HPLC) [6, 7]. 2-PY in urine has been measured by a fluorometric method [8], colorimetric methods [9, 10], spectrophotometry [11], and HPLC [12]. The present HPLC method measures both niacin metabolites simultaneously after separate ion-exchange extractions of the two metabolites and their two internal standards.

EXPERIMENTAL

Reagents

N-1-Methylnicotinamide chloride, trigonelline · HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl, and nicotinic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium acetate · $3H_2O$, potassium ferricyanide, boric acid, sodium hydroxide, ammonium chloride, sodium carbonate, and methyl iodide were analytical reagent grade and were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Sodium heptane sulfonate was purchased from Regis (Morton Grove, IL, U.S.A.). Glacial acetic acid was electronic grade from DuPont (Wilmington, DE, U.S.A.). Omnisolve® methanol and reagent grade concentrated HCl were obtained from MCB Manufacturing Chemists Inc. (Cincinnati, OH, U.S.A.). Bio-Rex 70 resin (100—200 mesh, Na^+), AG-1-X4 (100—200 mesh, Cl^-), and prepacked 4 cm × 0.7 cm I.D. polypropylene Econo-Columns® filled with either AG-50W-X8 (200—400 mesh, Na^+) or AG-1-X8 (200—400 mesh, AcO^-) were all purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Norit "A" neutral pharmaceutical grade decolorizing carbon was obtained from Amend Drug and Chemical Co. (Irvington, NJ, U.S.A.). Thionyl chloride was obtained from Aldrich (Milwaukee, WI, U.S.A.). Methyl ethyl ketone, *n*-butanol, and ethanolamine were reagent grade and were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). N,N-Dimethylformamide (DMF) was obtained from Pfaltz and Bauer (Stanford, CT, U.S.A.). The ethanolamine and DMF were redistilled before use.

The compound 1-methyl-5-carboxyl-2-pyridone was synthesized by the method of Huff [13]. The synthesis of niacin metabolite 1-methyl-5-carboxylamide-2-pyridone, internal standards 1-methyl-5-carboxyl-[N-(2-ethoxy)]-amide-2-pyridone and 1-methyl-3-carboxyl-[N-(2-ethoxy)]amidepyridine chloride, and internal standard precursor 3-carboxyl-[N-(2-ethoxy)]amidepyridine are described below. Melting points were performed on a micro hot stage melting point apparatus from Arthur H. Thomas Company (Philadelphia, PA, U.S.A.) and are uncorrected. Elemental analyses were done by Dow Chemical Western Division Analytical Lab. (Walnut Creek, CA, U.S.A.).

1-Methyl-5-carboxylamide-2-pyridone. 1-Methyl-5-carboxy-2-pyridone (5.00 g, 0.0327 moles) in 15 ml of water was adjusted to pH 5.9 with 5 *N* NaOH. Ammonium chloride (1.76 g, 0.0327 moles) was then added to the sodium salt solution followed by a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl (6.89 g, 0.0360 moles) in 11 ml of water. The resulting solution was stirred at 4° C for three days during which time the pH was held below 6.5 by the occasional addition of 1 *N* HCl. The reaction mixture was purified by passage through a 16.5 cm × 11 cm I.D. AG-1-X4 (100—200 mesh, Cl^-) ion-exchange column eluted with water. Fractions containing the product were

distilled under reduced pressure to dryness giving an off-white solid which was recrystallized from *n*-butanol utilizing a decolorizing charcoal hot filtration. After vacuum drying, 2.20 g of a white crystalline solid, m.p. 209—210° C, was obtained. Yield was 44%.

Elemental analysis $C_7H_8N_2O_2$. Theoretical: C 55.25%, H 5.30%, N 18.41%. Found: C 55.25%, H 5.35%, N 18.48%.

1-Methyl-5-carboxyl-[N-(2-ethoxy)]amide-2-pyridone. 1-Methyl-5-carboxy-2-pyridone (14.0 g, 0.0914 moles) was refluxed under nitrogen with 70 ml of thionyl chloride for 1 h, at which time the excess $SOCl_2$ was distilled off under reduced pressure. The resulting solid acid chloride was dissolved in 70 ml of DMF and added dropwise to a suspension consisting of sodium carbonate (9.69 g, 0.0914 moles), ethanolamine (5.61 g, 0.919 moles) and 280 ml of DMF stirring under nitrogen at 4°C. After the 45-min addition, the reaction mixture was stirred for another 0.5 h at 4° C, at which time the reaction mixture was filtered. The filtrate was distilled under reduced pressure to dryness giving a yellowish solid which was recrystallized from *n*-butanol utilizing a decolorizing charcoal hot filtration. One more *n*-butanol recrystallization without the charcoal gave a white crystalline solid which after vacuum drying weighed 12.3 g, a yield of 69%. The m.p. was 173—174°C.

Elemental analysis $C_9H_{12}N_2O_3$. Theoretical: C 55.09%, H 6.17%, N 14.28%. Found: C 54.83%, H 6.06%, N 14.19%.

3-Carboxyl-[N-(2-ethoxy)]amidepyridine. Nicotinic acid (10.0 g, 0.0813 moles) and ethanolamine (4.98 g, 0.0813 moles) were dissolved in 20 ml of water. The pH of the solution was adjusted to 6.0 with concentrated HCl. A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl (17.2 g, 0.0894 moles) in 16.5 ml of water was added to the reaction mixture stirring at 4°C. The pH of the reaction mixture was held at 6.0 by the occasional addition of 6 *N* HCl. After 24 h at 4°C the reaction mixture, a light yellow solution, was purified by passage through a 16.5 cm × 11 cm I.D. AG-1-X4 (100—200 mesh, Cl^-) ion-exchange column eluted with water. Fractions containing the product were distilled under reduced pressure to dryness resulting in a yellow solid. This was recrystallized from methyl ethyl ketone utilizing a decolorizing charcoal hot filtration. After vacuum drying, 9.78 g of a white crystalline solid, m.p. 88—90° C, were obtained. The yield was 72%.

Elemental analysis $C_8H_{10}N_2O_2$. Theoretical: C 57.84%, H 6.07%, N 16.86%. Found: C 57.67%, H 6.11%, N 17.00%.

1-Methyl-3-carboxyl-[N-(2-ethoxy)]amidepyridine chloride. 3-Carboxyl-[N-(2-ethoxy)]amidepyridine (11.0 g, 0.0662 moles), methyl iodide (94 g, 0.66 moles), and 110 ml of methanol were refluxed for 7 h, at which time the excess methyl iodide and the methanol were distilled off under reduced pressure. The resulting oil was diluted with 11 ml of water and then purified by passage on a 23 cm × 2.5 cm I.D. AG-1-X4 (100—200 mesh, Cl^-) ion-exchange column eluted with water. The fractions containing the product were distilled under reduced pressure to dryness giving a light yellow solid. This was recrystallized from *n*-butanol utilizing a decolorizing charcoal hot filtration. After vacuum drying, 10.7 g of a white crystalline compound, m.p. 161—162° C, were obtained. The yield was 75%.

Elemental analysis $C_9H_{13}N_2O_2Cl$. Theoretical: C 49.89%, H 6.05%, N 12.93%, Cl 16.36%. Found: C 49.73%, H 6.03%, N 13.04%, Cl 16.18%.

Working standard and controls. A working standard solution consisted of 15 μg/ml 2-PY and 10 μg/ml N1MN in a matrix composed of 0.25 *M* sodium acetate and 0.16 *M* boric acid. Controls were normal urine containing 0.16 *M* boric acid. The low control was a urine having a 24-h volume of greater than 2 l. The high control was a urine having a 24-h volume of less than 1 l.

Storage of reagents. Standards and controls were stored at −20° C. Mobile phase buffer and the two solutions containing the two working internal standards were stored at 4° C. Bio-Rad prepacked AG-1-X8 Econo-Columns were stored at 4°C. Bio-Rad prepacked AG-50W-X8 Econo-Columns, 2 *N* HCl, methanol, and Bio-Rex 70 resin were stored at room temperature.

Extraction of metabolites from standard solution and urine

One milliliter of urine or standard solution was mixed with 100 μl of 150 μg/ml 1-methyl-5-carboxyl-[N-(2-ethoxy)]amide-2-pyridone (2-PY internal standard) in water and 100 μl of 2 *N* HCl. This solution was added to a 4 cm × 0.7 cm AG-50W-X8 ion-exchange column which dripped directly into a 4 cm × 0.7 cm AG-1-X8 ion-exchange column. The urine solution was eluted with 7 ml of water added to the AG-50W-X8 column. The last 5 ml of the effluent from the AG-1-X8 column were collected, saved, and labeled "2-PY extract". One milliliter of urine or standard solution was diluted with 19 ml of water and added to a 4 cm × 0.7 cm Bio-Rex 70 ion-exchange column. The column was then washed with two 5-ml aliquots of water. This was followed by the addition of 5 ml of 0.2 *M* sodium acetate solution containing 2.4 μg/ml 1-methyl-3-carboxyl-[N-(2-ethoxy)]amidepyridine chloride (N1MN internal standard). The last 4 ml of effluent from the Bio-Rex 70 column were collected, saved, and labeled "N1MN extract." Fifty microliters of each 2-PY extract were mixed with 600 μl of the corresponding N1MN extract to make a solution for injection into the chromatograph.

High-performance liquid chromatography

The chromatography was performed on an Altex Model 330 isocratic liquid chromatograph (Altex Scientific, Berkeley, CA, U.S.A.) equipped with a 15 cm × 4.6 mm I.D. Ultrasphere Ion Pairing analytical column of 5 μm particle size (Altex Scientific) and a 3 cm × 4.6 mm I.D. Brownlee MPLC LiChrosorb C-18 guard column of 10 μm particle size (Rheodyne, Berkeley, CA, U.S.A.).

The temperature of the analytical column was kept at 35° C by an aluminum column temperature control block (Waters Assoc., Milford, MA, U.S.A.) connected to a Haake Model FJ temperature-controlled circulating water bath (Haake Instruments, Saddle Brook, NJ, U.S.A.). The volume of injection was 50 μl. The mobile phase used was 91% buffer (containing 0.15 *M* acetate, pH 5.0, and 0.01 *M* sodium heptanesulfonate) and 9% methanol. The flow-rate was 1.0 ml/min. Detection of the analytes and internal standards was by UV absorbance at 254 nm, 0.01 absorbance units full scale. Analytes and internal standards were quantitated by manually measuring peak heights as recorded by a Hewlett-Packard strip chart recorder, Model 7123A (Hewlett-Packard, Avondale, PA, U.S.A.).

RESULTS

HPLC separation

Typical chromatograms obtained from a standard, a low control, and a high control are shown in Fig. 1. The metabolite 2-PY eluted at 3.5 min, its internal standard eluted at 4.4 min, the metabolite N1MN eluted at 5.6 min, and its internal standard eluted at 8.0 min. The low control 2-PY level was 6.64 μg/ml of urine and its N1MN level was 1.90 μg/ml of urine. From the chromatogram it can be seen that detection limits of 0.3 μg/ml of urine for both metabolites were possible.

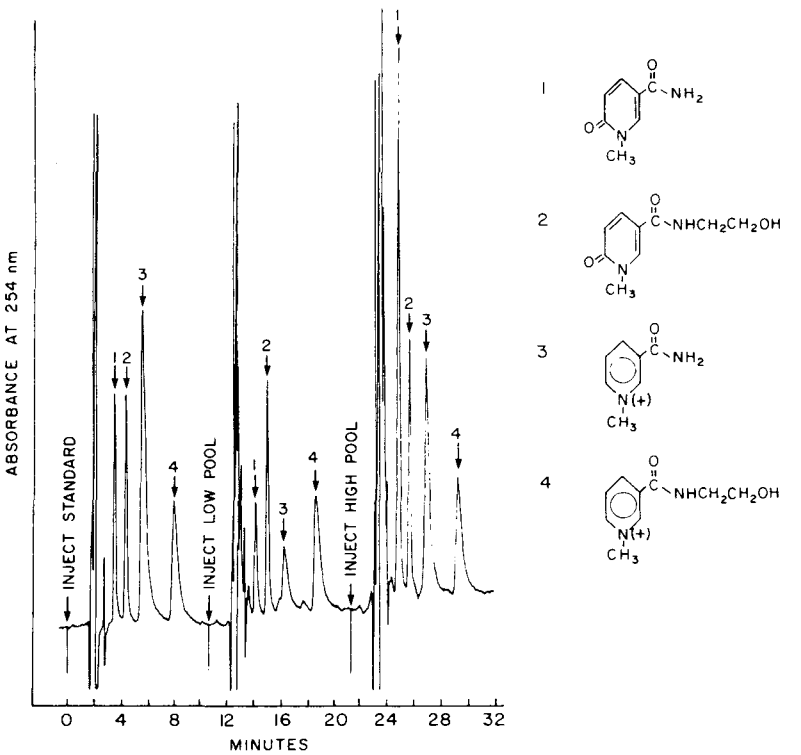

Fig. 1. Chromatogram of standard, low control, and high control with relevant structures. Compound 1 is niacin metabolite 1-methyl-5-carboxylamide-2-pyridone. Compound 2 is internal standard 1-methyl-5-carboxyl-[N-(2-ethoxy)]amide-2-pyridone. Compound 3 is niacin metabolite N-1-methylnicotinamide. Compound 4 is internal standard 1-methyl-3-carboxyl-[N-(2-ethoxy)]amidepyridine.

Method linearity

Linearity of the method was demonstrated using urine from a donor after ingestion of a vitamin preparation containing nicotinamide. The undiluted specimen contained 99 μg/ml of the 2-PY metabolite and 23 μg/ml of the N1MN metabolite. These concentrations were approximately four times higher than the mean of the reference ranges for these metabolites. The specimen (containing 0.16 *M* boric acid) was diluted with the same solution used as

the matrix for the 15 μg/ml 2-PY and 10 μg/ml N1MN standard. A linear relationship was observed for both metabolites with up to a five-fold dilution of this high specimen when the peak height ratio of metabolite to its internal standard was plotted against the dilution of the urine sample.

Method precision

Precision of the assay was studied by running three different controls in duplicate with one standard in each of ten different batches. Table I shows the mean, intra-run coefficient of variation (C.V.), and the inter-run coefficient of variation for the metabolites in each control. The inter-run C.V. of the 2-PY metabolite assay was approximately 3% and the inter-run C.V. of the N1MN metabolite assay was approximately 5%. The C.V. was relatively constant with concentration for both metabolites and the ratio of intra-run C.V. to inter-run C.V. was approximately 0.5.

TABLE I

METHOD IMPRECISION

Data are based on ten runs with two determinations per run.

	Low control	Medium control	High control
2-PY metabolite			
Mean, $\overline{X}$ (μg/ml)	6.64	14.91	32.33
Intra-run C.V. (%)	1.6	1.1	1.3
Inter-run C.V. (%)	2.7	2.5	2.8
N1MN metabolite			
Mean, $\overline{X}$ (μg/ml)	1.90	4.17	8.04
Intra-run C.V. (%)	2.9	2.2	2.5
Inter-run C.V. (%)	5.1	4.7	4.7

Metabolite extraction study

The elution of the niacin metabolites and their internal standards from the ion-exchange columns was studied by collecting 0.5-ml fractions of effluent from the ion-exchange columns and analyzing 50 μl of each 0.5-ml fraction with the HPLC system. Peak heights of each metabolite or internal standard detected in the 0.5-ml fractions were measured in millimeters and plotted against volume of effluent coming from either the Bio-Rex 70 columns or the AG-1-X8 column. Fig. 2 and 3 show the elution profile for the standard and a spiked control containing 14.9 μg/ml 2-PY and 10.2 μg/ml N1MN. Based on Fig. 2, the 2—7-ml fraction of the distilled water eluant contained most of the 2-PY and its internal standard and this fraction was used for the assay. Likewise, most of the N1MN was eluted in the 1—5-ml fraction of the 0.2 *M* sodium acetate eluant. It should be noted that the elution curve of the N1MN internal standard was not a "peak". This was a consequence of adding the N1MN internal standard to the eluting buffer rather than to the specimen prior to its introduction onto the Bio-Rex 70 column. The N1MN internal standard could not be added to the specimen prior to the Bio-Rex 70 extraction because of erratic recovery.

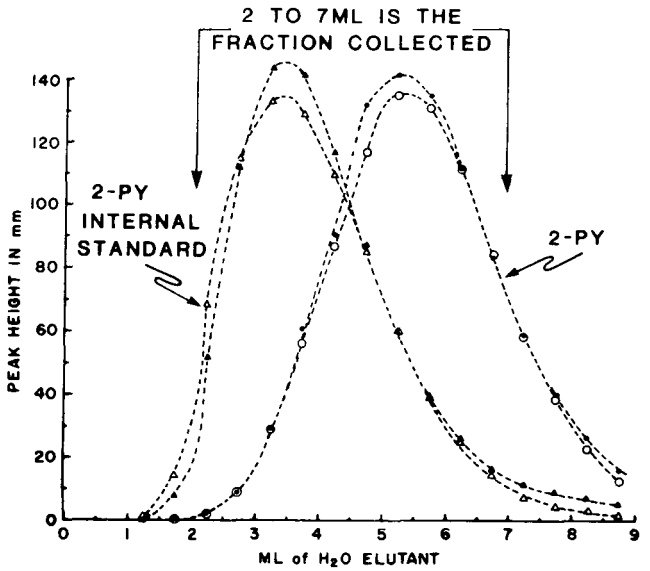

Fig. 2. Elution profile of 2-PY metabolite and 2-PY internal standard from sequential AG-50W-X8 and AG-1-X8 columns. (▲), 2-PY internal standard, 15 μg, spiked into 1 ml of medium urine control; (●), 2-PY metabolite, 14.9 μg/ml, in medium urine control; (△), 2-PY internal standard, 15 μg, spiked into 1 ml of standard; (○), 2-PY metabolite, 15.0 μg/ml, in standard.

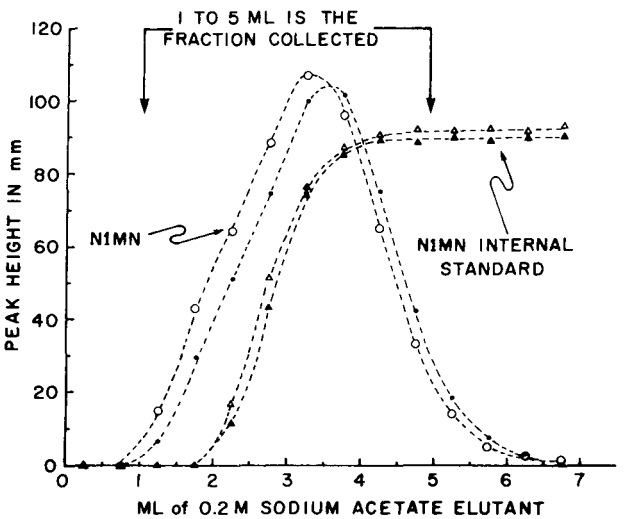

Fig. 3. Elution profile of N1MN metabolite and N1MN internal standard from Bio-Rex 70 columns. (▲), N1MN internal standard, 4.8 μg/ml, in 0.2 *M* sodium acetate buffer used to elute spiked medium control; (●), N1MN metabolite, 10.2 μg/ml, in spiked medium urine control; (△), N1MN internal standard, 4.8 μg/ml, in 0.2 *M* sodium acetate buffer used to elute standard; (○), N1MN metabolite, 10.0 μg/ml, in standard.

Recovery studies

An experiment was designed to determine whether urine ionic strength affected recovery since normal random urines range in osmolality from 50 to 1200 mosm/kg H_2O. N1MN (5 and 10 μg/ml) and 2-PY (10 and 20 μg/ml) were added to five different sodium acetate solutions (containing 0.16 *M* boric acid) varying from 200 to 1000 mosm/kg H_2O.

The extraction recoveries and the recovery ratios of metabolites to internal standards were calculated. The recovery of 2-PY ranged from 71% to 78% and the 2-PY internal standard recovery ranged from 87% to 89%. The recovery of the N1MN internal standard ranged from 54% to 61%. The recovery of N1MN ranged from 82% to 90% at matrix osmolality 200—800 mosm/kg H_2O but fell to about 77% at 1000 mosm/kg H_2O. The result was that the recovery ratio of N1MN to N1MN internal standard was around 1.5 at matrix osmolality 200—800 mosm/kg H_2O, but was only 1.3 at a matrix osmolality of 1000 in osm/kg H_2O (see Table II).

TABLE II

RECOVERY RATIOS OF METABOLITES TO INTERNAL STANDARDS VS. MATRIX OSMOLALITY

Each matrix consisted of the proper amount of sodium acetate to produce the desired osmolality in addition to 0.16 *M* boric acid.

Matrix osmolality (mosm/kg H_2O)	Recovery ratios			
	2-PY spike 10 μg/ml	2-PY spike 20 μg/ml	N1NM spike 5 μg/ml	N1MN spike 10 μg/ml
200	0.82	0.82	1.52	1.54
400	0.85	0.85	1.46	1.55
600	0.84	0.85	1.52	1.61
800	0.86	0.88	1.40	1.46
1000	0.84	0.83	1.32	1.29

The recovery of 2-PY and N1MN from three different urines having osmolalities of 201, 561, and 945 mosm/kg H_2O was determined. The three urines were spiked with both 2-PY and N1MN to add an additional 10 μg of each metabolite to 1 ml of urine. After adding boric acid to a level of 0.16 *M*, the three baseline urines and the three spiked urines were run through the assay in duplicate with a single standard solution. Based on the standard solution, recovery of the 2-PY metabolite ranged from 93% to 96% while recovery of the N1MN metabolite ranged from 93% to 95% (see Table III).

An experiment was performed to establish whether the recoveries of the N1MN and its internal standard would vary with the bed heights of the Bio-Rex 70 columns. Unlike the prepacked AG-50W-X8 and AG-1-X8 ion-exchange columns, the Bio-Rex 70 columns were individually packed just prior to starting the assay and the bed heights were found to vary slightly. The resin bed heights in the experiment ranged from 36 mm to 44 mm. Percentage recovery and the ratio of N1MN peak height to that of its internal standard

TABLE III

RECOVERY OF 2-PY AND N1MN FROM THREE SPIKED URINES WITH DIFFERENT OSMOLALITIES

Specimens and their spikes were run in duplicate. Recovery was calculated against a standard solution run singly.

	Specimen 1	Specimen 2	Specimen 3
Urine osmolality (mosm/kg H_2O)	201	561	945
Baseline urine, 2-PY level (μg/ml)	4.6	17.4	31.5
10 μg/ml spiked urine, 2-PY level (μg/ml)	13.9	26.8	41.1
Recovery of 10 μg/ml 2-PY spike (%)	93	94	96
Baseline urine, N1MN level (μg/ml)	1.1	5.8	10.9
10 μg/ml spiked urine, N1MN level (μg/ml)	10.4	15.1	20.4
Recovery of 10 μg/ml N1MN spike (%)	93	93	95

TABLE IV

RECOVERY OF N1MN METABOLITE AND INTERNAL STANDARD VS. BIO-REX 70 RESIN BED HEIGHT

Data based on a solution containing 10 μg/ml N1MN, 0.25 *M* sodium acetate, and 0.16 *M* boric acid.

Resin bed height (mm)	Recovery (%)		N1MN:N1MN internal standard peak height ratio
	N1MN metabolite	N1MN internal standard	
36	95.5	62.7	3.0
38	95.1	60.3	3.1
40	93.5	57.1	3.0
42	83.7	50.0	3.3
44	75.6	42.9	3.4

peak height are shown in Table IV. As the resin bed height in the column increased, the recoveries of both the metabolite and its internal standard decreased. The N1MN metabolite to N1MN internal standard ratio was relatively constant. An ideal internal standard would have given a constant recovery ratio, but bed heights of 36—40 mm provided acceptable recovery and ratios.

Sample stability

Seven-day stability of urine specimens was achieved for both niacin metabolites at 30° C, 4° C, and —20° C by the addition of 1 g of boric acid per 100 ml (0.16 *M* boric acid) of urine. Each stability study had a total of sixteen data points accumulated over four independent runs. Table V lists the calculated rectangular regression lines and the correlation coefficient for the six separate stability studies.

TABLE V

STABILITY STUDY RESULTS FOR NIACIN METABOLITES (μg/ml) IN URINE PRESERVED WITH 0.16 *M* BORIC ACID AT 30° C, 4° C, AND —20° C

Metabolite	Storage condition	Calculated rectangular regression line*	Correlation coefficient
2-PY	30° C	$Y = 1.003X + 0.11$	0.9922
2-PY	4° C	$Y = 0.997X - 0.09$	0.9942
2-PY	—20° C	$Y = 0.973X + 0.06$	0.9925
N1MN	30° C	$Y = 0.991X + 0.06$	0.9952
N1MN	4° C	$Y = 0.984X + 0.10$	0.9945
N1MN	—20° C	$Y = 0.969X + 0.01$	0.9934

*X is day 0 and Y is day 7.

Reference range

A reference range study was conducted on 27 female and 23 male adults. All 50 donors in this study stated that to their knowledge they had no liver damage, were not in the second or third trimester of pregnancy, and did not take oral contraceptives or B vitamins. Boric acid (1 g) was used to preserve 100 ml of each 24-h urine collection. Normal range histograms for mg 2-PY per 24 h, mg N1MN per 24 h, and the 2-PY to N1MN weight ratio over 24 h are shown in Figs. 4—6. All three normal ranges form skewed (non-gaussian) distributions with normal range estimates [14] of 6.0—51.3 mg 2-PY per 24 h, 1.6—14.8 mg N1MN per 24 h, and a 2-PY to N1MN weight ratio of 1.76 to 5.90 (95% confidence limits).

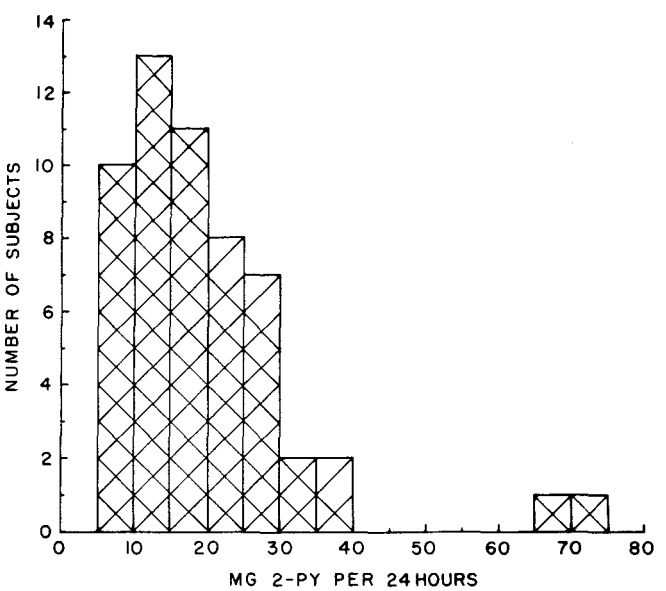

Fig. 4. Reference range histogram of 24-h 2-PY excretion. For 50 normals, reference range estimate (95% confidence limits) was 6.0—51.3 mg of 2-PY per 24 h.

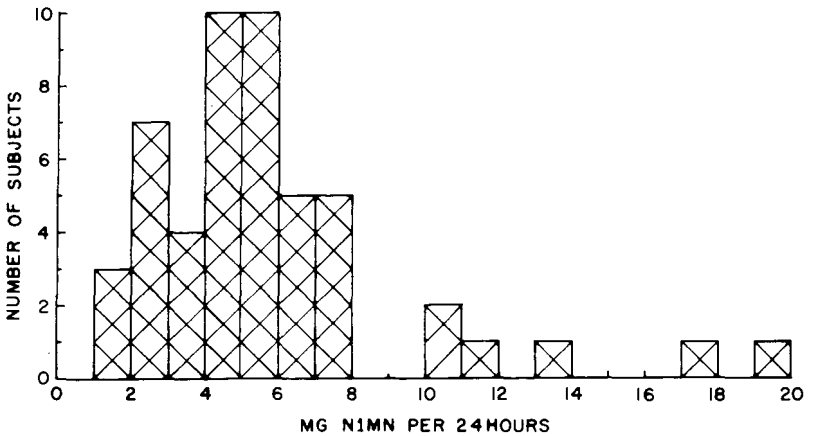

Fig. 5. Reference range histogram of 24-h N1MN excretion. For 50 normals, reference range estimate (95% confidence limits) was 1.6—14.8 mg of N1MN per 24 h.

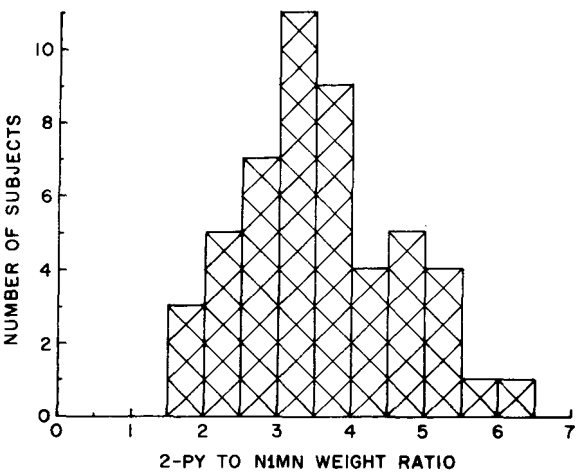

Fig. 6. Reference range histogram of 24-h 2-PY to N1MN weight ratio. For 50 normals, reference range estimate (95% confidence limits) was 1.76—5.90.

Interference study

A total of 37 drugs and drug metabolites were examined for interference (Table VI). No interference with any of the niacin metabolites or internal standards was seen.

DISCUSSION

The simultaneous measurement of both major human metabolites of niacin represents a novel step forward in the determination of niacin status. The present method utilized a modification of the 2-PY extraction of Price [11] and an approach similar to that used by Shaikh et al. [6] in the extraction of N1MN. The result was a very clean niacin metabolite extract providing an ex-

TABLE VI

COMPOUNDS TESTED THAT DO NOT INTERFERE WITH THE ASSAY

Compound	Concentration (μg/ml)	Compound	Concentration (μg/ml)
Acetylisoniazid	200.0	Isoniazid	100.0
Amitriptyline	5.0	Lidocaine	2.5
Brompheniramine	2.5	Meperidine	2.5
Caffeine	20.0	Meprobamate	20.0
Chlorpheniramine	5.0	Methadone	5.0
Chlorpromazine	10.0	Methadone metabolite	5.0
Codeine	10.0	Methapyrilene	5.0
Desalkylflurazepam	10.0	Methaqualone	5.0
Desipramine	5.0	Methyprylon	5.0
N-Desmethyldiazepam	10.0	Norpropoxyphene	10.0
N-desmethyldoxepin	5.0	Nortriptyline	5.0
Dextromethorphan	10.0	Oxycodone	10.0
Diazepam	10.0	Pentazocine	10.0
Diphenhydramine	2.5	Phenacetin	20.0
Doxepin	5.0	Phencylidine	2.5
Doxylamine	5.0	Promazine	5.0
Flurazepam	10.0	Propoxyphene	5.0
Glutethimide	20.0	Trihexyphenidyl	5.0
Imipramine	5.0		

cellent baseline for HPLC. The selectivity gained in the double ion-exchange extraction procedure was demonstrated by the lack of interference from the 37 compounds tested.

The excellent precision of the 2-PY analysis was due to the consistency of the prepacked Bio-Rad AG-50W-X8 and AG-1-X8 ion-exchange columns. These columns exhibited unusual uniformity with respect to resin volume. However, efforts to utilize prepacked Bio-Rex 70 (100—200 mesh, Na^+) ion-exchange columns were unsuccessful because they contained substantial quantities of "fines" causing unacceptable variation in flow-rate.

Recovery experiments showed that concentrated urine with osmolalities greater than 1000 mosm/kg H_2O caused recovery of the N1MN metabolite to be reduced. Concentrated urines with 24-h total volumes near 500 ml (as compared with more commonly found 1000—1500-ml outputs) should be diluted 1 plus 1 with distilled water containing 0.16 *M* boric acid prior to the 1 plus 19 dilution of the urine with distilled water. This results in a urine solution which gives a good recovery of the N1MN metabolite when run on the 4 cm × 0.7 cm I.D. Bio-Rex 70 columns.

With so many different analytical techniques used in the past to measure the two major niacin metabolites it was not surprising that reference ranges published in the literature vary considerably. Table VII shows some reported reference ranges for comparison with Figs. 4—6 along with three pathological subpopulations selected from the literature. Relatively good agreement was evident in the normal daily N1MN excretion. However, different researchers have reported varying 24-h urine values for the 2-PY metabolites of niacin as well as the 2-PY to N1MN weight ratio.

Prinsloo et al. [21] studied eleven children with pellegra and showed clearly that they had low urinary levels of niacin metabolites. A metabolite ratio of 0.6 ± 0.4 was extremely low compared to the reported normal values shown in Table VII. Alcoholics and people with cirrhosed livers may also exhibit low 2-PY to N1MN metabolite weight ratios. This may be due to the fact that their damaged livers were slow to oxidize the N1MN to the 2-PY metabolite and consequently the N1MN was excreted before the oxidation could occur. The difference between a well-fed alcoholic and a true pellagrin would be that the absolute 24-h excretion values of niacin metabolites are low for a pellagrin and high for the well-fed alcoholic. A 24-h urine collection is required for the analysis of niacin status because conversion of the results from an untimed, random specimen to 24-h excretion values on the basis of creatinine concentration can lead, in the case of a pellagrin, to an incorrect interpretation. This is due to the abnormally low creatinine values which may be found in cases of pellagra [21].

TABLE VII

REFERENCE RANGES WITH STANDARD DEVIATIONS

Author Date	2-PY (mg per 24 h)	N1MN (mg per 24 h)	2-PY:N1MN by weight	Sample population
Present 1982	20.1 ± 12.9	5.8 ± 3.6	3.60 ± 1.06	50 normals
Vivan et al. [15] 1958	9.1 ± 2.5	5.9 ± 2.1	1.57 ± 0.16	4 normals
Motegi [16] 1960	11.8	4.6	—	24 normals
Joubert and DeLange [17] 1962	13.9 ± 5.7	7.7 ± 2.3	—	10 normals
DeLange and Joubert [1] 1964	—	—	2.06 ± 0.74	12 normals
Marnardi and Tenconi [18] 1964	12.2	6.7	—	50 normals
Leklem et al. [19] 1975	17.8 ± 3.5	6.0 ± 1.8	3.00 ± 0.72	9 normals
Joubert and DeLange [17] 1962	4.4 ± 3.9	7.0 ± 3.2	—	10 alcoholics
Gabuzda and Davidson [20] 1962	—	11.3 ± 3.0	—	10 cirrhosis patients
Prinsloo et al. [21] 1968	1.0 ± 0.7	1.7 ± 1.0	0.6 ± 0.4	11 pellagra children

In summary, a new HPLC method has been developed to measure niacin metabolites in urine. The method showed excellent precision (C.V. 2.5—5%), recovery and linearity. Samples could be stored for seven days at 30°C, 4°C or −20°C. No interfering peaks were found from 37 drugs tested and the reference range was consistent with others that have been reported.

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CHROMBIO. 1381

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC PROCEDURE FOR THE QUANTITATION OF CHLORPROMAZINE IN PLASMA AND ITS COMPARISON WITH A NEW HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A gas chromatographic—mass spectrometric assay using selected ion monitoring is compared with a high-performance liquid chromatographic assay using an electrochemical detector for single-dose studies of the psychotherapeutic phenothiazine drug chlorpromazine. Measurements were made after extraction of chlorpromazine and the internal standard, prochlorperazine, from basified plasma with an isopropanol—pentane solvent mixture. Following evaporation of the organic solvents the residue was reconstituted in a small volume of methanol and subjected to gas chromatographic—mass spectrometric selected ion detection. The residual sample was then evaporated and made up in a larger volume of acetonitrile and analyzed by high-performance liquid chromatography using an electrochemical detector. These specific methods display excellent correlation for plasma concentration determinations in the range of 0.25—10 ng ml^{-1} and will allow for the study of the pharmacokinetics of chlorpromazine following single low doses of the drug.

INTRODUCTION

Chlorpromazine is the most widely used phenothiazine antipsychotic, and as such has been researched in the most detail with regard to analysis, metabolism, pharmacokinetics and plasma concentrations versus clinical response correlations. It is extensively metabolized to numerous metabolites, and several of these are psychoactive. The major metabolites include the sulfoxide, N-oxide, 7-hydroxy, N-desmethyl and N-didesmethyl compounds. The quantitative analysis of chlorpromazine in plasma has been performed by several methods; the first reported was the gas chromatographic—electrochemical detection (GC—ElCD) procedure of Curry [1]. This method has undergone several

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modifications to improve the sensitivity and ease of application, as well as to include the quantitation of metabolites such as the sulfoxide, 7-hydroxy and N-desmethyl compounds [2—5]. Other chemical methods of analysis include radioassay [6], fluorometry [7], gas chromatography—nitrogen—phosphorus detection (GC—NPD) [8, 9], high-performance liquid chromatography (HPLC) [10—13], and gas chromatography—mass spectrometry (GC—MS) [14—17]. Most of these methods have adequate sensitivity so as to quantitate chlorpromazine plasma concentrations following chronic therapeutic doses of the drug. However, except for GC—MS these chemical procedures generally lack the sensitivity needed for single-dose pharmacokinetic studies.

Furthermore, there is a special need for systematic comparison of the various methods proposed by different investigators for the determination of chlorpromazine, since it has been shown in the past that unexplained discrepancies were observed when the analytical results of different laboratories were compared with each other [18]. It is believed in the author's laboratory that the best quality control consists in comparing different sensitive and specific methods for the determination of chlorpromazine within the same laboratory. Once the valid comparisons are made, then, and only then can reliable pharmacokinetics and plasma levels—clinical response correlations be studied meaningfully.

Therefore, in the past a radioimmunoassay method [19] reported from this laboratory, which had the necessary sensitivity and reproducibility for single dose pharmacokinetic studies [20], was certified for its specificity by an HPLC—UV detection method [10]. This latter HPLC method, which was only sensitive down to 1 ng of chlorpromazine per ml of plasma, was recently improved by the use of an electrochemical detector and improved extraction recovery so that 0.25 ng ml^{-1} of the drug using a 2-ml plasma sample can be quantitated [21]. This HPLC—ElCD method is now compared with a newly developed GC—MS procedure, the latter method being reported here. In the GC—MS procedure the drug and the internal standard, prochlorperazine, following extraction from basified plasma are chromatographed on an OV-1 column. Use of a selected ion technique, monitoring the molecular ions for chlorpromazine at *m/z* 318 and prochlorperazine at *m/z* 373, provided sensitivity down to 0.25 ng ml^{-1} of plasma. For comparison of this procedure with the HPLC—ElCD method plasma samples were obtained from healthy volunteers administered single 50-mg oral doses of chlorpromazine.

EXPERIMENTAL

Materials

Heparinized evacuated tubes (Vacutainer®) were obtained from Becton Dickinson and Co. (Mississauga, Canada). All solvents were distilled in glass and all other chemicals were analytical grade used without further purification.

Chlorpromazine was obtained in the form of Thorazine® tablets, 25 mg, as chlorpromazine·HCl from Smith Kline and French Labs. (Philadelphia, PA, U.S.A.). Prochlorperazine mesylate was a gift from Rhone Poulenc Pharma (Montreal, Canada). All assays and sample manipulations were performed in subdued light. Standard solutions of chlorpromazine and prochlorperazine were

prepared by dilution in double distilled deionized water. Appropriate dilutions of the standard solutions were made in pooled fresh plasma obtained from blood collected from healthy volunteers.

Plasma level study

Five healthy, overnight fasted, male volunteers weighing between 56 kg and 86 kg were each given orally a single 50-mg dose of chlorpromazine, two 25-mg tablets (Thorazine SKF) with 250 ml tap water. Blood samples were collected at scheduled intervals over a 24-h period in evacuated glass tubes (Vacutainers), centrifuged, and the separated plasma was stored at −20°C until analysis. During collection of the venous samples care was taken to avoid contact of the blood with the rubber stopper of the evacuated tube. This precaution was taken in order to avoid distortions in plasma concentrations as reported for phenothiazines [22, 23].

Extraction of samples

To a 10-ml PTFE-lined screw-capped test tube were added 2 ml of plasma and 1 ml of aqueous internal standard solution (prochlorperazine, 100 ng ml^{-1}). The sample was gently mixed (Vortex Genie, Fisher Scientific Company, Edmonton, Canada) and 0.5 ml of saturated sodium carbonate solution was subsequently added. The mixing was then repeated and 5 ml of 3% isopropanol in *n*-pentane was added. The tube was tightly capped and mixed (Evapomix, Fisher Scientific) for 15 min at speed 6.5 and centrifuged (T-J6 centrifuge, Beckman Instruments, Toronto, Canada) at room temperature at 1720 *g* for 5 min. The upper organic layer was transferred by pasteur pipette to another 10-ml PTFE-lined screw-capped test tube. The aqueous layer was re-extracted with a further 5-ml of 3% isopropanol in *n*-pentane, which was again transferred by pasteur pipette into the test tube containing the first aliquot. After the addition of antibumping granules the organic solvents were evaporated in a dry bath at 65°C. The dried residue was reconstituted for GC—MS analysis with 30 μl of methanol by vortex mixing and subsequent centrifugation. Aliquots of 4 μl were injected for GC—MS analysis. After GC—MS analysis the residual sample was evaporated in a dry bath at 65°C, reconstituted in 200 μl of acetonitrile and a 100-μl aliquot was used for injection into the HPLC—ElCD system.

Instrumentation

The GC—MS mass fragmentography was performed on a V.G. Micromass MM 16F mass spectrometer interfaced via a single stage jet separator to a Hewlett-Packard 5711A gas chromatograph and equipped with a V.G. 2025 data system. The instrument was operated in the electron impact (EI) mode with the interface and ion source at 280°C and 220°C, respectively.

The chromatographic column was a coiled glass tube 1.22 m × 2 mm I.D. packed with 3% OV-1 on acid-washed, dimethyldichlorosilane treated Gas-Chrom Q, 100—120 mesh. The injection port and the column oven temperatures were 300°C and 280°C, respectively and the helium carrier gas flow-rate was 30 ml min^{-1}. Additional MS conditions were: emission current, 200 μA and electron multiplier 2.1 kV. A column bleed ion at *m/z* 281 was used as a

computer reference lock mass, in order to compensate for any instrumentational drift, and the molecular ions of chlorpromazine *m/z* 318 and the internal standard, prochlorperazine *m/z* 373 were alternately monitored by the computer with a dwell time on each ion of 200 msec.

HPLC—ElCD [21] was performed using a Waters M45 liquid chromatographic pump (Waters Assoc., Mississauga, Canada) fitted with a Rheodyne Model 7125 valve loop injection system utilizing a 500-μl loop (Technical Marketing Associates, Calgary, Canada). The column used was a 250 mm × 4.6 mm I.D. column packed with Spherisorb CN 10 μm (Beckman Instruments). The mobile phase consisted of 0.1 *M* ammonium acetate—acetonitrile (10:90), degassed before use by Millipore filtration. The column was maintained at ambient temperature with a flow-rate of 4 ml/min. Detection was achieved using an electrochemical detector (Bioanalytical Systems Model LC4A, Technical Marketing Associates). The detector was fitted with a glassy carbon electrode set at +0.9 V in the oxidation mode with a fixed 10-nA feed going to a Perkin-Elmer Model 056 recorder (Perkin-Elmer, Montreal, Canada). All changes in attenuation were made only with the recorder to avoid baseline stabilization problems.

RESULTS AND DISCUSSION

The low-resolution EI spectra of chlorpromazine and prochlorperazine (not shown) indicated that the best ions to monitor for single ion chromatograms of these two compounds, without significant interference or contribution to each other, were their molecular ions at *m/z* 318 and *m/z* 373 for chlorpromazine and prochlorperazine, respectively.

Using the GC—MS conditions as outlined in the Experimental section the chlorpromazine monitored ion at *m/z* 318 eluted with a retention time of 1 min 12 sec, while the monitored ion at *m/z* 373 for prochlorperazine eluted with a retention time of 2 min 58 sec.

Typical single ion chromatograms of chlorpromazine and the internal standard prochlorperazine are shown in Fig. 1. In order to obtain clean chromatograms, free of interfering peaks, such as those shown in Fig. 1, it is essential that the plasma used to prepare chlorpromazine standards, as well as the plasma used to dilute unknown plasma samples, be fresh plasma stored in glass containers. This requirement was made obvious when plasma stored in plastic was first employed as a diluent for patient plasma samples. Under these circumstances a large peak interfering with chlorpromazine was seen with a retention time of approximately 1 min 10 sec. This interfering peak may be some form of phthalate ester from the plastic, since fresh plasma stored only in glass did not contain this interfering peak. Fig. 1B shows a chromatogram of a spiked plasma sample containing 10 ng of chlorpromazine per ml of plasma. Fig. 1C is a chromatogram of a plasma sample taken 2 h after the oral administration of 50 mg of chlorpromazine to a healthy male volunteer; the chlorpromazine concentration was estimated to be 3.2 ng ml^{-1}. Fig. 1D shows a typical chromatogram for the internal standard at a concentration of 50 ng ml^{-1} of plasma.

The mean overall recovery for chlorpromazine at 1 and 5 ng ml^{-1} was 86% for both concentrations, while the recovery of prochlorperazine at 50 ng ml^{-1}

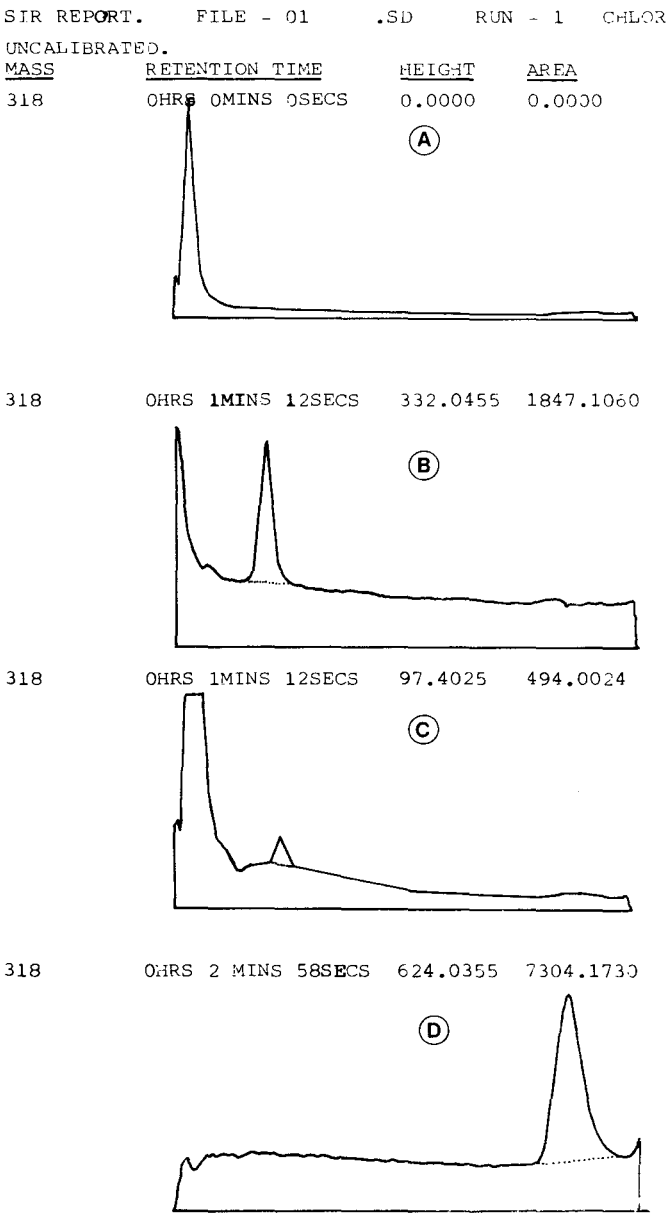

Fig. 1. Selected single ion chromatograms of chlorpromazine and prochlorperazine plasma samples. (A) Blank plasma sample; (B) spiked plasma for chlorpromazine at 10 ng ml^{-1}; (C) plasma from a healthy volunteer 2 h after receiving a single 50-mg oral dose of chlorpromazine, the concentration of chlorpromazine was estimated to be 3.2 ng ml^{-1}; (D) prochlorperazine peak obtained from the sample plasma used in (C), the concentration of prochlorperazine was 50 ng ml^{-1}.

was 86% [21]. Data obtained for the construction of a calibration curve for chlorpromazine analyzed using the described GC—MS procedure are shown in Table I. The calibration curve was linear from 0.25 to 10 ng ml^{-1}, with a coefficient of variation of 6.7% at 0.25 ng ml^{-1}, the lowest concentration analyzed. This coefficient of variation is comparable to that obtained by the HPLC—ElCD procedure (5.1% at 0.25 ng ml^{-1}) described earlier from these laboratories [21].

Both the GC—MS method described here and the earlier reported HPLC—ElCD procedure were applied to the pharmacokinetic analysis of plasma samples obtained from volunteers who had received a single 50-mg oral dose of chlorpromazine. A comparison of the plasma concentration versus time profile for a typical volunteer analyzed by both the GC—MS and HPLC—ElCD procedures is shown in Fig. 2. As can be seen, both methods, which are specific, demonstrate sufficient sensitivity to follow plasma concentrations of chlor-

TABLE I

CALIBRATION CURVE DATA FOR CHLORPROMAZINE GC—MS

Concentration (ng/ml)	n	Mean peak height ratio	Standard deviation	C.V. (%)
0.25	4	0.0161	0.0011	6.73
0.50	4	0.0293	0.0020	6.97
1.0	5	0.0583	0.0048	8.15
2.5	5	0.1579	0.0060	3.80
5.0	5	0.2787	0.0177	6.35
10.0	5	0.6205	0.0571	9.70

$y = 0.0614x - 0.0035; r^2 = 0.9970$

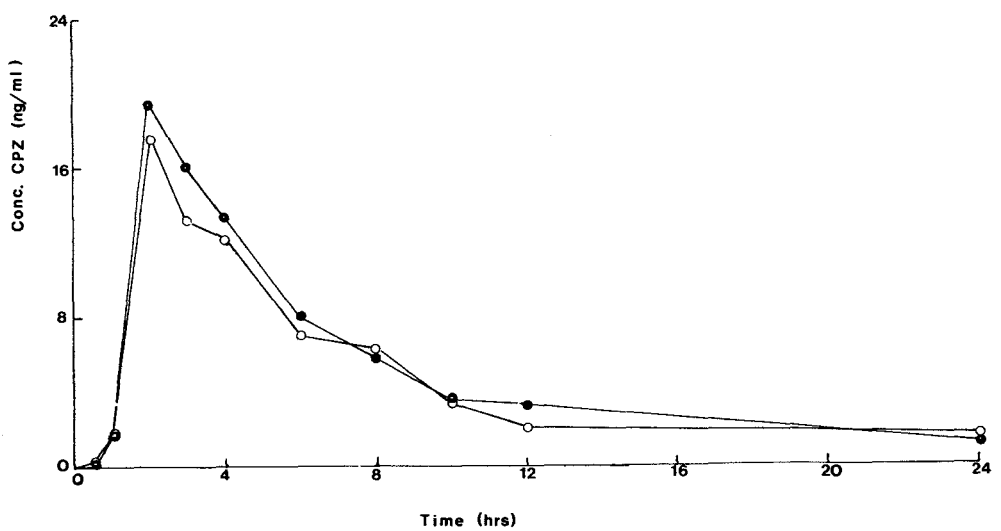

Fig. 2. Plasma concentration—time profiles for a single volunteer after ingesting 50 mg chlorpromazine (CPZ). Comparison of methods of analysis by HPLC—ElCD procedure [21] (●—●) and GC—MS procedure (present method) (○—○).

promazine to as late as 24 h following this low dose. The area under the curve from 0 to 24 h (AUC_0^{24}) based on the GC—MS procedure was determined to be 106.53 ng h ml^{-1} while the AUC_0^{24} for this same volunteer using the HPLC—ElCD procedure was 120.64 ng h ml^{-1}.

A further comparison of the HPLC—ElCD procedure and the described GC—MS procedure is shown in Fig. 3. In this figure the concentrations of chlorpromazine determined at various time intervals by both procedures is shown for the five volunteers who received a 50-mg oral dose of chlorpromazine. The slope of the regression line for this comparison had a value of 0.9713 with a correlation coefficient of 0.9617. These results indicate that the two methods compare favourably.

The described GC—MS method is precise, accurate and specific for chlorpromazine. The established sensitivity and specificity of GC—MS procedures for chlorpromazine and other drugs have made methods of analysis of this type the yardstick to which other methods are often compared. As such, the excellent agreement between the previously described HPLC—ElCD procedure [21] and the newer GC—MS procedure indicates that both methods accurately determine plasma chlorpromazine concentrations. In addition the results show that both methods have the necessary sensitivity to follow plasma concentrations

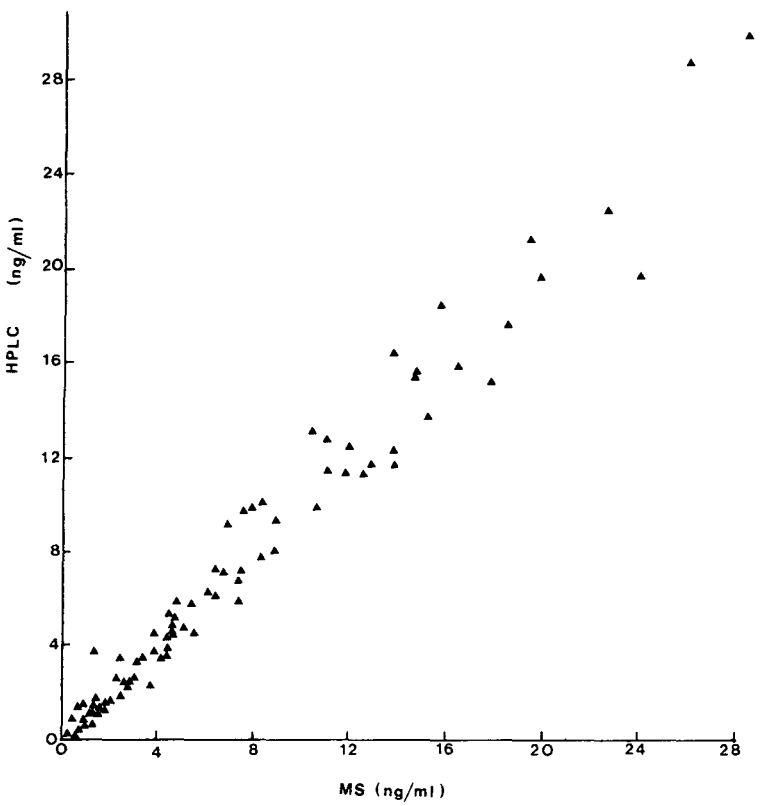

Fig. 3. Comparison of plasma concentrations determined for five volunteers after ingestion of 50 mg of chlorpromazine and analyzed by the described GC—MS procedure as well as by an HPLC—ElCD procedure [21]; $n = 90$, $r^2 = 0.9617$, and slope = 0.9713.

of chlorpromazine in patients undergoing chronic treatment, as well as even for the assessment of bioavailability/pharmacokinetic parameters of chlorpromazine in healthy volunteers receiving low single oral doses. These methods will now be used to determine reliable and meaningful clinical pharmacokinetics of chlorpromazine, a drug for which studies of plasma levels—clinical response correlations have not been conclusive [2, 3, 5, 17, 24—27].

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CHROMBIO. 1378

SIMULTANEOUS DETERMINATION OF NICOTINE AND COTININE IN PLASMA USING CAPILLARY COLUMN GAS CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

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SUMMARY

A rapid and sensitive method is described for the simultaneous determination of nicotine and its principal metabolite, cotinine, in plasma. A one-step extraction procedure is employed and the quantitative analyses are performed by capillary column gas chromatography using a thermionic specific detector. Other special measures to avoid contamination from external sources such as atmosphere, solvents and laboratory equipment, which constitutes the major limiting factor of nicotine assay, were also undertaken. The structural analogues of nicotine and cotinine, N-methylanabasine and N-ethylnorcotinine, are used as internal standards. Moreover, a micromethod, which requires only 0.1 ml of plasma and found to be suitable for analysis of cotinine in finger-tip samples of blood, is described. Linearity over the concentration ranges 5—100 ng of nicotine per ml of plasma and 5—500 ng of cotinine per ml of plasma is demonstrated. The precision of the method has been investigated by determining the reproducibility at different levels of nicotine and cotinine within the working ranges, for both 1-ml and 0.1-ml samples of plasma.

INTRODUCTION

A large number of publications dealing with the quantitative determination of nicotine and/or cotinine in plasma have appeared in the literature during the past decade. Although some of the methods described are based on radioimmunoassay [1, 2] or liquid chromatography [3, 4], the vast majority are founded on gas chromatography using electron-capture [5], flame ionization [6, 7] or thermionic specific detectors [8—14], or a mass spectrometer in a low- or medium-resolution mode [15, 16].

The specificity and sensitivity of the gas chromatographic methods depend largely on the work-up procedures, the gas chromatographic separation and the

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detector characteristics. Since their accuracy and reproducibility are primarily influenced by the work-up techniques, most recent studies have been focused on this part of the analysis. Thus, Kogan et al. [14] have presented a method for the simultaneous extraction and determination of nicotine and cotinine, Feyerabend and Russell [10] have introduced a single-step micro-extraction procedure for the analysis of nicotine, and Jacob et al. [13] have demonstrated that the use of internal standards closely analogous to nicotine and cotinine is advantageous, and lowers the limits of detectability. The fact remains, however, that all methods presented so far for the determination of nicotine and cotinine in one sample involve several extraction steps and require at least 1 ml of blood. The main reason for this is the interference from extraneous nicotine, derived from the environment and from contaminated solvents and glassware. Thus, one-step rather than multi-step extraction procedures should be employed and the loss in separation thereby experienced compensated by more efficient gas chromatography and/or higher detector selectivity.

In view of this and the fact that all workers but Dow and Hall [16] have used packed columns for the gas-chromatographic separation, we have developed an assay for the simultaneous determination of nicotine and cotinine which is applicable to both 1-ml and 0.1-ml samples of plasma. It is based on a one-step single extraction of nicotine and cotinine from plasma, more suitable internal standards, and capillary column gas chromatography utilizing a thermionic specific detector.

MATERIALS AND METHODS

Glassware

All glassware for the extraction procedure was cleaned by overnight immersion in a Deconex 11 detergent solution, washed consecutively with hot tap water and ethanol in an ultrasonic bath. The glassware was kept in an oven at 100—200°C for some hours before use.

Chemicals and reagents

(−)-Nicotine, which was purchased from Fluka, Buchs, Switzerland, was purified by distillation under nitrogen before the preparation of standard solutions. N-Methylanabasine was a gift from Stockholm University, Sweden. (−)-Cotinine was synthesised by the method of Bowman and McKennis [17] and double distilled under nitrogen before use. N-Ethylnorcotinine was prepared using adopted methods of synthesis, i.e. condensation of N-vinylpyrrolidone with ethyl nicotinate to form myosmine by the method of Brandänge and Lindblom [18], reduction of myosmine to nornicotine [19], ethylation of nornicotine to N-ethylnornicotine [20] followed by oxidation to N-ethylnorcotinine according to the method of Bowman and McKennis [17]. N-Ethylnorcotinine was purified by liquid chromatography over silica gel. The structures and purities of the alkaloids used in the assay were confirmed by gas chromatography, proton nuclear magnetic resonance and mass spectrometry.

Dichloromethane (IR spectroscopic grade) was purchased from Fluka. The purity of every new bottle was checked by gas chromatography of the evapora-

tion residue from 10 ml of dichloromethane. Ethanol (99.9%) was of spectroscopic quality. The sodium hydroxide solution, which was prepared from pellets (Fluka) previously heated to 200°C, was extracted twice with dichloromethane just before use.

Instrumentation

The gas chromatographic analyses were performed on a Varian Model 3700 instrument, equipped with an all-glass capillary injector (Varian direct injector) and a thermionic specific detector containing an electrically heated ceramic-alkali bead. The detector was operated at a bias voltage of −4 V and the bead heating current potentiometer was set between 3.0 and 3.8 A, depending upon the age of the bead. Optimal sensitivity and specificity for nitrogenous compounds was achieved at flow-rates of hydrogen, air and make-up helium of 4.5, 175 and 35 ml/min, respectively. The flow-rate of helium carrier gas through the column was 40 cm/sec. A fused-silica capillary column (25 m × 0.32 mm I.D.) wall-coated with a free fatty acid phase, SP-1000 (film thickness 0.15 μm), and purchased from Orion Analytica, Finland, was used throughout these studies. Sharp peaks with minimal tailing were obtained by introducing the outlet of the column through the detector liner directly into the flame-tip of the detector. The column temperature was 135°C during the nicotine analyses and 200°C during the cotinine analyses. The injector and detector temperatures were 250°C and 290°C, respectively.

Using the chromatographic conditions described above, the retention times for nicotine and N-methylanabasine were 2.3 and 3.2 min, respectively, and for N-ethylnorcotinine and cotinine, 4.6 and 5.0 min, respectively.

The plotting of the chromatograms and the integration of the peak areas were carried out by a Hewlett-Packard Model 3388 A plotter/integrator or by a Perkin-Elmer Model 561 recorder coupled to a Varian Model CDS-111 integrator.

Extraction procedure

Plasma (1 ml) and internal standards (100 ng of N-methylanabasine and 100 ng of N-ethylnorcotinine in 100 μl of ethanol) were pipetted into a 10-ml tube fitted with a screw cap having a PTFE—faced rubber liner, and thoroughly mixed. The sample was made alkaline by addition of aqueous sodium hydroxide (5 *M*, 1 ml), extracted with dichloromethane (2 ml) for 5 min using a Vortex-Genie mixer and then centrifuged at 3000 *g* for 15 min in an MSE-GF-6 universal centrifuge to break the emulsion. Most of the aqueous layer was discarded and the organic and remaining aqueous layers were separated by freezing the tube in a dry-ice—acetone bath and subsequently transferring the unfrozen organic layer to a conical 2-ml septum-cap vial. The dichloromethane was evaporated under a gentle stream of nitrogen, keeping the tubes in an electrically heated sample concentrator (Tecam-Dri Block DB-3) at 40°C. The dichloromethane was exchanged with ethanol by addition of the latter solvent (100 μl) during the concentratiom step, which was continued until less than 20 μl of solution remained. Part (0.1—0.3 μl) of the remaining ethanolic solution was injected onto the chromatographic column.

Micro-extraction

Plasma (0.1 ml) was mixed with an ethanolic solution (10 μl) of internal standards (N-methylanabasine 1 ng/μl, N-ethylnorcotinine 1 ng/μl) in a conical 2-ml septum-cap vial. The sample was made alkaline by the addition of aqueous sodium hydroxide (5 *M*, 100 μl) and then extracted with dichloromethane (500 μl). The work-up procedure was the same as that described in the paragraph above.

Plasma blanks

Human plasma was obtained from volunteers who had fasted overnight before donating blood. Bovine plasma has been used for calibration purposes, as problems were encountered in obtaining true blank human plasma. Extracts from bovine plasma and from the true blank of human plasma were found to give identical gas chromatograms.

Calibration procedure

Ethanolic nicotine standard solutions of different concentrations (0.05—1.0 ng/μl) containing the same concentration of the internal standard (1.0 ng/μl) were prepared for calibration purposes. These standard solutions (100 μl, 10 μl) were added to bovine plasma (1 ml, 0.1 ml) to provide samples containing 5, 10, 20, 50, 80 and 100 ng of nicotine and 100 ng of the internal standard, N-methylanabasine, per ml of plasma.

Similarly, bovine plasma (1 ml, 0.1 ml) was spiked with ethanolic standard solutions having different concentrations of cotinine and the same concentration of the internal standard, to provide samples containing 5, 10, 20, 50, 80, 100, 250 and 500 ng of cotinine and 100 ng of the internal standard, N-ethylnorcotinine, per ml of plasma.

The ethanolic standard solutions were stored in the absence of light at 4°C and were found to be stable for at least one year.

The samples were taken through the extraction procedure and analysed by gas chromatography. Calibration curves were constructed by plotting the amount of nicotine versus the peak area ratio of nicotine and N-methylanabasine and by plotting the amount of cotinine versus the peak area ratio of cotinine and N-ethylnorcotinine.

Recovery

Extraction efficiency. Human plasma (1 ml) was spiked with nicotine and cotinine (4 μg of each), and extracted at alkaline pH with dichloromethane (2 ml). A portion of the organic layer (0.2 ml) was transferred to a tube containing internal standards (0.4 μg of each) in ethanol (2 ml), and subsequently analysed as described below.

Overall recoveries. Human plasma (1 ml) was spiked with nicotine and cotinine (100 ng of each), and extracted with dichloromethane (2 ml) at alkaline pH. A portion of the organic layer (1 ml) was transferred to a conical tube and evaporated. The internal standards (50 ng of each) were added as ethanolic solutions at the end of the evaporation step.

Part of the ethanolic solutions were injected into the gas chromatograph. The peak area ratios of nicotine/N-methylanabasine and cotinine/N-ethyl-

norcotinine were estimated and the recoveries were calculated by comparison with the peak area ratios of non-extracted standard solutions. Similarly, the recoveries of N-methylanabasine and N-ethylnorcotinine were estimated by spiking human plasma with these compounds and using nicotine and cotinine as external standards.

RESULTS AND DISCUSSION

Contamination

A peak having the same retention time as nicotine has been observed by several authors during the analysis of plasma from non-smokers and has caused some confusion. We have also encountered this peak and have unambiguously identified the corresponding compound as nicotine by high-resolution mass spectrometry. It derives from extraneous nicotine present in the environment, which is incorporated into the plasma either by ingestion by the non-smoker or by contamination during sampling, sample work-up, or chromatographic analysis.

This interference, which originates from sources such as contaminated atmosphere, reagents and apparatus, has been minimized by rigorous care and control of all steps in the present assay. It has thus been possible, by undertaking the precautions discussed below, to reduce the nicotine concentration of "zero nicotine" plasma samples to between 0.3 and 0.6 ng/ml (Fig. 1A and B). Many of the contamination problems associated with the nicotine analysis do not occur in the cotinine analysis, since in tobacco smoke the concentration of the latter is much lower than that of nicotine.

In accordance with results of Feyerabend and Russell [21], we found that the atmosphere is the most serious source of contamination, and hence the entire analytical procedure was performed in a sealed laboratory, all air passing through an active carbon filter. All work-up of samples, i.e. extraction and evaporation, was carried out in a flow-hood. Smoking was strictly forbidden in the area surrounding this laboratory and the analyst had to be a non-smoker.

To avoid contamination from reagents, their purity was checked before use. Gas chromatographic examination of evaporation residues derived from different grades of solvents showed that dichloromethane (IR-spectroscopic grade from Fluka) and ethanol (Spectroscopic grade) could be employed without purification. However, the purity of every new batch of dichloromethane had to be checked as the quality of this solvent varied considerably. The sodium hydroxide solution, which is easily contaminated by nicotine, had to be prepared and stored with care and its purity examined before use. Pure sodium hydroxide solutions were prepared by dissolving pellets, heated beforehand to 200°C, in deionized water and extracting the resulting solution with dichloromethane just prior to use.

Cleaning of laboratory equipment, such as tubes, pipettes and syringes, was found to be of great importance since nicotine as well as the other bases used in the assay readily bind to glass and metal surfaces. All glassware was meticulously washed and subsequently kept in an oven at 200°C until required.

Besides extraneous nicotine, the plasma samples could also be contaminated by other compounds interfering with the chromatographic analysis. Thus blood

samples treated with heparin in the course of the preparation of plasma, were found to contain a component giving rise to a peak interfering with the nicotine peak in the chromatographic analysis. Therefore, this anticoagulant was replaced by sodium citrate. Interfering peaks were also encountered when plastic bags or tubes were used for storage of plasma.

We have also investigated a microtechnique for the analysis of nicotine and cotinine, the major advantage of which is a faster and simpler sampling procedure. In agreement with results of Feyerabend and Russell [10], we found this technique applicable to samples of blood obtained in the normal way but not to those obtained by finger-tip puncture, since these samples were heavily contaminated by nicotine derived from the skin. However, this technique was found to be suitable for the analysis of cotinine, where contamination is much less of a problem.

Extraction

Interference by extraneous nicotine is mainly due to incorporation during the extraction procedure. In order to minimize the exposure of the samples to the atmosphere, reagents and glassware, we have developed a simplified extraction technique, which involves a single extraction of nicotine and cotinine from plasma by dichloromethane at alkaline pH. Dichloromethane was found to be the most suitable organic solvent for a simultaneous extraction of nicotine and cotinine and their internal standards as the recoveries of these compounds from a single extraction were found to be between 95 and 100% (Table I). Purification of the samples by time-consuming back-extraction steps could be omitted as high-resolution gas chromatography was used in the analytical step. This direct extraction procedure, which involves high recoveries and low interference by extraneous nicotine, enabled accurate quantification of small amounts of nicotine and cotinine in plasma. Thus, this extraction procedure was a prerequisite for the analysis of 0.1-ml plasma samples.

TABLE I

RECOVERIES FROM HUMAN PLASMA

Compound	Extraction recovery (%)	Overall recovery (%)
Nicotine	96	83
N-Methylanabasine	99	100
Cotinine	97	96
N-Ethylnorcotinine	103	95

Gas chromatography

The sensitivity and selectivity of the thermionic specific detector for nitrogen-containing compounds allowed a relatively precise determination of small amounts of nicotine and cotinine. The detection limit at a signal-to-noise ratio of four was estimated to be 5 pg of nicotine and 20 pg of cotinine. The detector response was found to be linear and reproducible down to 5 pg of nicotine and 20 pg of cotinine, which corresponds to a minimal detectable amount of 0.1 ng of nicotine and 0.4 ng of cotinine per ml of plasma.

The use of the simplified extraction procedure described above, required a refined separation technique in the analytical step. This was achieved by using high-resolution capillary column gas chromatography. By optimization of critical parameters and instrumental configuration, excellent resolution and sensitivity were obtained; of particular importance were the use of a direct injector, insertion of the outlet of the fused-silica column directly into the flame-tip, and use of helium as carrier gas. A single injection of a plasma extract gave no significant interference by any endogenous material present in normal human plasma, but in the nicotine analysis interfering peaks appeared in the chromatograms after ten consecutive injections. These were readily removed by conditioning the column at 220°C for about 10 min. Typical gas chromatograms obtained after extraction of 1 ml of blank human plasma and of 1 ml of an authentic human plasma sample from a heavy smoker, containing 35 ng of nicotine and 350 ng of cotinine, are shown in Figs. 1 and 2.

As shown, the gas chromatographic analyses of nicotine and cotinine were performed isothermally at different column temperatures. The compounds were eluted at low k' values and sharp peaks with minimal broadening were obtained. Under these conditions the full resolution capability of the column was not utilized, but the chromatographic resolution was still sufficient to achieve good baseline separations of nicotine and cotinine from their internal standards. The sensitivity and reproducibility were also improved by this gas

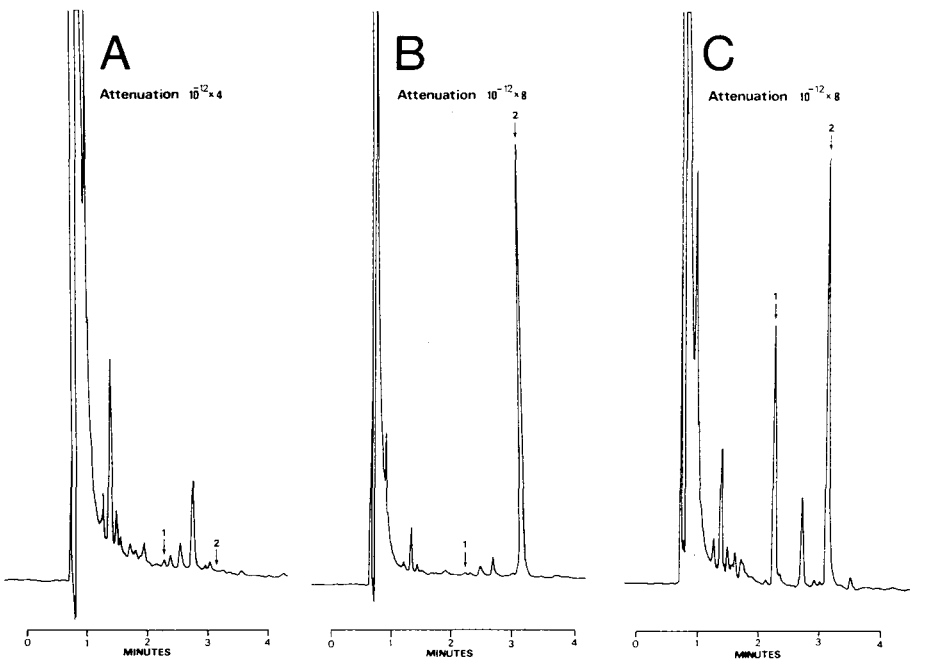

Fig. 1. Gas chromatograms obtained from extracts from human plasma samples (1 ml). (A) Extract of a non-smoker's plasma. (B) Extract of a non-smoker's plasma spiked with 100 ng/ml of the internal standard, N-methylanabasine. (C) Extract of an authentic plasma sample from a smoker containing 35 ng of nicotine and spiked with 100 ng of N-methylanabasine. Nicotine (1) and the internal standard, N-methylanabasine (2), were eluted isothermally at 135°C.

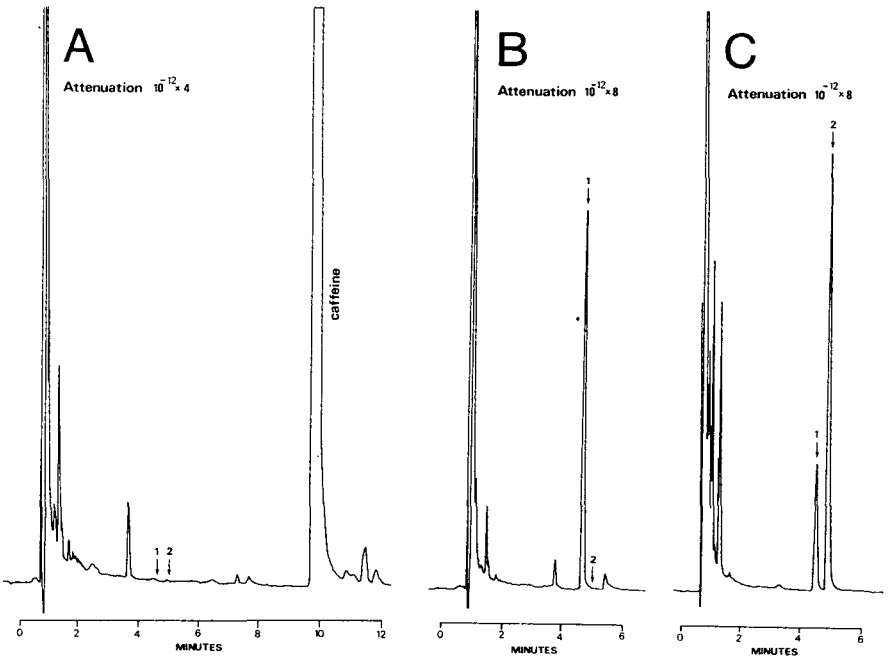

Fig. 2. Gas chromatograms obtained from extracts of human plasma samples (1 ml). (A) Extract of a non-smoker's plasma. (B) Extract of a non-smoker's plasma spiked with 100 ng/ml of the internal standard, N-ethylnorcotinine. (C) Extract of an authentic plasma sample from a smoker containing 350 ng of cotinine and spiked with 100 ng of N-ethylnorcotinine. N-Ethylnorcotinine (1) and cotinine (2) were eluted isothermally at 200°C.

chromatographic procedure and, moreover, the total analytical time was shortened, especially when the two analyses were performed on separate gas chromatographs.

A factor which limits the accuracy of quantification is the reduction in sensitivity which results from repeated injections of plasma extracts. However, the sensitivity could be restored by cleaning the injector insert and disposing of 10 cm of the column inlet to remove accumulated residues.

Internal standards

The choice of internal standards is important for the sensitivity and the precision of the method. Since there are great differences in solvent partitioning and chromatographic properties of nicotine and cotinine, separate internal standards have been used for the analysis of these compounds. Our initial attempts to use quinoline as standard for nicotine and lidocain for cotinine, as described in previous methods, failed. In accordance with Jacob et al. [13], we observed large variations in the peak area ratios of nicotine to quinoline and of cotinine to lidocain, results which are probably due to different relative losses during work-up and/or during gas chromatography.

These difficulties were circumvented by using the structural analogues N-methylanabasine and N-ethylnorcotinine as internal standards (Fig. 3). Their extractability into dichloromethane, volatility and affinity to glass and metal

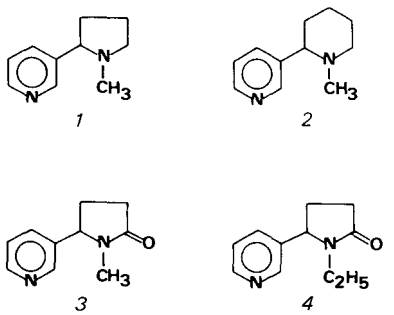

Fig. 3. Structures of nicotine (1), N-methylanabasine (2), cotinine (3) and N-ethylnorcotinine (4).

surfaces are similar to those of nicotine and cotinine, as demonstrated by their high overall recoveries (Table I). Moreover, the use of these analogues as internal standards also improved the precision of the chromatographic analysis as their detector responses and retention times are close to those of nicotine and cotinine.

Precision and calibration

Both 1-ml and 0.1-ml samples of blank bovine plasma were spiked with 100 ng of each of the internal standards and with nicotine and cotinine to give samples of different concentrations within the range 5—100 ng/ml nicotine and within the range 5—500 ng/ml cotinine. Ten samples at each concentration were prepared and taken through the extraction procedure. The precision of

TABLE II

PRECISION OF DETERMINATION OF NICOTINE AND COTININE APPLIED TO 1-ml SPIKED BOVINE PLASMA SAMPLES

	Amount added (ng/ml)	Mean amount found* (ng/ml, $n=10$)	Standard deviation (S.D.)	Coefficient of variation (C.V. %)
Nicotine	5	5.1	0.4	8.6
	10	9.7	0.5	4.8
	20	19.1	0.6	2.9
	50	47.9	2.0	4.6
	80	78.6	3.5	4.4
	100	99.3	2.6	2.7
Cotinine	5	5.4	0.7	13.6
	10	10.4	0.6	6.2
	20	20.5	1.3	6.4
	50	50.4	1.2	2.3
	80	80.0	2.0	2.5
	100	98.7	1.5	1.5
	250	257.2	1.0	0.4
	500	496.8	3.5	0.7

*The results of the nicotine analyses have been corrected for a background level of nicotine.

TABLE III

PRECISION OF DETERMINATION OF NICOTINE AND COTININE APPLIED TO 0.1-ml SPIKED BOVINE PLASMA SAMPLES

	Amount added (ng/ml)	Mean amount found* (ng/ml, $n=10$)	Standard deviation (S.D.)	Coefficient of variation (C.V. %)
Nicotine	5	5.4	0.6	11.2
	10	10.1	0.5	5.4
	20	20.4	1.2	5.8
	50	49.5	1.3	2.7
	80	78.6	2.5	3.2
	100	102.4	3.7	3.6
Cotinine	5	5.1	0.6	11.2
	10	11.2	0.7	6.4
	20	21.0	1.0	4.7
	50	52.1	1.5	3.0
	80	80.2	1.3	1.6
	100	98.6	1.7	1.7
	250	252.4	5.0	2.0
	500	489.9	10.3	2.1

*The results of the nicotine analyses have been corrected for a background level of nicotine.

the method was evaluated by calculating the coefficient of variation at each concentration of nicotine and cotinine. The mean values, standard deviations and coefficients of variation of each plasma level of nicotine and cotinine are given in Tables II and III. In the case of nicotine analysis, the background level obtained from blank plasma samples was subtracted (see Fig. 1). The results show that plasma levels could be accurately determined down to 5 ng of nicotine and cotinine per ml of plasma.

Ten calibration curves were constructed for each of the ranges 5—100 ng of nicotine and 5—500 ng of cotinine per ml of plasma with both 1-ml and 0.1-ml samples. The calibration curves were linear over the entire range investigated and the mean correlation coefficient of the ten curves were in all cases better than 0.9995. The calibration curves are reproducible from day to day, which is demonstrated by the small coefficients of variation (0.5—3.5%) of the slopes.

TABLE IV

CONCENTRATION RANGES AND MATHEMATICAL EXPRESSIONS OF THE NICOTINE AND COTININE CALIBRATION CURVES

Concentration ranges	Amount of plasma (ml)	Regression equation ($y = ax + b$)		Correlation coefficient (r)	n
		a	b		
Nicotine (5—100 ng/ml)	1.0	0.989	0.210	0.9997	10
	0.1	1.006	1.389	0.9995	10
Cotinine (5—500 ng/ml)	1.0	0.990	1.086	0.9996	10
	0.1	0.982	1.532	0.9998	10

The concentration ranges of nicotine and cotinine, the coefficients of correlation and the mathematical expression of the calibration curves are summarized in Table IV.

CONCLUSION

A rapid and sensitive method has been developed for the simultaneous determination of nicotine and cotinine at nanogram levels using as little as 0.1 ml of plasma. The high degree of precision achieved is attributable to the exclusion of extraneous contaminants, the use of a single-step extraction procedure, the choice of suitable internal standards, and the use of high-resolution capillary gas chromatography and thermionic specific detection. It is well suited for routine analysis of nicotine and cotinine in plasma not only from smokers and users of snuff, but also from "passive smokers".

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IMPROVED METHOD FOR ROUTINE DETERMINATION OF NICOTINE AND ITS MAIN METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

Extrelut^{R} extraction and glass capillary gas chromatography were applied to the routine determination of nicotine and its metabolites cotinine, nicotine-1′-N-oxide and cotinine-1-N-oxide in urine and plasma. After extraction of nicotine and cotinine both N-oxides and phendimetrazine-N-oxide (used as internal standard) were reduced to their bases by SO_2 on-column and eluted by a mixture of diethyl ether and dichloromethane. The minimum detectable concentrations are 0.03 μg/ml for urinary nicotine and cotinine and 0.1 μg/ml for the N-oxides. In plasma samples the corresponding values are 5 ng/ml and 15 ng/ml, respectively, with sample values as small as 2 ml. The advantage of the direct determination of all four compounds of interest in one sample reduced the amount of plasma required. The straightforward and rapid extraction and reduction procedure as well as the long-term stability of the gas chromatographic separation system make the method suitable for routine application.

INTRODUCTION

The determination of nicotine and its metabolites in biological fluids is of particular interest to investigators studying correlations between the biological effects of tobacco smoking and smoking habits (active, passive, frequency, type of cigarette, etc.).

Nicotine taken up from cigarette smoke is primarily metabolized in the human organism to cotinine and nicotine-1′-N-oxide [1]. Both compounds are formed by independent pathways [2]. Which of them predominates depends on the age of the test person and the urinary pH [3]. Cotinine undergoes further oxidation to cotinine-1-N-oxide [4, 5]. All these metabolites are excreted in urine together with the non-metabolized nicotine.

The expected concentrations for nicotine, cotinine and nicotine-1′-N-oxide are below 1 μg/ml in most urine samples [3]. Disregarding heavy cigarette

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smokers, plasma contains nicotine below 60 ng/ml and cotinine below 200 ng/ml. The low concentration as well as the minute amounts of plasma available demand a sensitive method permitting the determination of all compounds in one sample. The analysis of nicotine and cotinine in urine described by Beckett and Triggs [6] has been modified and completed by several workers [3, 6—11]. All these methods are time consuming and therefore not suitable for a large number of samples. As a rule they include liquid—liquid extraction, determination of nicotine-1′-N-oxide after reduction by $TiCl_3$, and gas chromatographic (GC) analysis. However, ether extraction of nicotine and successive extraction of cotinine with dichloromethane [7] results in unavoidable losses of cotinine [12]. Furthermore, the experimental error is increased by the determination of the N-oxide as the difference between the total nicotine content after reduction of the oxide and the original nicotine amount. Finally, the analysis of nicotine-1′-N-oxide in plasma proved to be rather difficult by this method.

If the GC analysis of the extracts is carried out on packed columns, the high elution temperature of cotinine considerably limits the lifetime of the column. For that reason, as well as for their high separation power and inertness, glass capillary columns proved to be superior [13].

The advantages of the nitrogen-sensitive alkali flame ionisation detector have been reported [10, 14], but its low long-term stability limits its routine application. The mass spectrometer, used as a highly specific GC detector [13, 15] is too expensive for routine analysis.

In our view a method for routine analyses should fulfil the following criteria: rapid and straightforward extraction and clean-up procedure; simultaneous analysis of nicotine and cotinine; determination of N-oxides independent of nicotine and cotinine in the same sample, not only for urine but also for plasma; efficient and stable separation and detection. These requirements were met by the application of Extrelut^R columns for the extraction step, the on-column reduction of the N-oxides in the presence of phendimetrazine-N-oxide as internal standard, and the use of glass capillary columns for separation.

EXPERIMENTAL

Materials

Cotinine (70% aqueous solution) and nicotine-1′-N-oxide (purity ca. 80%) were purchased from R. Hallermayer, Augsburg, G.F.R. Lidocaine, lidocaine hydrochloride and phendimetrazine hydrochloride were kindly supplied by Gerot Pharmazeutika, Vienna, Austria. Quinoline (purity 99%) purchased from Fluka (Buchs, Switzerland) and nicotine (synthesis grade, 98%) purchased from Merck-Schuchardt (Munich, G.F.R.) were distilled shortly before use.

Phendimetrazine-N-oxide was prepared from phendimetrazine by H_2O_2 oxidation. Phendimetrazine was obtained by adding 1 ml of 5 *N* NaOH to 0.54 g of phendimetrazine hydrochloride, extracted three times with 3 ml of ethyl ether and dried over Na_2SO_4. After evaporation of the solvent the resulting 0.43 g of phendimetrazine were oxidized with 4 ml of 5% H_2O_2 at room temperature for 18 h and at 40°C for a further 20 h. Finally, the unreacted phendimetrazine was removed by extraction with diethyl ether and the sample con-

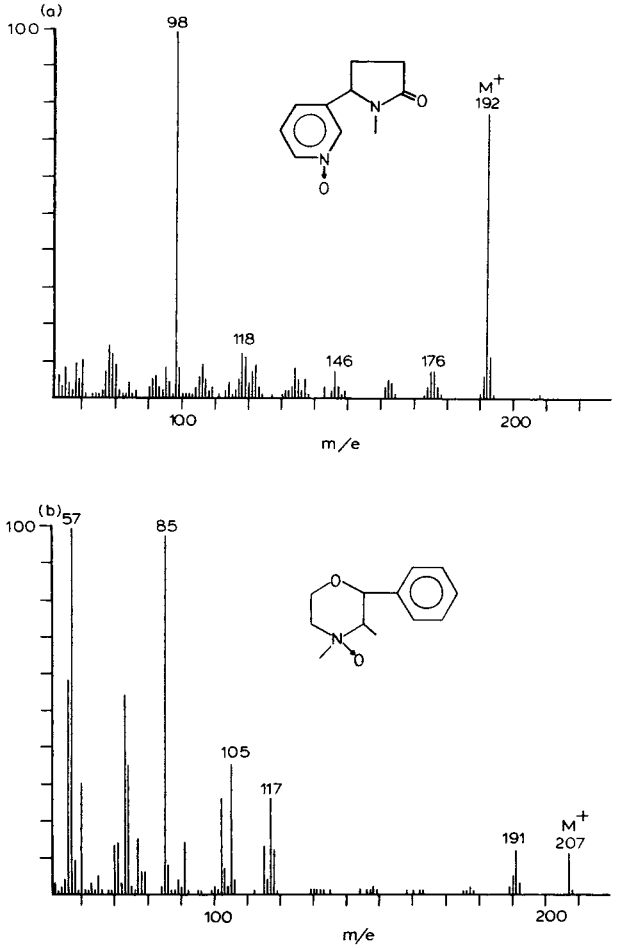

Fig. 1. Mass spectra of cotinine-1-N-oxide (a) and phendimetrazine-N-oxide (b). Varian CH 5 DF, 70 eV, direct probe 120°C.

taining the N-oxide was freeze-dried. The procedure yielded 0.44 g of phendimetrazine-N-oxide. The structure was verified by mass spectrometry (Fig. 1).

Cotinine-1-N-oxide was synthesized in a similar way. The oxidation was carried out with a 10% solution of H_2O_2 at 60°C for 72 h. The residual H_2O_2 was degraded by a short heat treatment and the unreacted material removed by repeated extraction with dichloromethane. The mass spectrum is shown in Fig. 1.

For Extrelut extraction two types of columns (dimensions 23 mm × 93 mm and 7 mm × 10 mm) were filled at a packing density of 0.30 g/cm^3 with different loadings of Extrelut (Merck) according to the sample size [16].

Procedures

Urine samples. To 4.0 ml of urine 0.5 ml of 0.5 *N* NaOH and the internal standard solution (quinoline, lidocaine, phendimetrazine and phendimetrazine-

N-oxide, 5.0 μg of each dissolved in 0.1 ml of acetone) were added. The sample was applied to a column filled with 3.0 g of Extrelut. After 30 min, 13 ml of the solvent mixture (diethyl ether—dichloromethane, 1:4, v/v) were poured into the column. The eluate (ca. 10 ml), containing nicotine, cotinine, quinoline, lidocaine and phendimetrazine, was concentrated in a water bath at room temperature under a stream of nitrogen to a volume of 1 ml and analyzed by GC.

Afterwards, the N-oxides of nicotine, cotinine and phendimetrazine were reduced by passing a stream of SO_2 through the column; 30 min after saturation of the column with SO_2 the sample was made alkaline by passing a stream of NH_3-saturated air through the column. The pH of the gaseous effluents in both steps was monitored by an indicator strip. The neutralization with NH_3 was carried out slowly to avoid warming up beyond 40°C. The

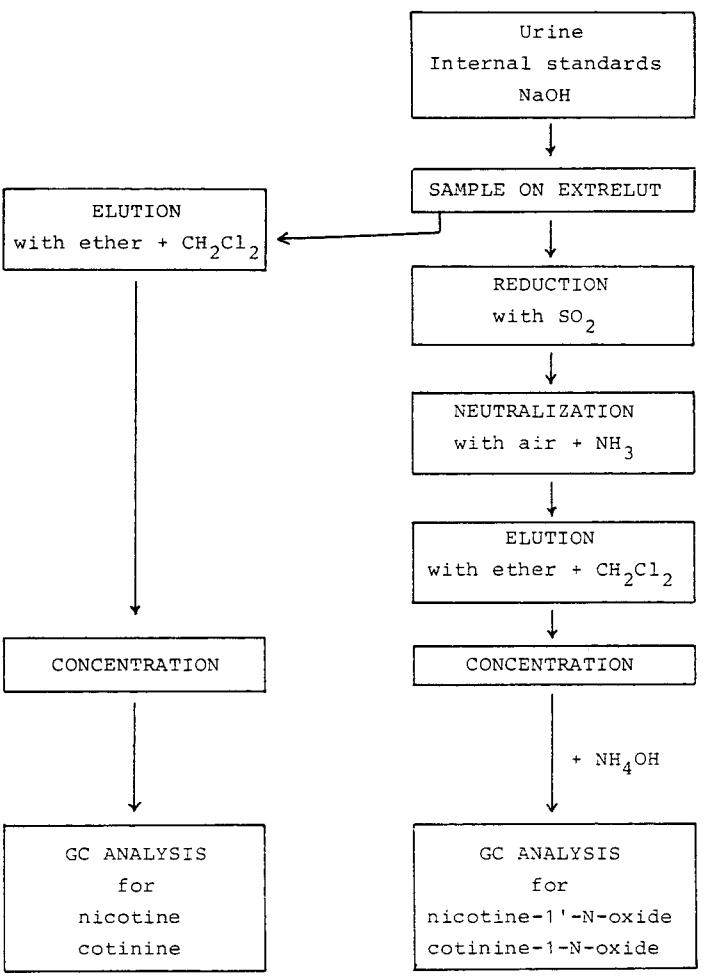

Fig. 2. Flow diagram of urine processing for the determination of nicotine, cotinine and their N-oxides.

resulting bases were eluted and concentrated as described above. Prior to GC analysis 5 μl of concentrated NH_4OH were added to each sample. A flow diagram of the procedure is shown in Fig. 2.

Plasma samples. To 2.0 ml of plasma 2.0 ml of 0.1 *N* NaOH and the internal standard solution (quinoline, lidocaine, phendimetrazine and phendimetrazine-N-oxide, 200 ng of each dissolved in 10 μl of acetone) were added. The sample was applied to a column filled with 2.5 g of Extrelut. After 30 min 13 ml of the solvent mixture were poured onto the column. The eluate (ca. 10 ml), containing nicotine, cotinine, quinoline, lidocaine and phendimetrazine, was collected into a calibrated conical tube. After addition of 0.5 ml of 0.2 *N* HCl, mixing and concentrating to a volume of about 6 ml, the sample was ultrasonicated for 15 min and centrifuged at 550 *g* for 5 min. The aqueous layer (ca. 0.45 ml) was quantitatively transferred with a syringe to a smaller vial and further purified by washing with 2 ml of diethyl ether. Then the aqueous sample was made alkaline by adding a drop of 6 *N* NaOH and applied to a column of the smaller type [16], filled with 0.25 g of Extrelut. After an equilibration time of 30 min, nicotine, cotinine and the corresponding standards were eluted with 1.5 ml of the solvent mixture. The eluate (ca. 1.1 ml) was concentrated to a volume of 100 μl, mixed with 5 μl of concentrated NH_4OH and analyzed by GC.

Following the elution of the bases the N-oxides remaining on the first column (2.5 g of Extrelut) were reduced, made alkaline and eluted as described for the urine sample. The next step followed was as described for the plasma sample, including re-extraction with 0.5 ml of 0.2 *N* HCl. A flow diagram of the procedure is shown in Fig. 3.

GC instrumentation and working conditions

For GC a Carlo Erba instrument Model 2150, equipped with a Dani automatic liquid sampler ALS 3641 (modified to hold syringes with 70 mm needle length) was used. The detector signals were quantitated by a Spectra-Physics Integrator System I. The gas chromatograph was equipped with a split/splitless injection port, containing a glass insert filled in a length of 30 mm with Carbowax 1000 deactivated glass beads (100—120 mesh). This precolumn kept the non-volatile sample components off the capillary column [17].

The raw capillaries were drawn from borosilicate glass to a length of 28 m with an inner diameter of 0.3 mm. After formation of an intermediate layer of $BaCO_3$ [18] they were coated with a 0.1 μm thick film of the polyglycol phase Pluronic F 68 (Fluka).

The analyses were carried out under the following conditions: injection port temperature 250°C; oven temperature program: starting isothermal 40°C for 1 min, first heating rate 30°C/min, intermediate isothermal 80°C for 2 min, second heating rate 10°C/min, final isothermal 230°C for 10 min; detector, FID, base temperature 250°C; carrier gas hydrogen, inlet pressure 0.55 bar (linear gas velocity 50 cm/sec at room temperature); injection volume (splitless injection) 1.0 μl.

The evaluation was carried out by the internal standard method. The standards were quinoline and lidocaine for nicotine and cotinine, respectively. In addition, phendimetrazine was used as an overall indicator of the adsorptivi-

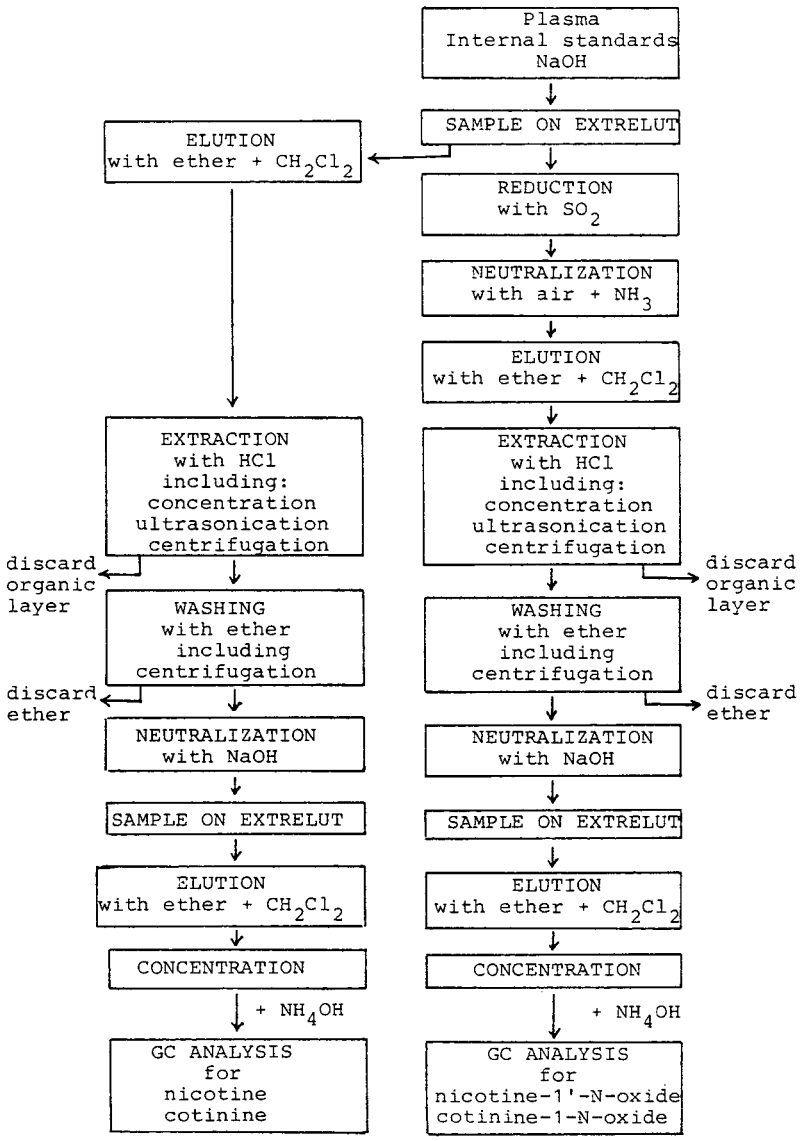

Fig. 3. Flow diagram of plasma processing.

ty of the system. If the phendimetrazine peak showed a broadening or a retardation the injection was repeated after addition of 5 μl of concentrated NH_4OH. The determination of the nicotine- and cotinine-N-oxides was based on phendimetrazine-N-oxide as internal standard.

RESULTS AND DISCUSSION

Figs. 4 and 5 show gas chromatograms of plasma samples processed as described above. The good separation of the peaks of interest from coextracted impurities and the adequate sensitivity of the flame ionisation detector

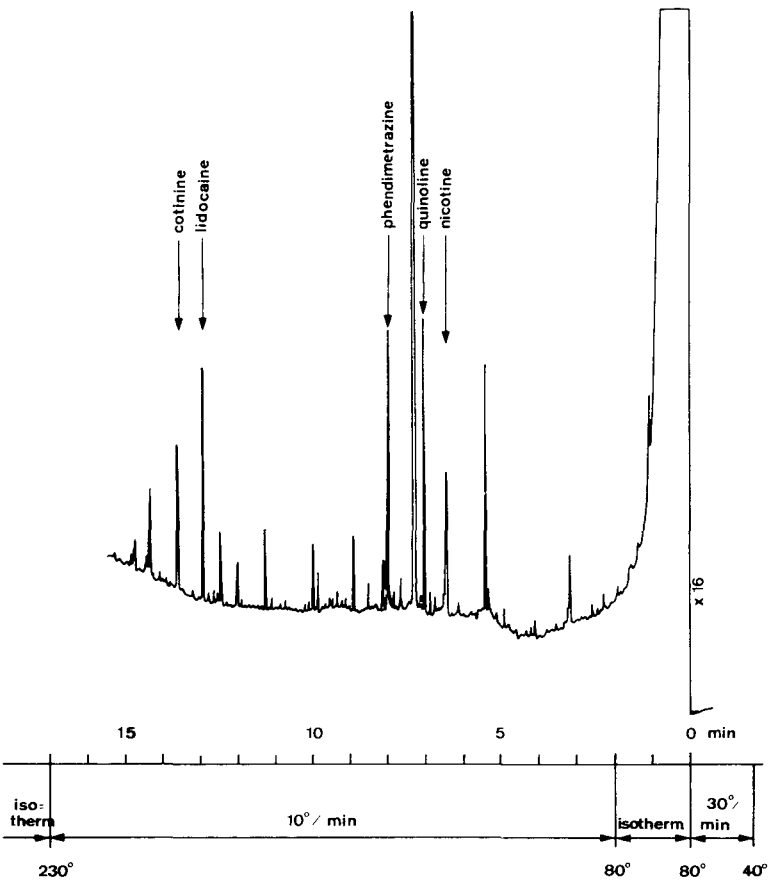

Fig. 4. Gas chromatogram of an extract of a 2-ml plasma sample containing nicotine and cotinine (each 60 ng/ml) and processed as described in the text.

rendered specific detectors like alkaline flame ionisation or mass spectrometry unnecessary. The use of a precolumn as well as the relatively mild working conditions guaranteed a satisfactory lifetime of the capillary columns. Thus the GC system exhibited the high long-term stability needed for routine application.

The minimum detectable concentrations using the urine procedure were 0.03 μg/ml for nicotine or cotinine and 1.0 μg/ml for nicotine-1′-N-oxide. The recovery was at least 85% for the bases and about 60% for the N-oxides. The plasma-processing procedure was more sensitive, permitting the detection of about 5 ng/ml nicotine or cotinine and 15 ng/ml N-oxides. In this case the recovery was at least 80% for the bases and about 30% for the N-oxides. Calibration curves for the overall procedure, represented by linear regression equations, are given in Table I.

During extraction, especially in the case of plasma samples, extreme care has to be taken to keep the laboratory air free from nicotine. There is ample experimental evidence that even trace amounts of cigarette smoke in the laboratory air significantly increase the nicotine values.

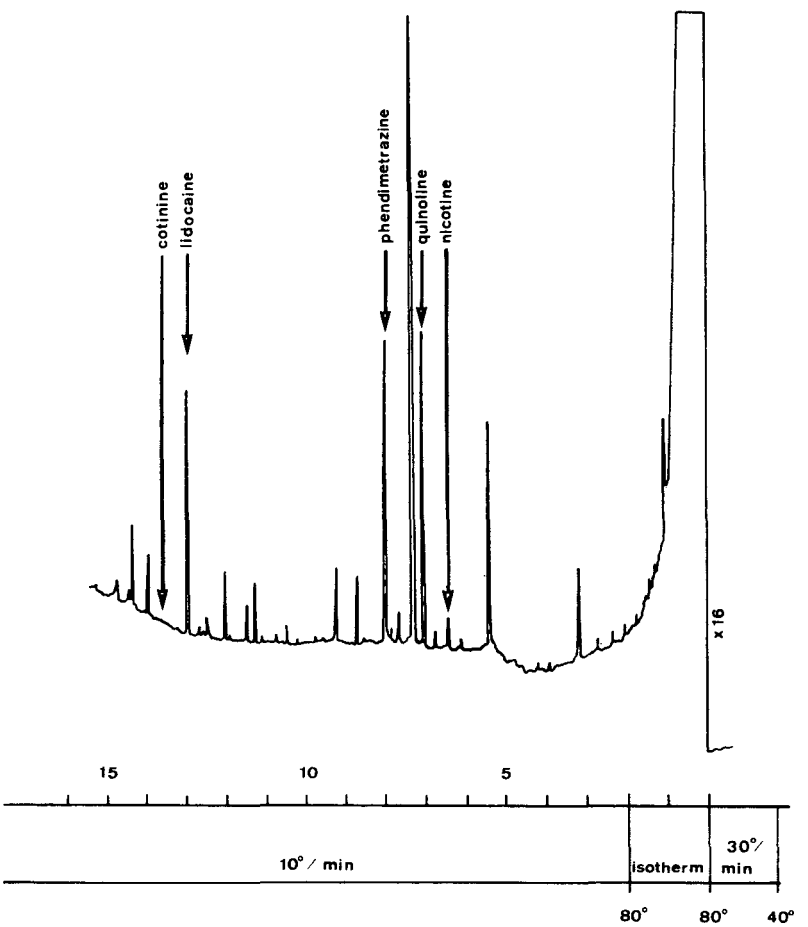

Fig. 5. Gas chromatogram of an extract of a 2-ml plasma blank taken from a non-smoker working in a smoking environment.

In samples reduced with SO_2 a broadening and/or retardation of the phendimetrazine peak and, to a lesser extent, of the nicotine peak was sometimes observed. This effect could be suppressed by the addition of 5 μl of concentrated NH_4OH to the 1-ml sample prior to GC analysis. NH_4OH prevents adsorption of alkaline compounds like nicotine on glass surfaces [19] and also neutralizes traces of SO_2 which might be present in the eluent.

The application of Extrelut considerably simplified the extraction procedure, thus substantially reducing the analysis time. The internal standard phendimetrazine-N-oxide, which is reduced and eluted under the same conditions as nicotine-1'-N-oxide or cotinine-1-N-oxide, allowed easy monitoring of the overall process. Moreover, only one internal standard mixture for both bases and N-oxides had to be added.

The solvent mixture used (diethyl ether—dichloromethane, 1:4, v/v) eluted nicotine as well as cotinine, thus excluding the uncontrolled cotinine losses which usually occur during solvent—solvent extraction. The Extrelut extraction

TABLE I

LINEAR REGRESSION OF COMPOUND/INTERNAL STANDARD PEAK HEIGHT RATIO AGAINST CONCENTRATION

Range: 0—15 μg/ml for urine, 0—250 ng/ml for plasma.

	Regression coefficient	Intercept on y-axis	Correlation coefficient
Urine			
Nicontine	0.6062	0.0075	0.9999
Cotinine	0.6660	—0.0070	0.0089
Nicotine-1'-N-oxide	0.3273	0.0020	0.9958
Cotinine-1-N-oxide	0.3450	0.0200	0.9980
Plasma			
Nicotine	0.0072	0.0233	0.9971
Cotinine	0.0101	—0.0154	0.9982
Nicotine-1'-N-oxide	0.0056	0.0240	0.9957
Cotinine-1-N-oxide	0.0064	0.0005	0.9957

allowed the determination of both the N-oxides independently from their bases in the same sample. The method is successful even for amounts of plasma as small as 2 ml.

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GAS CHROMATOGRAPHIC ANALYSIS OF KETAMINE AND NORKETAMINE IN PLASMA AND URINE: NITROGEN-SENSITIVE DETECTION*

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SUMMARY

A sensitive gas chromatographic method for quantitative analysis of ketamine and norketamine in human and animal biological fluids is described. The nitrogen-sensitive detection procedure used is more stable than electron-capture detection and reduced analysis time. The method used bromo-ketamine as an internal standard for quantitation and is linear from 10—25,000 ng/ml. No interferences were shown with drugs commonly associated with cardiac surgery with cardiopulmonary by-pass. This assay is sensitive, specific, using either native or derivatized drugs and can be used for routine analysis of ketamine and norketamine in plasma or urine.

INTRODUCTION

Ketamine (K), [2-(*o*-chlorophenyl)-2-(methylamino)] cyclohexanone, is used for induction and maintenance of anesthesia and analgesia [1, 2]. It is extensively metabolized in man and animals [1—4]. The present paper describes a sensitive and reliable gas chromatographic (GC) method for the quantitative assay of K and its major metabolite norketamine (NK) in plasma. Our single-dose pharmacokinetic studies of K require the measurement of

*Preliminary results reported in ref. 22.

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drug levels in the range of concentration (10—25,000 ng/ml) used in patients and animals. The method is linear over this range. Of the metabolites, we analyzed only NK, because it is the only one with proven significant pharmacologic activity [5—7]. 5,6-Dehydronorketamine was identified by GC—mass spectrometry (MS) [3, 4, 8--10] as a metabolite. This structure was questioned [11] and later evidence indicated that it was an artifact due to non-enzymatic dehydration of one or both isomers of the metabolite 5-hydroxynorketamine [12].

There are several assays for K in biological fluids [3, 4, 8—10, 12—19], some of which also measure NK and other metabolites [3, 4, 8—12]. But, it has been shown that in the routine analysis of K in biological fluids by a number of methods endogenous biological substances have often interfered [9, 10]. One group deemed it necessary to resort to GC—MS techniques which they compared to GC with electron-capture detection procedures [9, 10].

Our method utilizes nitrogen—phosphorus selective detection and has resulted in greater specificity, rapidity and linearity over the concentration range usually encountered in plasma; we optimized reaction conditions and used more suitable column materials than those employed previously [8]. The method was also adapted for the assay of K and NK excreted in urine; the extraneous biological material that is extracted with the organic solvent was largely eliminated by purification of the extract by thin-layer chromatography (TLC) prior to GC analysis.

EXPERIMENTAL

Standards and reagents

K, NK and bromo-ketamine, the internal standard (IS) (Fig. 1) were obtained as hydrochlorides, as a gift from Drs. A.J. Glazko and T. Chang (Parke-Davis, Ann Arbor, MI, U.S.A.). All solvents used were either glass-distilled or HPLC grade; glassware was treated prior to each use with 10% dimethyldichlorosilane (Aldrich, Milwaukee, WI, U.S.A.) in toluene by soaking

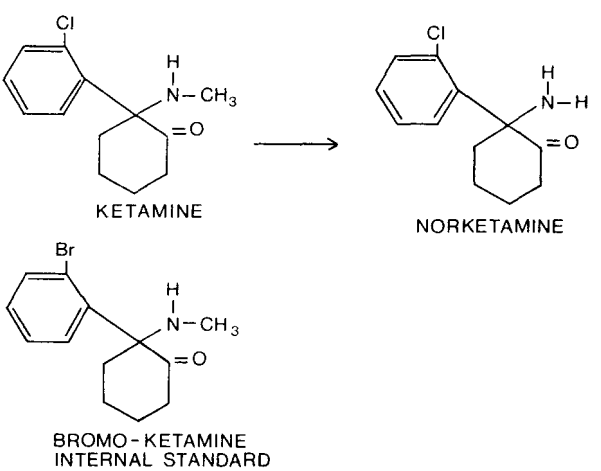

Fig. 1. Structures of ketamine, norketamine and the internal standard, bromo-ketamine.

overnight. After decanting, excess silane was removed with methanol and the tubes were dried at 110°C. Dimethylaminopyridine (DMAP) and heptafluorobutyric anhydride (HFBA) were purchased from Aldrich. Prior to first use and every six months DMAP was recrystallized with ethyl acetate and stored dry at 5°C. If DMAP becomes exposed to the atmosphere, it will decompose and cause interfering GC peaks. Solutions of DMAP and standards of K and analogue compounds were prepared monthly and stored at −20°C. To determine optimal conditions for the use of DMAP, standards of K, NK and IS were heated at different temperatures (45°C, 60°C and 80°C) and times (15–180 min) with HFBA to form the monoacyl derivatives. Identical experiments with pyridine [8] were carried out concurrently. Blood was collected in the presence of heparin in glass tubes or special Vacutainers (Becton-Dickinson, Rutherford, NJ, U.S.A.), fitted with stoppers without tris-2-butoxyethyl phosphate, TBEP; containing powdered Na_2EDTA, Lot OM 617. Plasma was obtained from blood by centrifugation at 500 *g* and was transferred into polyethylene capped tubes (Elkay Products, Shrewsbury, MA, U.S.A.) with a silanized borosilicate Pasteur pipet. Plasma and urine were stored at −70°C. Derivatized samples can be stored desiccated or in toluene at −20°C for at least six months.

Instruments

A 3711 Varian gas chromatograph equipped with a thermionic detector which is selective for nitrogen compounds was utilized. The parameters were: electrometer sensitivity, 10^{-12} A/mV; bead, 450; bias voltage, −4. Gas flow-rates were hydrogen, 4.6; air, 180; and carrier (nitrogen), 30 ml/min. Temperatures (Ultrabond 20M Carbowax column) were: injector 240°C, detector 250°C, and the column temperature was 180°C. The column temperature

TABLE I

RELATIVE RETENTION TIMES AND OPERATING TEMPERATURES FOR KETAMINE AND NORKETAMINE ANALYSIS AND THEIR HFBA DERIVATIVES

Column			Deriv-ative	Relative retention***		Retention (sec)	Correlation coefficient §
Type*	Temperature** (°C)			Ketamine	Norketamine	IS	(*r*)
	A	B					
Ultrabond	180	225	no	0.71	0.82	175	0.9956
	180	225	yes	0.75	0.60	278	0.9965
SE-30	155	275	yes	0.76	0.68	382	0.9992
OV-17	170	275	yes	0.68	0.36	585	0.9998

*Ultrabond 20M Carbowax, 100—120 mesh (Ultra, Mount Hope, RI, U.S.A.); SE-30, 2%, 800—100 mesh Chromosorb W HP; OV-17 2%, 100—120 mesh, Chromosorb W HP (Applied Science, State College, PA, U.S.A.). The bonded Carbowax had narrower peak widths, better symmetry and resolution than other stationary phases tested.

**A = Operating temperature; B = conditioning temperature.

***Relative to bromo-ketamine = 1.0.

§ Correlation coefficient of fit $y = mx + b$ for K (50—3000 ng/ml).

was maintained isothermally at 180°C for 8 min and then programmed to 225°C at 15°C/min and remained at the final temperature for 3 min. Data reduction was accomplished with a Spectra-Physics (Santa Clara, CA, U.S.A.) 4100 integrating microcomputer.

The dimensions of all columns were 1.8 m × 6.3 mm O.D., 2 mm I.D. The packings are given in Table I. In earlier experiments we also used 2% OV-17 and SE-30. Oxygen was excluded from the column to prevent deterioration. MS data were recorded on a Finnigan 3200 gas chromatograph—mass spectrometer in the chemical ionization mode. The column was 3% OV-17. The helium carrier gas flow-rate was 20 ml/min; column temperature, 180°C; electron energy, 70 eV; glass jet separator temperature, 210°C; reactant gas was methane.

Procedures

Blood (1—6 ml) was obtained from adults, children, monkeys and dogs given K. Blank specimens of blood and urine were obtained prior to the administration of K for control purposes. Analysis was carried out with plasma. The range of doses was 2—15 mg/kg. Plasma, 0.2—1.4 ml, was placed into a conical 13-ml ground glass centrifuge tube (Kontes, Vineland, NJ, U.S.A.). The appropriate amount of IS, in water (1 μg/ml), was added. The normal range of IS additions was 250—2000 ng per sample, added with a polypropylene Finn micro pipet. The IS usually was within 1/10 to 10 times the amount of the K and NK. Water was added to a total volume of 2 ml. The pH of the aqueous phase was brought to 9—10 with about 0.1 ml 0.1 *N* sodium hydroxide and 3 ml of *n*-heptane were added. In low level analysis, below 50 ng per sample, pH adjustments were made after the addition of heptane to prevent adsorption of the free bases. The mixture was shaken mechanically, 60 inversions per min for 15 min, and centrifuged at 500 *g* for 20 min at 5°C. The organic phase was transferred by a silanized pipet to a 5-ml centrifuge tube. The solvent was evaporated to almost dryness in vacuo with centrifugation (200-H Savant Speed-Vac, Hicksville, NY, U.S.A.). To ensure quantitative recoveries, two more serial extractions and evaporations were made. To the pooled material, 0.2 ml toluene was added in rinse fashion to concentrate the sample at the bottom of the tube and the mixture was evaporated to dryness in vacuo.

Derivative formation

The residue was dissolved in 1 ml of toluene and 0.1 ml of 0.1 *M* DMAP in toluene was added [20]. Then 20 μl of HFBA were introduced and the mixture was heated at 80°C for 2 h. Excess reagent and undesirable products were removed by two washes of 2 ml 5% ammonium hydroxide, followed by two washes of 2 ml of 1 *N* hydrochloric acid. Separation of phases after each wash was achieved by centrifugation at 500 *g* for 10 min. To the remaining organic phase, 1 ml of toluene was added in rinse fashion and the mixture was mixed and evaporated almost to dryness (Speed-Vac). The residue was dissolved in 10—50 μl toluene. It was then mixed by Vortex and 0.5—1.0 μl was injected into the gas chromatograph column.

For urine, the extract was purified by TLC before GC (Table II). Water,

TABLE II

THIN-LAYER CHROMATOGRAPHIC PARAMETERS

System A was used for purification of urine specimens prior to GC analysis; the appropriate zone was scraped and extracted twice with 2 ml warm methanol. The silica gel was separated by centrifugation; the pooled extracts were evaporated in vacuo. A separate side strip on the same plate was spotted with K and detected by exposure of the strip to iodine vapors in order to verify the migration of the drug. Radiochemical purification of [^{3}H]K was accomplished using System B (repeated three times), eluted as above, and evaporated to dryness with nitrogen. The residue was dissolved in 0.001 *M* hydrochloric acid—ethanolic solution. Purity was determined by all three Systems B, C and A.

System	Solvent	R_F value K + NK	TLC type*
A	Ethanol—methanol—water (6 : 6 : 1)	0.70	Silica gel 60
B	Ethanol—methanol—water (4 : 4 : 1)	0.81	Silica gel G
C	1,2-Dichloroethanol—ethanol (1 : 1)	0.77	Silica gel G

*Silica gel G was obtained from Supelco (Bellefonte, PA, U.S.A.) and silica gel 60 from E. Merck (Darmstadt, G.F.R.).

plasma or urine blanks from the experimental subjects and from drug-free volunteers were analyzed with each set of biological samples. Combined derivatized standards in toluene (at several concentrations; 50—2000 ng) were injected into the gas chromatograph at the beginning and end of runs. This was to check retention time and detector sensitivity. Recoveries were also analyzed with each set of samples. To verify the recovery of K and NK from biological fluids, blanks were spiked with aqueous standards of K, NK, IS and [^{3}H]K. These were randomly distributed among the unknown samples.

Synthesis of [^{3}H] ketamine · HCl

[^{3}H]K · HCl was synthesized by a modification of a published procedure [21]. K · HCl (50 mg) was labelled by the Wilzbach procedure (New England Nuclear, Boston, MA, U.S.A.). We carried out the purification as described [21], except that the exchange with 0.1 *N* sodium hydroxide at room temperature was carried out twice; others used heating with aqueous triethylamine [12]. Purification was carried out using TLC (Table II). The K carrier was added to the [^{3}H]K · HCl and it was recrystallized twice from ethanol. Purity was established by radio-TLC using three TLC systems (Table II). Radiochemical purity was greater than 99.3%. This was further confirmed by isotope dilution and high-performance liquid chromatography.

RESULTS AND DISCUSSION

Over 1000 plasma samples, mostly human and canine, were analyzed at least in duplicate by the described assay. The range of concentration was from 10—20,000 ng/ml. The calibration curves for K and NK extracted from plasma and urine were linear over the range studied (Table III). Reproducibility for

determination of K in plasma is given in Table IV. Details of the retention times and conditions are given in Table I; the coefficient of variation averaged 6% (Table IV).

TABLE III

CALIBRATION CURVES FOR K AND NK EXTRACTED FROM PLASMA AND URINE

Compound	IS (ng)	Range (ng/ml)	Regression equation $y = mx + b$*		r**	n
			m	b		
K	500	80—11,000	1.19	−0.037	0.9956	37
NK	500	10—700	0.761	−0.030	0.9965	41

*y = peak area ratio (K/IS and NK/IS); x = concentration ratio (K/IS and NK/IS).
**r = Correlation coefficient.

TABLE IV

REPRODUCIBILITY FOR DETERMINATION OF K IN PLASMA

Recovery over concentration range studied was 88 ± 3% (mean ± S.D., n = 8); precision was 4.0%

Added (ng/ml)	Found ± S.E.M.* (ng/ml)	S.E.M. (%)
50	54 ± 4	7.4
100	106 ± 7	6.5
150	153 ± 13	8.5
300	298 ± 6	2.0
450	423 ± 27	6.2
750	742 ± 38	5.3
1500	1530 ± 70	4.6
3000	3046 ± 126	4.1
		Average = 5.9

*S.E.M. = Standard error of the mean (n = 3).

Optimum conditions for derivatization were achieved with 40—60 min heating at 80°C; further heating for 68—180 min produced no significant change. Better yields and less interference with nitrogen detection and less deteriorization of the column were obtained with DMAP than with pyridine as the catalyst. Once derivatized, samples were stable for six months when kept dry and cold (−20°C). This is the first time DMAP was used as a catalyst for the formation of HFBA derivatives of K type compounds.

Recovery experiments using 100 ng of [^{3}H]K added to blank specimens of plasma and urine averaged 88%. This recovery is in agreement with the partition coefficient (K_p) > 100 for K and NK between aqueous buffer at pH 9 and heptane. With some plasmas containing high lipid concentrations and less than 150 ng/ml of K the recovery was not as complete; therefore, two extractions were needed. A number of drugs commonly used in a clinical anesthesia

were found not to interfere in the assays: morphine, diazepam, pancuronium and scopolamine. Quinidine partially co-chromatographed, but was minimally extracted. Propranolol and DMAP can interfere, if washes are omitted from either the derivatized or underivatized procedures. Blank equivalent for plasma and urine were less than 10 ng/ml. Specificity of the method was demonstrated by MS; it was shown that the individual peaks eluted with the retention times of K, NK, IS and their derivatives were due only to these individual compounds. MS data in the chemical ionization mode were the same as those published previously [4, 9, 10, 12]. The present method is a significant modification of a GC procedure with electron-capture detection [8]. By utilization of nitrogen-selective detection, it has been possible to achieve better linearity along with greater stability of the detector.

Two variants of the method have been used. For concentrations above 100 ng/ml the Ultrabond 20M column was suitable for underivatized samples. Below that level, the derivatization procedure should be used in order to increase the sensitivity and reduce low level noise commonly observed with

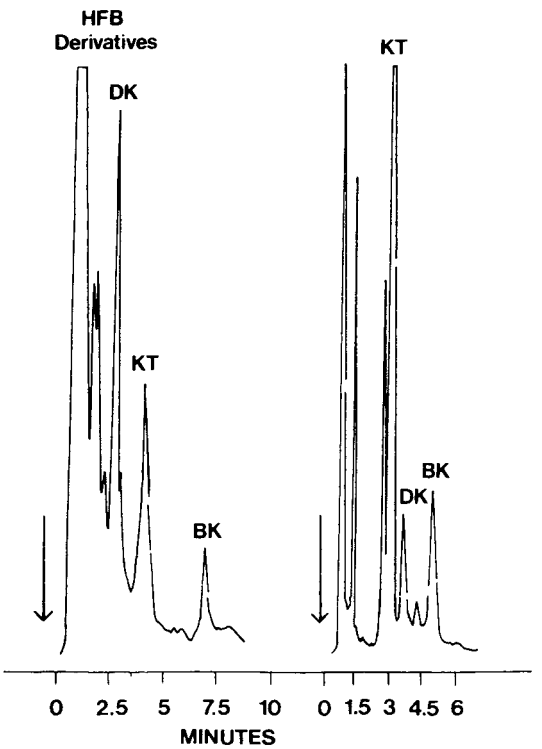

Fig. 2. (Left): gas chromatogram of an extract of plasma obtained from a dog (No. M63) given 10 mg/kg K (140 mg intravenously) not undergoing cardiac surgery with cardiopulmonary by-pass. (Right): gas chromatogram of an extract of plasma obtained from a patient (No. 6) given 2 mg/kg K (185.4 mg intravenously) and undergoing cardiac surgery with cardiopulmonary by-pass. Peaks: (left) DK = norketamine, 1025 ng/ml; BK = bromo-ketamine, 500 ng/ml; KT = ketamine, 1500 ng/ml; sample derivatized with HFBA. (right) DK = 185 ng/ml; KT = 1780 ng/ml; sample underivatized. Column: 20M Carbowax Ultrabond. Time in minutes.

biological samples. The derivatized samples are stable for at least six months.

While OV-17 and SE-30 columns provide separations of derivatized samples, the Ultrabond 20M column has several advantages. It gives still better resolution, results in symmetrical narrower peak widths, is more durable, and can be used without derivatization.

Attention was paid to the collection of blood samples. We had found that K is 20—45% bound to human plasma: 25% to 4% human albumin [22] and 28% to 0.1% alpha-1 acid glycoprotein. Also, TBEP is a constituent of stoppers of certain Vacutainer tubes and displaces K from the glycoprotein (unpublished results). The displaced K will penetrate red blood cells, thereby decreasing plasma levels, a falsely low plasma concentration is often obtained in the presence of TBEP [23—26].

Our studies of pharmacokinetics and distribution of K in man and animals employing the above-mentioned method have been described elsewhere [22, 27—29]. Typical data are given in Figs. 2, 3 and 4. The plasma concentration vs. time relationships in our patients were comparable to those found by other investigators [2, 3, 16]. The results in the dog were similar to those obtained with higher doses by Kaka and Hayton [30]. The method of analysis has also been applied by us to the study of the pharmacokinetics of K in infants and children (unpublished results). In these studies as little as 0.2 ml plasma (in some cases single specimens) were analyzed because of ethical limitations;

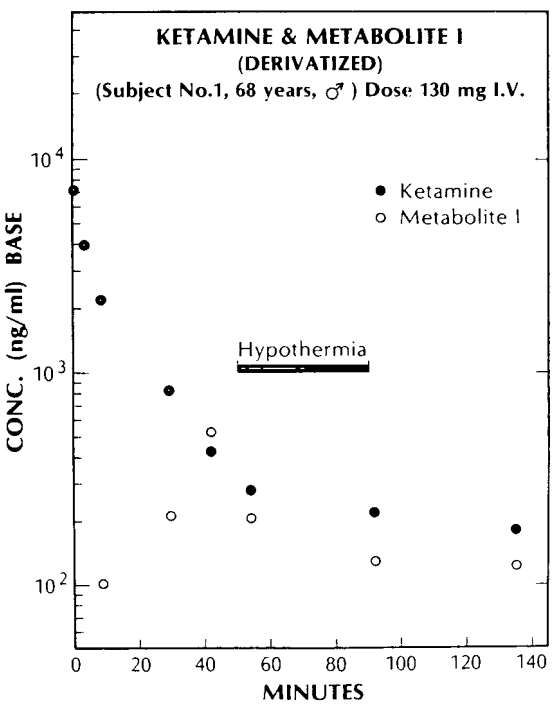

Fig. 3. Plasma concentrations of ketamine and norketamine (Metabolite I) in a patient over 2 h given 2 mg/kg (130 mg intravenously) ketamine, undergoing cardiac surgery with cardiopulmonary by-pass with hypothermia 55—90 min.

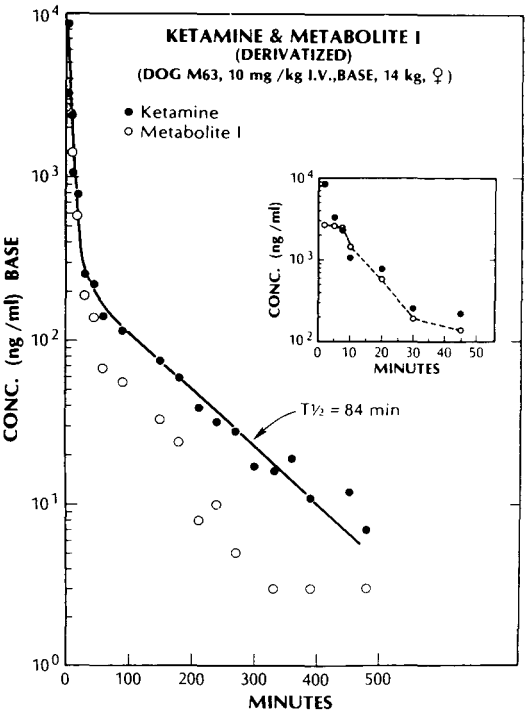

Fig. 4. Plasma concentrations of ketamine and norketamine (Metabolite I) in a dog over 6 h. Insert is for plasma levels for the first 45 min after the intravenous injection of K, 10 mg/kg samples were derivatized. Dose = 10 mg/kg intravenously of ketamine. Analyzed after derivatization. For abbreviations see Fig. 2.

the volume of blood withdrawn for analysis was less than 8% of the patients' estimated blood volume.

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PRE-COLUMN DERIVATIZATION WITH FLUORESCAMINE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS

APPLICATION TO TOCAINIDE

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SUMMARY

The quantitative determination of tocainide, a new antiarrhythmic agent, by high-performance liquid chromatography (HPLC) is reported. The drug and a chemically similar internal standard were extracted from blood plasma with acetonitrile under salting-out conditions obtained by saturation of the aqueous medium with sodium chloride—sodium carbonate. The organic extract, without evaporation, was treated with borate buffer (pH 8.2) and fluorescamine. The resulting derivatives were chromatographed on an ODS reversed-phase column using a methanol—phosphate buffer (pH 7.0) mixture as mobile phase and were detected fluorometrically by monitoring the emission at 485 nm, with excitation at 395 nm. The intra-assay coefficients of variation were 3.0 and 4.3% for ten replicate 0.25 and 1.00 μg/ml samples, respectively, and the inter-assay coefficient of variation was 3.6% for ten replicate 1.00 μg/ml samples. The procedure is simple, rapid, sensitive, and specific. Several other drugs and drug metabolites also were derivatized with fluorescamine and chromatographed successfully. Pre-column derivatization with fluorescamine followed by HPLC with fluorometric detection may have significant advantages in drug analysis.

INTRODUCTION

Tocainide (2-amino-2′,6′-propionoxylidide, Fig. 1), a new primary amine analogue of lidocaine, is an oral antiarrhythmic agent often effective in the treatment of chronic ventricular ectopy refractory to other modes of management [1]. Determination of the concentration of tocainide in blood serum is desirable during therapy since the therapeutic efficacy of the drug correlates with its concentration in serum [2], and is also needed in pharmacokinetic studies. Quantitative analysis of tocainide in biological fluids has been per-

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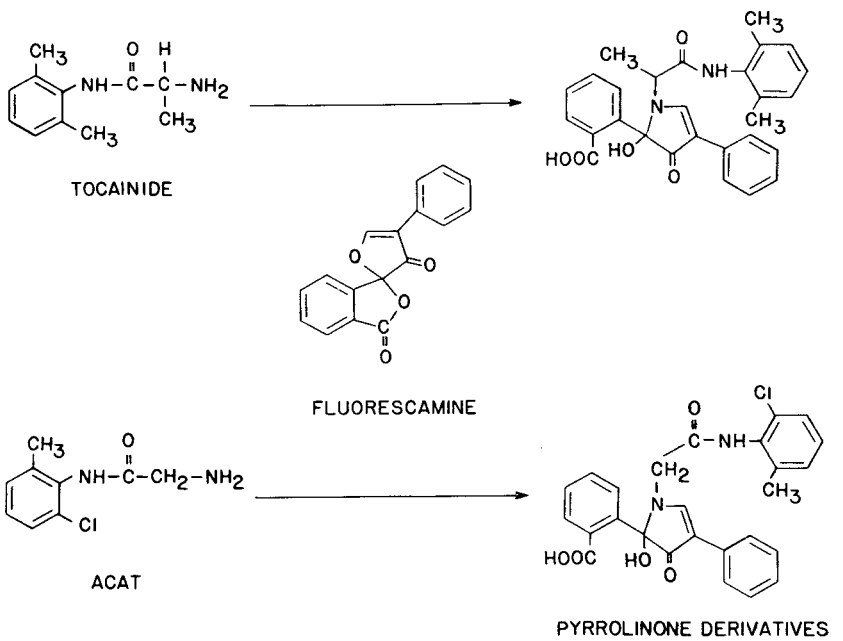

Fig. 1. The structures of tocainide and ACAT, and their reaction with fluorescamine.

formed with gas—liquid chromatography [3, 4] and with high-performance liquid chromatography (HPLC) [5—8]. The HPLC methods include the use of variable-wavelength detectors [5—7] and fluorescence detection [8].

An analytical method capable of determining the concentration of the drug at sub-therapeutic levels as low as 100 ng/ml was required for ongoing studies [9] on the disposition of tocainide. Two of the published HPLC procedures appeared to have the required sensitivity [6, 8] but neither method was suitable for our purposes: one procedure [6] required ultraviolet detection at 230 nm, not available to us, while the other [8], based on derivatization with dansyl chloride, is extremely elaborate and lengthy. Therefore we developed a new HPLC procedure for the determination of tocainide. The method is based on pre-column derivatization of the drug with fluorescamine followed by HPLC analysis with fluorescence detection. The assay is sensitive, simple, and rapid, and may have wide applicability in the analysis of drugs and drug metabolites possessing a primary amino group.

EXPERIMENTAL

Chemicals

Fluorescamine, the internal standard, 2-amino-6′-chloro-*o*-acetotoluidide (ACAT) hydrochloride, and 2,6-dimethylaniline were purchased from Aldrich (Milwaukee, WI, U.S.A.). Sulfamethoxazole, 5-hydroxytryptamine, procainamide, and *p*-chloroamphetamine were obtained from Sigma (St. Louis, MO, U.S.A.). Phentermine was purchased from Applied Science Labs. (State

College, PA, U.S.A.). The following compounds were kindly donated: tocainide (Dr. Robert Ronfeld, Astra Pharmaceuticals, Worchester, MA, U.S.A.); amphetamine and *p*-hydroxyamphetamine (Smith, Kline and French Labs., Philadelphia, PA, U.S.A.); amantadine (E.I. DuPont de Nemours Co., Wilmington, DE, U.S.A.); 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM) (Dr. Neal Castagnoli, Jr., School of Pharmacy, University of California); N-desisopropylpropranolol (Dr. John Thompson, School of Pharmacy, University of Colorado). Thyroxine was obtained from the Central Laboratory, University of Colorado Health Sciences Center, and *p*-methoxyamphetamine was synthesized as described previously [10].

Acetonitrile and methanol, distilled-in-glass grade, were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other reagents used were of analytical grade quality.

Working solutions

Fluorescamine was dissolved in acetonitrile to give a concentration of 3 mg/ml. Human plasma samples containing tocainide at several concentrations in the range of 0.1–20 μg/ml were prepared by appropriate dilutions of a 1.00 mg/ml stock solution. Other compounds to be derivatized with fluorescamine were dissolved in acetonitrile at 20—30 μg/ml. The derivatization buffer was saturated sodium borate, pH 8.2. The internal standard solution contained ACAT in acetonitrile at a concentration of 7.0 μg/ml.

Apparatus and conditions

The liquid chromatograph consisted of a Waters (Milford, MA, U.S.A.) Model 6000A pump, a Waters Model U6K injector, and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 204A spectrophotofluorometer equipped with a microflow cell.

Separations were carried out on a Beckmann Instruments (Berkeley, CA, U.S.A.) Ultrasphere ODS 5-μm 150 mm × 4.6 mm reversed-phase column. Chromatograms were recorded using a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3380A electronic integrator.

The mobile phase was prepared by diluting 425 ml of 0.067 *M* phosphate buffer, pH 7.0, to 1000 ml with absolute methanol, and was pumped at a flow-rate of 1.3 ml/min. The column effluent was monitored fluorometrically at an excitation wavelength of 395 nm and emission of 485 nm.

Solutions of tocainide and the internal standard at 5.0 μg/ml concentration in borate buffer (pH 8.2) were used to obtain the fluorescence spectra. The instrument was a Perkin-Elmer MFP-44A fluorescence spectrophotometer, operated at a sensitivity setting of 0.3 with excitation and emission slits set at 3.

Assay procedure

The plasma sample (500 μl) was placed in a 5-ml polypropylene centrifuge tube. An equal volume of internal standard solution was added, followed by ca. 200 mg of sodium chloride—sodium carbonate (4:1). The tube was capped and vortex-mixed for 60 sec, followed by centrifugation at 1700 *g* for 10 min. A 200-μl aliquot of the supernatant was transferred to another 5-ml centrifuge tube and an equal volume of borate buffer (pH 8.2) was added.

While vortex-mixing the contents of the tube, 50 μl of fluorescamine solution was added and the mixing was continued for 30 sec. A 30—50 μl aliquot of the solution was injected into the chromatograph. If a derivatized sample was not injected within 10 min after derivatization the tube was kept in ice until injection to minimize fluorophore degradation.

Quantitative evaluation of assay data

The tocainide:ACAT peak-area ratios were used to construct standard curves. The slope, intercept, and correlation coefficient were determined by linear least-squares regression analysis. The data were also analyzed by linear least-squares regression through the origin.

RESULTS

Fluorescence of derivatives

Fig. 2 shows the fluorescence spectra of the fluorescamine derivatives of tocainide and ACAT.

Chromatography

Fig. 3a shows the chromatogram of the fluorescamine derivatives of tocainide and ACAT. Analysis of a plasma sample from a patient receiving tocainide gave the chromatogram in Fig. 3b.

Recovery studies

Plasma samples containing 10.0 μg/ml tocainide were analyzed and the peak areas obtained for tocainide compared to those obtained from analysis of

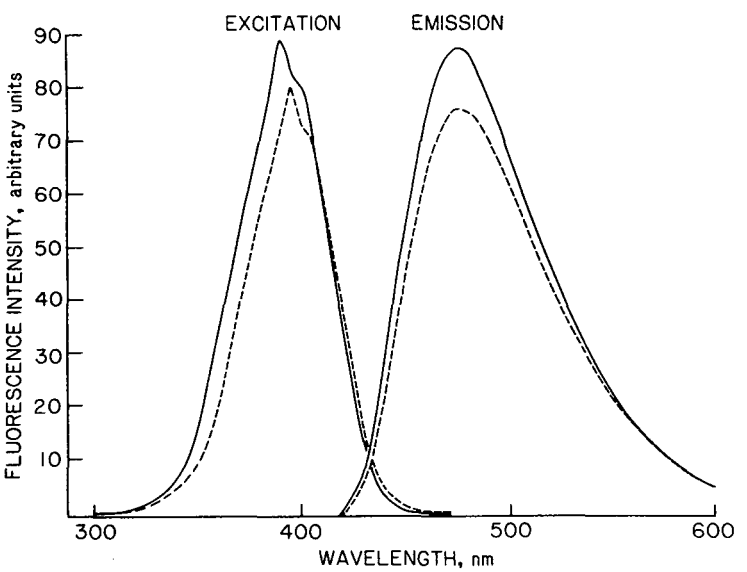

Fig. 2. Excitation—emission spectra of the fluorescamine derivatives of tocainide (—) and ACAT (– – –).

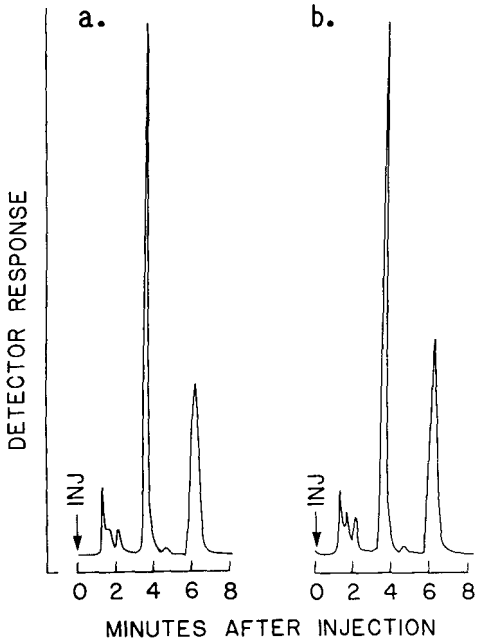

Fig. 3. Chromatograms of (a) tocainide and ACAT standards derivatized with fluorescamine; (b) derivatized extract of the serum of a patient receiving tocainide. Tocainide concentration 6.2 μg/ml. Retention times: ACAT, 3.4 min; tocainide, 6.0 min.

acetonitrile samples containing known, comparable, concentrations of the drug. The peak areas for ACAT were similarly analyzed. The recovery of both tocainide and ACAT was found to be 92 ± 5% ($n=5$).

TABLE I

TYPICAL CALIBRATION DATA*

Tocainide concentration (μg/ml)		Relative error (%)***
Amount added	Observed**	
0.100	0.109	+9.0
0.250	0.256	+2.4
0.500	0.514	+2.8
1.25	1.24	−0.8
2.50	2.52	+0.8
5.00	4.99	−0.2
10.0	9.85	−1.5
20.0	20.1	+0.5

*Least-squares line through origin; tocainide:ACAT peak area ratio = 168.1 (tocainide).
**From least-squares equation.
***[(observed − amount added)/amount added] × 100.

Assay linearity and precision

Standard curves were constructed by analyzing a series of plasma samples of known tocainide concentration. Each concentration was studied in triplicate. The data are given in Table I. The detector response was linear over the range of 0.1—20 μg/ml tocainide concentration. Linear least-squares regression analysis gave a line with an intercept which was not statistically different from zero. Therefore the standard curve through the origin was used in subsequent calculations.

Intra-assay precision was determined by analyzing a set of ten replicate plasma samples containing 0.25 μg/ml and a set containing 1.00 μg/ml tocainide. Inter-assay precision was determined by analyzing aliquots of a 1.0 μg/ml sample on ten separate days. The intra-assay coefficients of variation (C.V.) were 3.0 and 4.3% for the 0.25 and 1.00 μg/ml samples, respectively. The inter-assay C.V. was 3.6% for the 1.00 μg/ml samples.

Fluorescence intensity vs. pH of derivatization buffer

To determine the effect of pH in the derivatization medium plasma samples containing 1.0 μg/ml tocainide were analyzed using borate buffers of pH 7.5—10.0. The results are shown in Fig. 4. The effect of pH on the yield of the fluorescent derivatives of tocainide and ACAT is small.

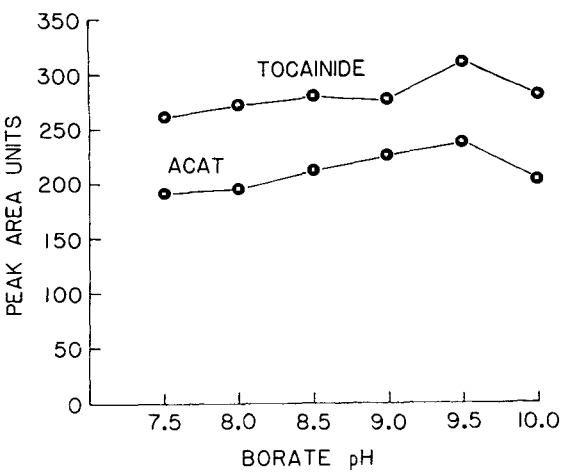

Fig. 4. Effect of pH of derivatization medium on the yield of the fluorescent derivatives of tocainide and ACAT. Each point is the mean of triplicate determinations.

Stability of fluorescence

Solutions of the fluorescamine derivatives of ACAT and tocainide were kept at room temperature and at 0°C to examine their stability. Several aliquots of each solution were injected into the chromatograph over a period of 5 h. A ca. 10% reduction in the tocainide peak area was observed at room temperature after 1 h. The rate of disappearance was slightly lower for ACAT, and increased with increasing pH for both derivatives. The decrease in peak area for both tocainide and ACAT was negligible at 0°C for 2 h.

Specificity

Serum samples containing no tocainide were carried through the analytical procedure without the addition of ACAT, and no interference from endogenous components was found. Propranolol, salicylate, and quinidine, all underivatized, gave no response. Dopamine, methyldopa, cycloserine, and tranylcypromine, all primary amines, gave no identifiable derivatives in the analysis. The hydrazine derivatives isoniazid, pheniprazine and 1,1-dibenzylhydrazine also gave no response. A series of other primary amines (Table II, see below) gave fluorescamine derivatives which did not interfere with the analysis of tocainide.

Fluorescamine derivatives of other drugs

A series of thirteen other primary amino compounds of pharmacological significance were derivatized with fluorescamine, and the derivatives were chromatographed under the conditions optimized for the derivatives of tocainide and ACAT. The results are shown in Table II. The chromatography

TABLE II

RETENTION TIMES OF THE FLUORESCAMINE DERIVATIVES OF SOME PRIMARY AMINE DRUGS AND DRUG METABOLITES

Chromatographic and derivatization conditions are given in the Experimental section.

Compound derivatized with fluorescamine (identification number)	Retention time of derivative (min)
Sulfamethoxazole (1)	1.5
5-Hydroxytryptamine (2)	2.1
Procainamide (3)	3.1
2,6-Dimethylaniline (4)	5.0
p-Hydroxyamphetamine (5)	5.6
Phentermine (6)	5.7
p-Methoxyamphetamine (7)	6.4
p-Chloroamphetamine (8)	8.8
Thyroxine (9)	8.8
N-Desisopropylpropranolol (10)	9.0
1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane (11)	9.1
Amphetamine (12)	11.8
Amantadine (13)	18.9

of the fluorescamine derivatives of N-desisopropylpropranolol (10) was also monitored at the excitation wavelength of 295 nm and emission of 350 nm, conditions suitable for the fluorometric analysis of propranolol and closely related compounds. No response was obtained.

DISCUSSION

Fluorescamine (4-phenylspiro(furan-2[3H],1′-phthalan)-3,3′-dione, Fig. 1) was synthesized ca. ten years ago [11]. This remarkable reagent, itself non-fluorescent, reacts rapidly with primary amines to give highly fluorescent

pyrrolinone derivatives (Fig. 1), and excess reagent is rapidly converted by water to non-fluorescent products [12, 13]. The reagent quickly found application in automated amino acid determinations [14, 15] and in protein [16] analysis. Subsequently, the use of fluorescamine was extended to a wide range of biophysical and biochemical applications. Fluorescamine has also been used in various analytical procedures for drugs. Interest in this area has produced detailed studies on the nature of the reaction of pharmaceuticals with fluorescamine and the factors affecting the reaction [17, 18]. It was shown [19] that conditions can be found for the selective reaction of aromatic amines with fluorescamine in the presence of aliphatic amines, and this finding prompted the development of an assay for procainamide in blood plasma [20]. Since this procedure does not involve chromatographic separation of the derivatives, interference from other aromatic amines may occur, and this was recognized by the authors [20]. Fluorescamine has been applied in the thin-layer chromatographic analysis of some therapeutic agents, drugs of abuse, and toxic substances [17, 18, 21–25].

Advances in HPLC in recent years have resulted in the use of fluorescamine to enhance the detection sensitivity of this technique. Analyses of antibiotics using post-column derivatization have been described [26–29], but pre-column derivatization of drugs followed by HPLC separation of the derivatives has received little attention. Only two reports have appeared on the pre-column derivatization and LC analysis of drugs. One of these described the quantitative determination of aminocaproic acid in serum [30], and the other concerns the analysis of the antidepressants clovoxamine and fluvoxamine in plasma [31].

For the determination of tocainide as its fluorescamine derivative by HPLC, ACAT (Fig. 1) was selected as the internal standard. This compound is a primary amine with a chemical structure closely related to that of tocainide. ACAT is readily available from a commercial source, is inexpensive, and has extraction and chromatographic properties compatible with those of tocainide.

The procedure developed for the isolation of tocainide and the internal standard from the biological fluid is based on saturation of the aqueous medium with sodium chloride–sodium carbonate and extraction into acetonitrile. This solvent is normally miscible with water, but becomes immiscible under the salting-out conditions used. This procedure, using different salt mixtures, was recently shown to have advantages in the isolation of several acidic and neutral drugs for HPLC analysis [32, 33]. With the incorporation of sodium carbonate the extraction of basic compounds was readily achieved in our procedure, and the recovery of tocainide and ACAT was nearly quantitative. The extraction procedure gave "clean" chromatograms, and appeared superior to the procedure used by De Jong [31], in which no extraction was performed and the serum samples were treated directly with fluorescamine. Significant interference from endogenous serum components was found under such conditions.

The extracts containing tocainide and ACAT were treated with fluorescamine in a procedure patterned after published derivatization methods [34, 35]. In our procedure, fluorescamine was added in acetonitrile instead of acetone [31, 34, 35]. Since the reaction between primary amines and fluores-

camine is extremely rapid [12], Schiff's base formation between the amine and acetone cannot compete with the derivatization reaction in most cases. Nevertheless, we selected acetonitrile as the solvent since it has been shown to be suitable for the derivatization [13] and since it was more convenient than acetone due to its lower volatility.

It has been suggested that pH 8—8.5 is optimal for the derivatization of aliphatic amines [13]. We examined the effects of pH in the derivatization medium on the fluorescence intensity of the tocainide and ACAT derivatives (Fig. 4). In the range of pH 7.5—10.0, the variation was small, with an apparent peak at pH 9.5. Since the decomposition of the fluorophores was more rapid at higher pH the derivatization pH selected was 8.2.

We recommend that the derivatization reaction be carried out immediately before HPLC analysis. The prepared derivatives may be stored, cooled in ice, for several hours. At room temperature the fluorescence intensity decreases significantly in 1—2 h. It has been stated that the fluorophores formed from primary amines and fluorescamine are stable for several hours [12], but a systematic study of their stability at neutral and alkaline pH has not been reported. Under sufficiently acidic conditions the fluorescence intensity of the derivatives decreases, and this has been shown to be the result of acid-catalyzed lactone formation between the hydroxy and carboxy groups of the derivatives [36].

Pre-column derivatization has significant advantages since it obviates the need for the more complex conditions and equipment required by the post-column derivatization procedures.

The fluorescamine derivatives were chromatographed on a reversed-phase ODS column, and gave good peak shapes (Fig. 3). The pH of the mobile phase was 7.0, a value compatible with both the stationary phase and the fluorescence properties of the derivatives [13]. The fluorescence spectra (Fig. 2) of the fluorescamine derivatives of tocainide and ACAT were very similar to those of the derivatives of other aliphatic primary amines [13, 17, 18]. Based on the spectra, 395 and 485 nm were selected as the excitation and emission wavelengths, respectively.

The product of the reaction of fluorescamine with racemic tocainide (Fig. 1) contains two asymmetric centers and, therefore, two diastereomeric derivatives are formed. There was no hint of separation of the diastereomers (Fig. 3). This may be due to unsuitable chromatographic conditions, or to a rapid epimerization around the carbinolamine chiral center [37].

A sample size of 0.5 ml is used in the assay procedure. If a smaller size is desired, the method may be scaled down, since only a small portion of the final derivatization mixture is injected into the HPLC. The procedure displayed good intra- and inter-assay reproducibility. Since the procedure is specific for primary amines, a variety of other, non-primary amine, drugs potentially present in patients receiving tocainide do not interfere. Amino acids are not extracted under the conditions used. Salicylic acid, a fluorescent compound, is also not extracted. Quinidine and propranolol do not interfere, since their fluorescence and chromatographic properties are different from those of the fluorescamine derivatives. In summary, the combination of specificity for primary amines, organic solvent extraction, specific fluorometry, and

chromatographic separation assures that many endogenous and exogenous compounds do not interfere. On the other hand, potential interference from primary amines must be carefully evaluated.

Therapeutically effective plasma concentrations of tocainide are in the 3.5–10 μg/ml range [2]. The lowest concentration of the drug to be measured in our pharmacokinetic studies is 100 ng/ml, and therefore the analytical procedure was not evaluated below this concentration. With appropriate modifications, e.g. concentration of the extracts, lower limits of sensitivity may be achieved. The procedure by De Jong [31], for example, is capable of determining the concentration of clovoxamine in blood plasma in the range of 10—1000 ng/ml, and has a limit of detection of 3 ng/ml. It must be noted, however, that the sensitivity attainable is also a function of the fluorescence quantum yield of the specific derivative.

Many therapeutic agents and other xenobiotic compounds are primary amines. Furthermore, many non-primary amine compounds are metabolized to primary amine derivatives via such biotransformations as N-dealkylation, reduction of azo, nitro, nitroso and hydroxylamino groups, and hydrolysis of amides, isocyanates and carbamates. Clearly, fluorescamine could play a significant role in the analysis of such metabolites. We have derivatized a series of pharmacologically significant primary amines with fluorescamine, and have chromatographed the derivatives (Table II). The chromatographic conditions were those used in the analysis of tocainide, and no attempt was made to optimize the chromatography for the compounds listed in Table II. Both aromatic (1, 3, 4) an aliphatic (2, 5–13) amines, including an amino acid (9) were studied. Several licit (5, 6, 8) and illicit (7, 11) derivatives of amphetamine (12) were included. 2,6-Dimethylaniline (4) is metabolite of lidocaine [38] formed by amide hydrolysis. N-Desisopropylpropranolol 10 is a N-dealkylated metabolite of propranolol [39]. The fluorescamine derivative of (10) was non-fluorescent at wavelengths normally used in the analysis of propranolol and its derivatives [40, 41]. It is clear that in drug disposition studies in which analytical specificity for a primary amine drug or drug metabolite is needed, precolumn derivatization with fluorescamine combined with liquid chromatography may have significant advantages.

1,1-Dibenzylhydrazine and the monosubstituted hydrazines isoniazid and pheniprazine were derivatized with fluorescamine, but derivatives could not be identified by HPLC with fluorescence detection. More unexpectedly, neither cycloserine nor tranylcypromine, both primary amines, gave an identifiable derivative. This may be due to unsuitable chromatographic conditions, although we believe this explanation is unlikely, since a wide variety of compounds gave derivatives readily chromatographed (Table II). Alternatively, the chemical structures of the two amines may give rise to derivatives with fluorescence properties different from those of other amines. It has been observed [17] that some primary amines produce non-fluorescent derivatives with fluorescamine. The catecholamines dopamine and methyldopa also failed to give identifiable derivatives. This failure was most likely due to the presence of borate in the medium, since it has been observed that borate suppresses the fluorescence of the fluorescamine derivatives of catecholamines [34].

In summary, the analytical method described possesses simplicity, speed,

sensitivity and specificity which make it very attractive for the analysis of tocainide, and, potentially, for many other primary amine drugs and drug metabolites.

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CHROMBIO. 1382

DETERMINATION OF SALBUTAMOL IN HUMAN PLASMA WITH BIMODAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND A ROTATED DISC AMPEROMETRIC DETECTOR

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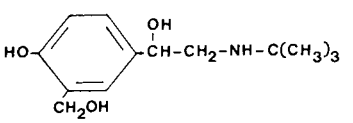
SUMMARY

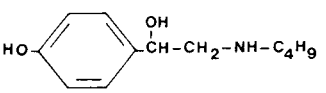
The sensitivity of electrochemical detection was combined with the selectivity of a bimodal high-performance liquid chromatographic system for the successful determination of salbutamol in human plasma. Following initial sample clean-up using Sep-Pak® cartridges, analytes were passed first through a cation-exchange column, and then, after column switching, through a reversed-phase column. An amperometric detector with a rotated disc working electrode was used for detection. The detection limit was 0.5 ng/ml when 1.0 ml of plasma was used. The coefficient of variation was 9.8% at an average concentration of 4.7 ng/ml. The method was adequate for pharmacokinetic studies and for clinical applications.

INTRODUCTION

Salbutamol, 2-*tert.*-butylamino-1-(4-hydroxy-3-hydroxy-methylfenyl)-ethanol (Fig. 1) is a sympathicomimetic drug with a selective effect on β-2-adrenoceptors. Its pharmacological action is well documented [1], and as a bronchodilator the drug is widely used in the treatment of respiratory diseases.



salbutamol



bamethan

Fig. 1. Chemical structure of salbutamol and of bamethan.

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However, pharmacokinetic information on salbutamol is restricted, apparently due to the rather limited analytical techniques currently available for its determination [2, 3]. Nevertheless, the pharmacokinetics of this drug could be of clinical relevance in various disease states and in connection with problems concerning its first-pass metabolism [2]. The high-performance liquid chromatographic (HPLC) method presented in this paper may facilitate investigations in this field.

MATERIALS AND METHODS

Chemicals

All aqueous solutions were prepared with double-distilled water.

Salbutamol sulphate and bamethan sulphate were kindly supplied by Glaxo (Hoofddorp, The Netherlands) and Boehringer Ingelheim (Alkmaar, The Netherlands), respectively. All other chemicals were of analytical reagent grade and obtained from Merck (Darmstadt, G.F.R.). They were used as received.

Aqueous solutions of standards were prepared containing 0.5 μg/ml salbutamol or 1.0 μg/ml bamethan. For purposes of preservation these solutions were slightly acidified with formic acid (± 0.01%, v/v).

Sep-Pak® cartridges were from Waters (Etten-Leur, The Netherlands). Spektralkohle Ringsdorff RW-A (Ringsdorff-Werke, Bonn–Bad Godesberg, G.F.R.) was used for the preparation of carbon paste.

Sep-Pak manipulation

Sep-Pak cartridges were connected to 5-ml disposable PVC syringes, from which the plunger had been removed, to serve as an eluent reservoir. The outlets of the cartridges were fixed to 10-ml reagent tubes as recipients for the eluate, leaving a vent for the air to be displaced (Fig. 2). In this form twelve cartridges could be handled in a Sorvall centrifuge in one run.

Chromatographic system

A schematic representation of the chromatographic system is given in Fig. 3. Eluents were delivered by two Kipp 9208 HPLC pumps (Kipp Analytica, Emmen, The Netherlands). Valve 1 was a Rheodyne 7120 injection valve, equipped with a 1.0-ml loop. Valve 2 was a Rheodyne 7000 six-port rotatory valve. Column 1 (50 × 4.6 mm) was home-packed with Partisil SCX (10 μm). Packing was performed by suction of a Partisil SCX suspension in water into the empty column at a vacuum bottle. Column 2 (250 × 4.6 mm) contained LiChrosorb RP-2 (10 μm) and was obtained as a prepacked column from Brownlee Labs. (Santa Clara, CA, U.S.A.). The composition of the eluent solutions was as follows. Eluent 1: phosphate buffer (pH 7.5) containing 2.58 g of $Na_2HPO_4 \cdot 2H_2O$ and 0.3 g of KH_2PO_4 per liter. Eluent 2: sodium perchlorate (40 g/l) and 2-propanol (45 ml/l) in a phosphate buffer (pH 7) containing 14.0 g of $Na_2HPO_4 \cdot 2H_2O$ and 7.5 g of KH_2PO_4 per liter. The flow-rate of pump 2 was 2.0 ml/min.

Electrochemical detection

The electrochemical detector used in this method has been described earlier

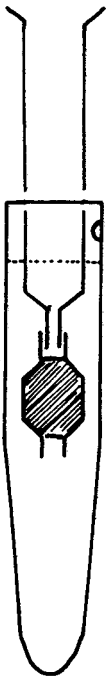

Fig. 2. Construction for the handling of Sep-Pak columns in a centrifuge.

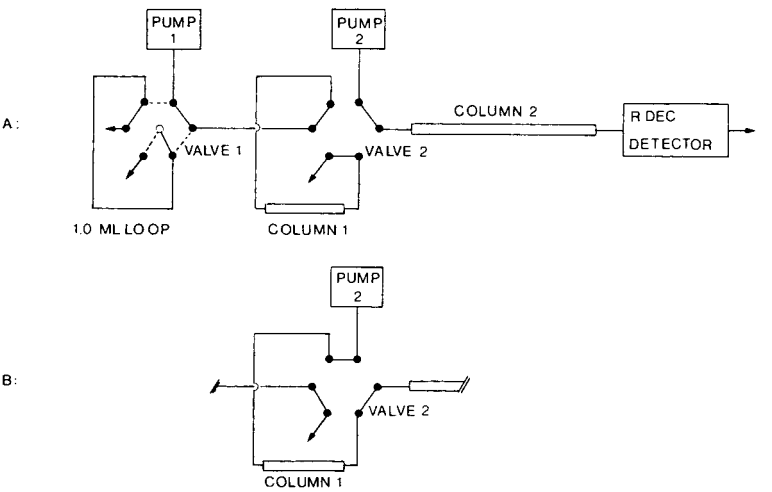

Fig. 3. Schematic representation of the bimodal HPLC system.

[4]. It differs from more common types of electrochemical detectors in that the working electrode is a rotated disc electrode. Carbon paste was used as the electrode material. The paste was prepared by mixing 15.0 g of graphite powder with 9.0 g of nujol [5]. A saturated calomel electrode (SCE; K 401, Radiometer, Copenhagen, Denmark) was used as the reference electrode and a platinum wire as the auxiliary electrode.

The three-electrode potentiostat was home-made and comparable in performance with commercially available instruments. The potential of the working electrode was maintained at +950 mV vs. SCE.

Each time the system was started up, the electrode was initially conditioned at a potential of +1600 mV vs. SCE for 15 min with eluent 2 flowing.

Sample preparation

In small glass tubes plasma samples (1.0 ml) were spiked with 20 μl of the internal standard solution.

Before use Sep-Pak columns were washed with 5.0 ml of methanol followed by 5.0 ml of water. The spiked samples were transferred with pasteur capillary pipettes and passed through the prepared columns. Sample tubes and pasteur pipettes were rinsed with 1.0 ml of water, which was then passed through the columns. The columns were then centrifuged at 1500 *g* for 10 min. The aqueous eluates were discarded and the columns transferred to clean glass tubes, to be eluted with 5.0 ml of a mixture of methanol—diethyl ether (25:75). The eluates were evaporated under nitrogen at about 40° C.

Calibration curves for salbutamol in the range 2.5—20 ng/ml were obtained by adding 5—40 μl of the standard solution ot 1.0 ml of blank human plasma samples and utilizing the above procedure.

Bimodal HPLC procedure

The residue from the Sep-Pak procedure was redissolved in 500 μl of eluent 1. Of this solution 250 μl were injected via valve 1 while valve 2 was in position A and the flow-rate of pump 1 was set at 3.0 ml/min. After about 5 min pump 1 was stopped. Valve 2 was switched to position B when the previous chromatogram was finished. When the solvent front appeared, valve 2 was switched back to position A and pump 1 was started again. A further sample could then be injected.

RESULTS

Representative chromatograms of plasma samples obtained with the method are shown in Fig. 4. When the elution time for column 1 was chosen correctly no interfering peaks were observed. Blank human plasma from several pools was tested in this respect.

The constancy of the detector response towards a test mixture containing salbutamol and bamethan was examined during a four-day period and a coefficient of variation of 6.9% was found (n = 8). The average of seven calibration plots made during a nine-day period was described by the equation $y = 0.0769x + 0.0213$. The standard deviations of the slope and intercept were 0.0063 and 0.00022, respectively. The coefficient of correlation ranged from 0.9987 to 0.9999 with a mean of 0.9994.

The standard deviation of the method was calculated from duplicate measurements using the formula S.D. = $\sqrt{d^2/2n}$, in which d is the difference between the duplicates and n is the number of duplicates. Thus the coefficient of variation was found to be 9.8% (n = 13) at an average concentration of 4.7 ng/ml.

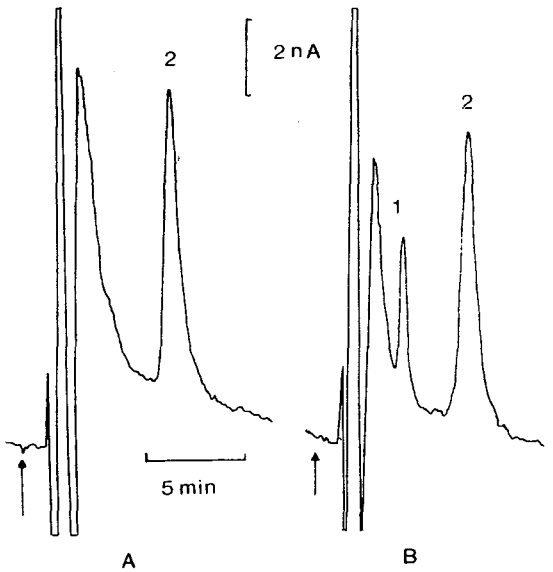

Fig. 4. Chromatograms of a blank plasma sample (A) and a plasma sample containing 5.8 ng/ml salbutamol (1 = salbutamol, 2 = internal standard).

The signal-to-noise ratio indicates a detection limit of about 0.5 ng/ml plasma.

The recovery of salbutamol and the internal standard was in all instances between 95 and 101%.

Ten plasma samples from a healthy volunteer after oral administration of 8 mg of salbutamol were analysed with the present method and a newly modified gas chromatographic—mass spectrometric (GC—MS) method in which salbutamol was converted to its trimethylsilyl derivative [3, 6]. The correlation plot of the GC—MS results (y) vs. the results of the present method (x) is described by the equation $y = 1.0002x + 1.02$ ($r = 0.9925$; $n = 10$; concentration range 4—25 ng/ml), the GC—MS method consistently giving somewhat higher values (Table I).

TABLE I

RESULTS OF THE PRESENT HPLC METHOD COMPARED WITH GC—MS

Sample time (h)	HPLC result (ng/ml)	GC—MS result (ng/ml)
0 (blank)	Not detectable	Not detectable
1.5	13.6	13.0
2.0	8.8	10.4
2.5	8.1	10.0
3.0	12.6	14.0
3.5	22.7	23.9
4.0	17.3	18.4
5.0	11.8	12.3
7.5	7.0	8.3
12.0	3.8	5.5

No interference with our method was observed from the following drugs when they were given to patients whose salbutamol levels were monitored: theophylline, prednisone, beclomethason, furosemide, triamterene, atenolol, methyldopa, acenocoumarol and nitrazepam.

A plasma concentration—time curve for a patient who received 2 mg of salbutamol orally is shown in Fig. 5.

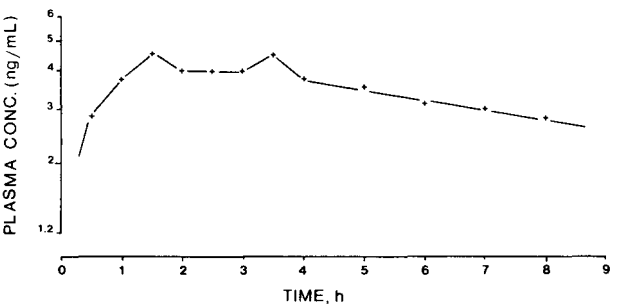

Fig. 5. Semilogarithmic plot of plasma concentration vs. time in a patient who received 2 mg of salbutamol orally at $t = 0$.

DISCUSSION

Due to the low therapeutic concentrations of salbutamol a sensitive detection method is required for its determination. Since the salbutamol molecule contains a phenolic hydroxyl group, electrochemical detection was a viable choice.

Preliminary voltammographic experiments with a rotated disc carbon-paste electrode showed in principle sufficient sensitivity (Fig. 6).

An anodic shift of the half-wave potential was observed with decreasing pH. This shift was expected as protons are liberated upon oxidation of the phenolic hydroxyl group. Therefore a pH of 7.0 was chosen for eluent 1, being near the upper limit allowed by the silica-based column material. The rotation speed of the electrode was so chosen that the current was under kinetic control [5].

When a constant mixture containing salbutamol and related drugs having a resorcinol group (orciprenaline and fenoterol) was injected repeatedly into column 2 over a longer period of time, it was observed that the detector response ratio of salbutamol towards the other compounds changed with the lifetime of the electrode. Hence it was concluded that in order to obtain reliable quantitative results the internal standard should contain an electroactive group very similar to that of salbutamol. This requirement was fulfilled by bamethan which also has a phenolic hydroxyl group.

The described pretreatment of the electrode at +1600 mV resulted in an improved sensitivity of the electrode, and reduced the conditioning period. The lifetime of an electrode varied from one to several weeks. A high electrode potential was required for the oxidation of salbutamol (Fig. 6). Thus selectivity had to be sacrificed for the purpose of sensitivity.

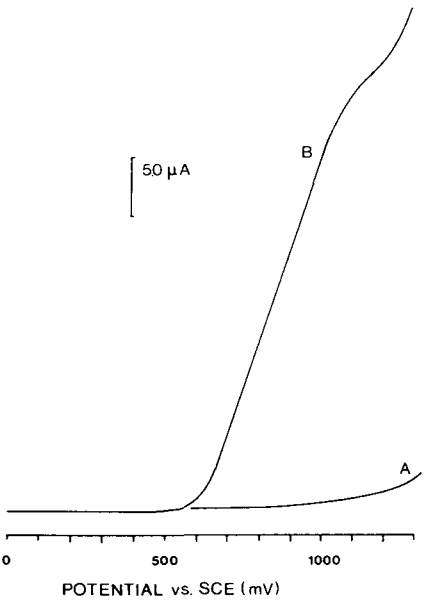

Fig. 6. Voltammograms as obtained with a rotated disc carbon-paste electrode. (A) pH 7.0 phosphate buffer; (B) pH 7.0 phosphate buffer containing 100 µg/ml salbutamol. (Rotation speed approx. 2000 rpm, scanning rate 5 mV/sec.)

Since it appeared very difficult to gain adequate selectivity with conventional sample preparation techniques, including ion-pair extraction [7], we have investigated the feasibility of mode-sequencing HPLC. Principles and applications of this concept have been reviewed recently [8]. In the approach used, compounds of interest remain on the ion-exchange column during elution with eluent 1, due to their high capacity factor in this system. Elution focusing is achieved on transferring the analytes to the reversed-phase column as a result of the high ionic strength of eluent 1. Resolution from an interfering compound in plasma was increased by ion-pair formation of the analytes with perchlorate ion [9]. After approximately every 40 injections column 1 needed to be repacked.

As salbutamol was eluted earlier than bamethan with eluent 1, its capacity factor on column 1 was determined at intervals by connecting the efflux of column 1 to the detector.

Our bimodal HPLC procedure can easily be automated with commercially available components. As sample pretreatment (column 1) and analysis (column 2) occur at the same time, five to seven samples can be analysed per hour.

The selectivity of the chromatographic system allowed direct injection of plasma samples after deproteinisation with perchloric acid and neutralization with sodium bicarbonate. However, a poor recovery was obtained due to co-precipitation of the analytes with plasma proteins. Furthermore, in the Sep-Pak procedure not only proteins were removed, but also electrolytes that would cause band spread on the ion-exchange column. Evaporation of the organic eluate from the Sep-Pak columns was facilitated as the aqueous eluate was almost quantitatively removed by centrifugation. Sep-Pak columns were reused

approximately 15 times with no measurable loss in performance.

The difference between the results obtained with GC—MS and the present method is not explained. However, we feel that the lower values found with our method (consistently about 1 ng/ml difference over the whole concentration range) cannot be due to standard solution errors, interfering peaks or recovery problems.

The above-described method can easily be modified for the assay of related drugs such as fenoterol and terbutaline, thus providing useful and relatively simple methods for pharmacokinetic studies of β-sympathicomimetics in man.

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CHROMBIO. 1391

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF IBUPROFEN AND ITS MAJOR METABOLITES IN BIOLOGICAL FLUIDS

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(First received February 16th, 1982; revised manuscript received June 21st, 1982)

SUMMARY

A sensitive and selective high-performance liquid-chromatographic assay for ibuprofen and its major metabolites in biological fluids is described. To ensure good chromatographic separation the drug and metabolites were run on a gradient elution system and detected with a variable wavelength detector set at 220 nm. A second, more rapid, isocratic system is also described for the detection of only ibuprofen.

INTRODUCTION

Ibuprofen [2-(4-isobutylphenyl)propionic acid] is an orally administered, non-steroidal anti-inflammatory agent used extensively in the treatment of arthritis. The literature concerned with the biochemical and toxicological studies carried out in man and animals has been reviewed [1]. The metabolism of ibuprofen in man and several animal species is documented [2]. Major metabolites in man are 2-[4-(2-hydroxy-2-methylpropyl)phenyl]-propionic acid (OH-ibuprofen) and 2-[4-(2-carboxypropyl)phenyl]propionic acid (COOH-ibuprofen). Among the techniques employed to quantify ibuprofen and its metabolites in biological samples are paper chromatography [3], gas—liquid chromatography (GLC) with prior derivatization [4], GLC with electron capture detection [5], GLC—mass spectrometry combinations [6] and high-performance liquid chromatography (HPLC) [7—10]. To the authors' knowledge all the HPLC assays reported for ibuprofen measure only the parent compound. The ability to quantify metabolite levels in urine and plasma can greatly aid in drug metabolism and pharmacokinetic studies. It was therefore considered appropriate to develop an HPLC assay capable of detection of

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ibuprofen and its major metabolites (Assay I). A second, more rapid assay for ibuprofen alone was also developed (Assay II).

MATERIALS AND METHODS

Standards

Ibuprofen, OH-ibuprofen, COOH-ibuprofen, and methyl prednisolone were provided by the Upjohn Company (Kalamazoo, MI, U.S.A.). Tolmetin was provided by McNeil Laboratories (Fort Washington, PA, U.S.A.)

HPLC apparatus for the simultaneous determination of ibuprofen, OH-ibuprofen and COOH-ibuprofen in biological samples, Assay I

Due to the more highly polar nature of the two metabolites relative to the parent compound, isocratic elution was found to be unsatisfactory and a gradient system had to be employed. The apparatus used consisted of two Waters Model 6000A pumps (Waters Assoc., Milford, MA, U.S.A.) controlled by a Model 660 solvent programmer, a Waters variable wavelength detector set at 220 nm and an Omniscribe chart recorder (Houston Instruments). Separation was achieved with a prepacked Whatman column (Whatman, Clifton, NJ, U.S.A.) (25 cm × 4.5 mm I.D., Partisil 10 ODS-3 packing). The eluents delivered by the two pumps were of the following compositions. Eluent A: acetonitrile—water (28:72). To each liter of eluent was added 500 μl of phosphoric acid and 500 μl of acetone. Eluent B: acetonitrile—0.05 *M* monobasic potassium phosphate (50:50). The delivery rate of the mobile phase was 2 ml min^{-1} and a typical run was performed in the following manner. The column was allowed to come to equilibrium with eluent A. At time zero the sample was loaded onto the column through a Waters loop injector. After 8 min the solvent programmer was switched from these initial conditions to the "run" mode. Over the next 6 min (i.e. 8—14 min after the injection) the percentage of eluent A in the eluting solvent was reduced from 100% to 0% in a linear manner with respect to time while the percentage of eluent B increased from 0% to 100%. Thus, from 14 min after the injection to the termination of the run the eluting solvent was 100% B. Fig. 1c graphically represents the change in eluent composition with respect to time.

HPLC apparatus for the determination of ibuprofen in biological samples, Assay II

The basic HPLC equipment used for Assay II was similar in all respects to that used in Assay I. However, due to the isocratic nature of the assay, the solvent delivery system consisted of a single pump. The mobile phase was methanol—water (70:30) with 1 ml of phosphoric acid added to each liter of eluent. The mobile phase was delivered at a flow-rate of 2.5 ml min^{-1}.

Sample preparation for Assay I

Assay I was primarily used to assay urine samples for unchanged drug and metabolites. A simple clean-up extraction was required before urine samples could be chromatographed. A 1-ml sample of urine or diluted urine was added to a screw-topped test tube. A 100-μl aliquot of internal standard solution

(methylprednisolone 1 mg/ml) was added followed by 1 ml of 1.5 *M* hydrochloric acid to reduce the pH below 1. Finally, 500 μl of water and 10 ml of methylene chloride were added and the tubes were capped and shaken for 20 min. The tubes were then gently centrifuged (250 *g*, 3 min, Sorvall RC3 centrifuge). The lower organic phase was transferred to a clean, dry test tube and was evaporated to dryness under a stream of prepurified nitrogen at 40° C. Samples were reconstituted with 200 μl of methanol and 10—30 μl of this were injected onto the column.

To enable free and conjugated metabolites to be assayed in the urine, all samples were assayed twice. Free drug and metabolites were assayed as described above. Total drug and metabolites (free plus conjugated) were assayed following alkaline hydrolysis. Hydrolysis was carried out by incubating 1 ml of urine or diluted urine with 500 μl of 1 *M* sodium hydroxide solution for 20 min at room temperature. Following this period the extraction was carried out as described above.

Sample preparation for Assay II

Assay II was primarily used to assay plasma samples for unchanged drug. Plasma samples were subjected to a clean-up extraction before being chromatographed. To 1 ml of plasma in a screw-topped test tube were added 100 μl of internal standard solution (tolmetin, 100 μg/ml internal standard solution). The samples was acidified with 500 μl of 1 *M* hydrochloric acid and 10 ml of methylene chloride were added. The tubes were capped, shaken for 10 min and then centrifuged at 1000 *g* for 5 min to ensure complete phase separation. The lower, organic layer was transferred to a clean, dry test tube and was evaporated to dryness under a stream of prepurified nitrogen at 40° C. Just prior to analysis the residue was redissolved in 200 μl of Assay II eluent and 10—30 μl of this sample were loaded onto the column.

Calibration procedure for Assay I

Blank urine was spiked with ibuprofen, OH-ibuprofen and COOH-ibuprofen in the range 5—200 μg ml^{-1}. The urine samples were subjected to the preparation procedures described above and were chromatographed in the normal manner. Peak heights of ibuprofen, metabolites and internal standard were measured from the resultant chromatograms. A peak height ratio (peak height of compound divided by peak height of internal standard) versus concentration curve was constructed for the parent drug and metabolites. The peak height ratios of unknown samples were compared to this standard curve, corrections being made for any dilutions involved.

Calibration procedure for Assay II

Blank plasma was spiked with ibuprofen over the range 1—140 μg/ml. Plasma prepared in this way was subjected to the normal extraction and chromatographic procedures. From the chromatograms obtained the ibuprofen:tolmetin peak height ratio was calculated and a calibration curve relating this ratio to the plasma concentration of ibuprofen was constructed. Unknown samples were quantified by reference to this standard curve.

RESULTS AND DISCUSSION

A typical HPLC trace for Assay I is shown in Fig. 1a. The relative order of peak retention and retention times were: OH-ibuprofen (8.0 min), COOH-ibuprofen (10.2 min), methylprednisolone (13.6 min) and ibuprofen (21.8 min). No interfering peaks were observed when blank urine was subjected to the assay (Fig. 1b). Calibration data (compound peak height ratio versus compound concentration, $\mu g\ ml^{-1}$) were best fitted by the power curve described by the equation

$$\ln Y = S\ln X + \ln D \quad (1)$$

where X and Y are concentration and peak height ratio, respectively. The relationship between X and Y was actually linear since S was essentially equal to unity. However, the use of eqn. 1 gave the lowest coefficient of variation of inversely estimated concentrations, apparently as a result of the different weighting of the points. The results of inversely estimating ibuprofen and metabolite concentrations from the calibration data generated from standards prepared over several months are shown in Table I. No systematic bias was observable over the concentration range studied. The intra-day reproducibility of Assay I is good, as is shown in Table II. Five independently prepared samples at three concentrations were run for each compound. The highest coefficient of variation (C.V.) observed was less than 14%, two other

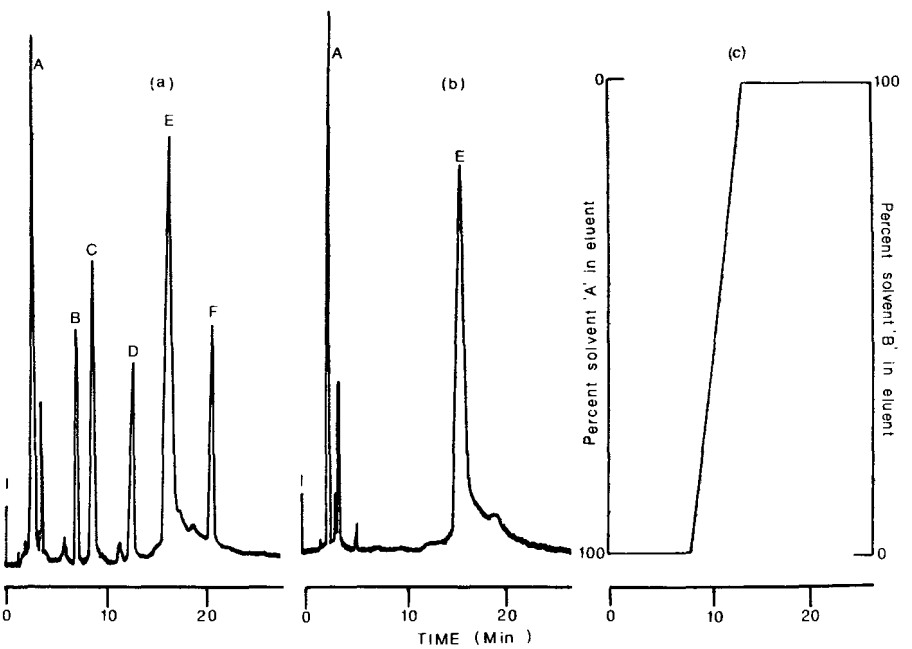

Fig. 1. (a) A typical chromatogram of ibuprofen and its metabolites extracted from urine. I = Injection; A = solvent front and highly polar contaminants; B = OH-ibuprofen; C = COOH-ibuprofen; D = methylprednisolone; E = low polar contaminants; F = ibuprofen. Blank urine (b) shows no interfering peaks. The change in eluent composition as a function of time is shown in part c.

TABLE I

PERCENTAGE THEORETICAL IBUPROFEN, OH- AND COOH-METABOLITE CONCENTRATIONS OBTAINED FROM THE URINARY CALIBRATION CURVES (ASSAY I)

Data presented in this table was collected over several months. Each inversely estimated concentration is the mean of at least 15 independently prepared calibration points assayed over this time span.

Ibuprofen concentration (μg/ml)	7.0	14.0	28.0	56.1	84.1	112.2	140.2
Mean (%)	102.2	98.9	100.6	105.5	98.6	93.7	107.6
Bias (%)	2.2	−1.1	0.6	5.5	−1.4	−7.3	7.6
C.V. (%)	13.6	15.1	14.6	11.6	10.2	10.5	8.3
OH-metabolite concentration (μg/ml)	9.1	18.3	36.6	73.2	109.7	146.3	184
Mean (%)	99.6	102.8	104.9	104	98.7	92.2	106.7
Bias (%)	−0.4	2.8	4.9	4	−1.3	−7.8	6.7
C.V. (%)	16.4	19.7	16.8	12.2	10.6	13.1	9.7
COOH-metabolite concentration (μg/ml)	9.3	18.6	37.2	74.5	111.7	149.0	186
Mean (%)	100.5	98.1	110.8	100.0	97.3	97.2	104.4
Bias (%)	0.5	−1.9	10.8	0	−2.7	−2.8	4.4
C.V. (%)	18.6	14.5	20.7	9.01	11.0	17.7	10.4

C.V. values were just greater than 10% while the remaining six were less than 10%. Urine spiked with ibuprofen and metabolites at 30 μg/ml was divided into 1-ml aliquots and frozen. On each assay day one urine aliquot was thawed and assayed. The stability of frozen samples and the inter-day reproducibility is shown in Table III. No trend towards sample degradation is seen but, as may have been expected over such a protracted period of time, slightly larger coefficients of variation are observed.

The lower limit of detection of Assay II as described for ibuprofen and its metabolites (around 5 μg ml^{-1}) was sufficient to enable detection of 1.0% of a 400-mg dose of ibuprofen in 1 l of urine. For the purposes for which the assay was used this proved to be adequate. If greater sensitivity is desired, extraction from a larger urine volume, or a decrease in internal standard and injection of a larger volume of the final extract would prove satisfactory.

A typical HPLC trace from Assay II is shown in Fig. 2a. The relative order of peak retention and retention times were tolmetin 3.2 min, and ibuprofen 7.7 min. No interfering peaks in blank plasma were observed (Fig. 2b). Calibration data were again fitted by a power curve described by eqn. 1. A summary of the calibration data obtained from 22 pooled calibration curves is summarized in Table IV. Again no systematic bias was noted over the concentration range studied. The intra-day reproducibility of Assay II is good as assessed by assaying six independently prepared plasma samples at three different concentrations (Table V). The coefficients of variation observed (all less than 10%) are of the same order as those reported by Kearns and

TABLE II

THE INTRA-DAY REPRODUCIBILITY OF ASSAY I

Each peak height ratio was obtained from an independently prepared calibration sample; all samples were assayed on the same day.

OH-metabolite concentration (μg/ml)	183	73.2	9.1
Peak height ratio	2.49	1.22	0.186
	2.87	1.30	0.182
	2.65	1.15	0.194
	2.88	1.20	0.179
	3.25	1.10	0.179
Mean	2.83	1.20	0.184
C.V. (%)	10.1	6.5	3.4
COOH-metabolite concentration (μg/ml)	186	74.5	9.3
Peak height ratio	2.08	0.876	0.150
	2.78	0.885	0.136
	2.09	0.886	0.137
	2.58	0.906	0.137
	2.18	0.808	0.117
Mean	2.34	0.872	0.135
C.V. (%)	13.5	4.3	8.7
Ibuprofen concentration (μg/ml)	140	56.1	7.0
Peak height ratio	2.83	1.19	0.134
	2.45	1.28	0.162
	2.74	1.09	0.158
	2.55	1.05	0.158
	2.95	0.999	0.147
Mean	2.70	1.121	0.152
C.V. (%)	7.4	10.4	7.5

TABLE III

INTER-DAY REPRODUCIBILITY OF ASSAY I AS INDICATED BY QUALITY CONTROL SAMPLES

	OH-Ibuprofen	COOH-Ibuprofen	Ibuprofen
Theory (μg/ml)	36.6	37.2	28.0
Range (μg/ml)	31.8—41.3	28.9—42.0	21.9—37.6
Mean (μg/ml)	36.5	36.8	28.2
S.D. (%)	3.1	4.0	4.9
C.V. (%)	8.4	10.8	17.3

Wilson [10] although the range of concentrations reported here is much greater. Plasma spiked with ibuprofen at 30 μg/ml was divided into 1-ml aliquots and frozen. On each assay day one of these quality control samples was thawed and assayed. The results are presented in Table VI. The low

Fig. 2. (a) A typical chromatogram of ibuprofen extracted from plasma. I = Injection; A = solvent front and highly polar contaminants; B = tolmetin; C = ibuprofen. Blank plasma (b) showed no interfering peaks.

TABLE IV

PERCENTAGE OF THEORETICAL IBUPROFEN CONCENTRATION ESTIMATED FROM STANDARD CURVES FROM ASSAY II

Data summarized in this table were collected over several months. Each inversely estimated mean is the result of at least 22 independently prepared calibration points assayed over this time span.

Ibuprofen concentration (μg/ml)	1.4	2.8	7.0	14.0	42.1	105	140
Mean (%)	106	95.7	105.9	100.3	98.1	101.5	100.4
Bias (%)	6	−4.3	5.9	0.3	−1.9	1.5	0.4
C.V. (%)	21.1	13.9	14.8	13.6	7.4	11.3	5.8

TABLE V

THE INTRA-DAY REPRODUCIBILITY OF ASSAY I

Each peak height ratio was obtained from independently prepared samples; all samples were assayed on the same day.

Ibuprofen concentration (μg/ml)	140	14	2.8
Peak height ratio	10.53	1.39	0.253
	10.20	1.36	0.264
	10.11	1.38	0.258
	11.36	1.30	0.273
	11.48	1.17	0.281
	10.97	1.11	0.279
Mean	10.77	1.28	0.268
C.V. (%)	5.4	9.2	4.3

TABLE VI

INTER-DAY REPRODUCIBILITY OF ASSAY II AS INDICATED BY QUALITY CONTROL SAMPLES

Theory (μg/ml)	30
Range (μg/ml)	25.9—34.4
Mean (μg/ml)	29.1
S.D. (%)	3.3
C.V. (%)	11.3

coefficient of variation (11.3%) indicates good stability of frozen plasma samples and reproducibility of the assay over several months.

The applicability of the assays reported in a clinical study has been demonstrated. Fig. 3a presents the mean plasma concentration—time curves obtained

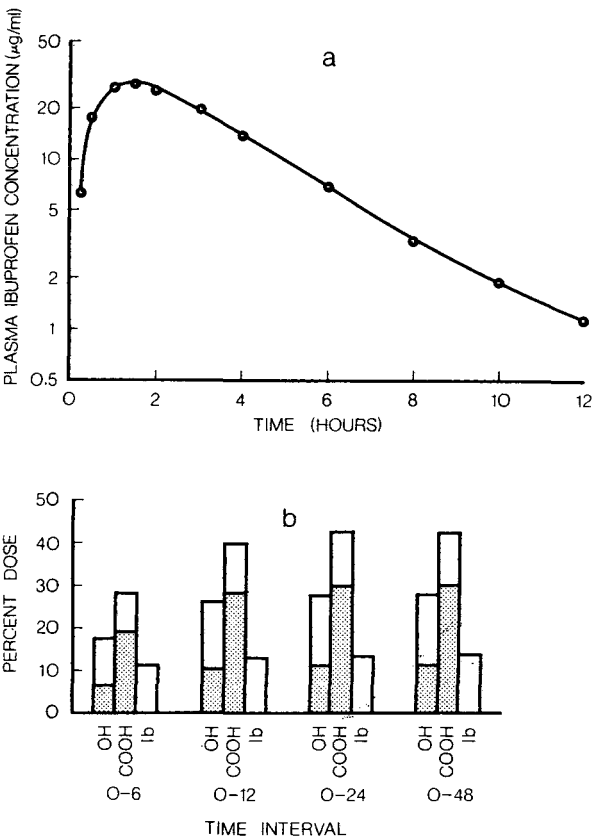

Fig. 3. A demonstration of the applicability of Assays I and II to pharmacokinetic studies. (a) Plasma concentration—time curve of ibuprofen after oral administration of a 400-mg tablet. (Each point is the mean of 15 subjects.) (b) Cumulative percentage of ibuprofen and its two major metabolites excreted in the urine after a 400-mg dose of ibuprofen. The larger bars represent the total percentage excreted in the measuring period. The smaller, shaded bars represent the percentage of free drug or metabolite excreted in the same interval. (All values are the means of 15 subjects.)

from 15 subjects after a 400-mg dose of ibuprofen orally. Assay II as reported here was ideally suited for following these plasma profiles since the concentration range encountered over the study was 110—1 μg/ml. It was generally considered to be unnecessary to lower the assay limits; however, since only about 1/10th of the reconstituted sample was injected on column the theoretical limits of the assay as described could easily be reduced to 0.1 μg/ml, thus making this assay comparable to or better than that of Ali et al. [9].

Urinary excretion data from the same study are presented in Fig. 3b to demonstrate the applicability of Assay I. Urinary recovery as assayed by this new HPLC method is in good agreement with literature values. It should be noted that Assay I is applicable to plasma samples but that the concentrations of metabolite present are nearly always at the limits of detection.

CONCLUSION

Assays have been developed to quantify ibuprofen or ibuprofen and its major metabolites in biological fluids. Adequate sensitivity and reproducibility of calibration data have been demonstrated. The applicability of the described assay methods has been shown. In future papers the applications of these assays to biological samples (plasma and urine) obtained in a four-phase clinical study involving ibuprofen will be discussed.

ACKNOWLEDGEMENT

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MEASUREMENT OF PLASMA MELPHALAN AT THERAPEUTIC CONCENTRATIONS USING ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive isocratic high-performance liquid chromatographic (HPLC) method for the measurement of melphalan in plasma is presented. It requires an extraction step using columns of XAD-2 resin before injecting the clarified methanol eluate directly into the HPLC system. The HPLC system uses an isocratic mobile phase containing an ion-pair reagent, and a sensitive fixed-wavelength (254 nm) monitor with a noise specification of $<2 \cdot 10^{-5}$ absorbance units peak to peak.

The concentration of melphalan was followed in a patient with multiple myeloma on day 1 and day 4 of a four-day course of the drug. Little difference was detected between the two curves with terminal half-lives of 71 and 68 min respectively and areas under the curve of 1.08 and 1.15 min $\cdot \mu$g/ml $\cdot$ (mg dose)$^{-1}$.

INTRODUCTION

Since melphalan [4-bis(2-chloroethyl)amino-L-phenylalanine; L-phenylalanine mustard; L-PAM] was synthesised by Bergel and Stock [1], it has been used with considerable success in treating multiple myeloma [2, 3]. It has also been used in treating carcinoma of the ovary, breast and testis [4]. Only recently, however, have there been methods available to measure melphalan in biological samples after the administration of therapeutic doses. Mass spectrometry has been used by a number of groups [5—7] but this technique is not readily available for routine analysis.

High-performance liquid chromatography (HPLC) has been used to measure melphalan in the mouse and dog [8] and in man [9, 10]. However, none of

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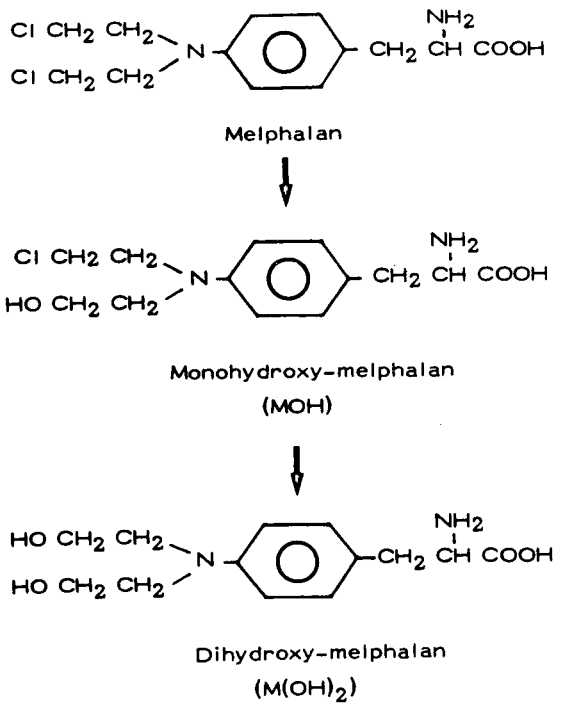

Fig. 1. Spontaneous degradation of melphalan in aqueous media.

these HPLC methods is capable of measuring plasma levels of the drug after normal oral therapeutic doses (5—15 mg) because peak plasma concentrations of melphalan are sometimes only 40—60 ng/ml [7, 11].

Despite the fact that a number of metabolites of melphalan have been identified in animals after intravenous and oral administration [12], none have been identified in man to date [5, 13, 14]. The drug does, however, degrade spontaneously yielding two hydrolysis products, monohydroxy-melphalan (MOH) and dihydroxy-melphalan [$M(OH)_2$; Fig. 1], although neither of these degradation products has cytotoxic activity [15].

This paper describes a sensitive assay for melphalan in plasma using a simple HPLC method. The separation of the biological material remaining after extraction from melphalan and an internal standard is accomplished by incorporating in the mobile phase a negatively charged ion-pair reagent (sodium dodecyl sulphate). This forms an ionic association (at low pH) with the positively charged amino group on melphalan giving a virtually hydrophobic ion-pair and the greater retention time necessary for separating it from the biological peaks. The method has been used to quantify the absorption of melphalan in multiple myeloma patients, after normal therapeutic doses were administered orally [11], and this work has led to the suggestion that melphalan should be taken after a meal to obtain greatest absorption [11].

EXPERIMENTAL

Materials

Melphalan was a generous gift from Burroughs Wellcome (Beckenham, Great Britain) and general labelled [^{3}H]melphalan was obtained from the Radiochemical Centre (Amersham, Great Britain). Sodium dodecyl sulphate and XAD-2 were purchased from BDH (Poole, Great Britain). Dns-arginine [DA, Nα(5-dimethylaminonaphthalene)-1-sulphonyl)-L-arginine], similar to Dns-proline [9] but eluting in a better position, was obtained from Sigma (Poole, Great Britain) and used as an internal standard. All materials were used as received except for the XAD-2 which was washed thoroughly with acetone and then with methanol until the absorbance (at 254 nm) of the washings was less than about 0.1 a.u. $M(OH)_2$ was prepared by the method of Furner et al. [8].

HPLC instrumentation

A dual reciprocating pulseless pump (Constametric IG, Laboratory Data Control (LDC), Stone, Great Britain) was used together with a Rheodyne injection valve (with a 200-μl loop), a 250 × 4.6 mm reversed-phase column packed with Spherisorb ODS (5 μm) and kept to 40 ± 1°C with a block heater (Jones Chromatography, Cardiff, Great Britain). A fixed-wavelength (254 nm) UV III monitor (LDC) with 0.002 absorbance units full scale (a.u.f.s.) maximum sensitivity was used rather than the possible alternative, the variable-wavelength Spectromonitor (LDC) set to a wavelength of 263 nm (melphalan's absorption maximum), due to the much reduced noise factor of the former. However, a new fixed-wavelength monitor did cause considerable problems for a time, when noise caused by a new type of digital display ruled out all sensitive work [16].

HPLC mobile phase

The mobile phase was made up by mixing one volume of 0.675 g/l solution of sodium dodecyl sulphate with four volumes of methanol and adjusting to approximately pH 3.0 with concentrated sulphuric acid (10—15 drops per 2.5 l; final concentration about 1.8 m*M* sulphuric acid). While the positions of the peaks seen in blank plasma extracts were virtually unaffected by pH, the positions of DA and melphalan were very dependent, and so final pH adjustment of the mobile phase was made by dropwise addition of acid. After each addition of acid, the mobile phase was used to chromatograph melphalan and DA to check whether the peaks were in satisfactory positions. The mobile phase was filtered through a 0.45-μm filter and degassed by ultrasonication prior to use.

Calibration

Every day the high-performance liquid chromatograph was calibrated with at least two 200-μl injections of a reference mixture of 0.4 μg/ml melphalan and 1.67 μg/ml DA in mobile phase. Any deviation from the expected peak heights and distances suggested faults in mobile phase or the chromatograph. The ratio (DA/melphalan) of the responses (measured in mm peak height at 0.002 a.u.f.s.

per ng material) was termed the response ratio.

The sample manipulation procedures were checked by extracting plasma samples containing known amounts of added melphalan and DA. Three standards were used with every extraction of one blank and eleven unknown plasma samples: 20 ng/ml melphalan, 200 ng/ml melphalan, and also a sample containing 200 ng/ml melphalan and 500 ng/ml DA to which no further DA was added. The results from these standards were used to calculate extraction efficiencies and thence a DA/melphalan extraction ratio.

Sample preparation

Patient management has been described previously [11], and plasma samples were stored at −40°C before analysis. As simple precipitation of protein [8, 9] was found to leave too much UV absorbing material in solution, extraction of the plasma was first carried out using XAD-2 resin. Columns of washed XAD-2 (25 × 5 mm, volume = 0.5 ml) were prepared in 5-ml pipettes and washed with 2 volumes (approx. 10 ml each) of acetone, 2 volumes of HPLC grade methanol and 2 volumes of degassed distilled water. DA (500 ng) was added to plasma samples (1.0 ml) and the mixtures applied to the columns. As soon as the plasma had run through, the column was washed with 1 volume of degassed distilled water, and the melphalan and DA eluted off with 1.5 ml methanol. Any slight precipitate was removed either by filtration through a 0.45-μm polytetrafluoroethylene filter or by microcentrifugation at 12,000 *g* for 3 min. The clarified eluate was stored at −40°C until 200-μl aliquots could be injected directly into the chromatograph.

Calculation

DA and melphalan peak heights were measured and a melphalan/DA peak height ratio (M/DA PHR) calculated. Melphalan concentrations were then calculated using the DA/melphalan response ratio (DA/M RR) and the DA/melphalan extraction ratio (DA/M ER; see Calibration) using the equation

$$\text{Concentration of melphalan (ng/ml)} = \frac{\text{M}}{\text{DA}}\ \text{PHR} \cdot \frac{\text{DA}}{\text{M}}\ \text{RR} \cdot \frac{\text{DA}}{\text{M}}\ \text{ER} \cdot \text{ng DA added per ml} \quad (1)$$

Data analysis

The data were then fitted to a biexponential equation of the form

$$C = -B(e^{-ka(t-t_0)} - e^{-\beta(t-t_0)}) \quad (2)$$

using the non-linear regression computer program NONLIN [11, 17] [where C = concentration of melphalan in plasma (ng/ml) at time t (min), B is a constant (ng/ml), β is an apparent first-order distribution rate constant measured in min^{-1}, ka is the apparent absorption rate constant (min^{-1}), and t_0 is the delay (min) before absorption starts].

Plasma half-lives ($t_{\frac{1}{2}}$; min) were calculated using the general formula:

$$t_{\frac{1}{2}}x = \frac{0.693}{x} \tag{3}$$

(where $x = \beta$ or ka), and the area under the curve [AUC; min·μg/ml·(mg dose)$^{-1}$] was calculated per mg dose of melphalan with the formula

$$\text{AUC} = \frac{10^{-3}}{\text{dose}} \cdot B\left(\frac{1}{\beta} - \frac{1}{ka}\right) \tag{4}$$

RESULTS

Fig. 2 shows the separation achieved between melphalan, Dns-arginine (the internal standard) and the two hydrolysis products of melphalan, MOH and $M(OH)_2$.

Fig. 3a shows the chromatogram of a blank plasma extract. The chromatograms shown in Fig. 3b and c are of extracts of a patient's plasma taken 237 and 15 min respectively after an intravenous dose of 25 mg of melphalan had been administered (the order was reversed so that the blank extract could more easily be compared to the sample containing low melphalan concentration). The plasma melphalan concentrations calculated from these chromatograms were 35 and 661 ng/ml respectively. $M(OH)_2$ and probably MOH are not extracted by this process.

Verification of the method was undertaken using both standard solutions of

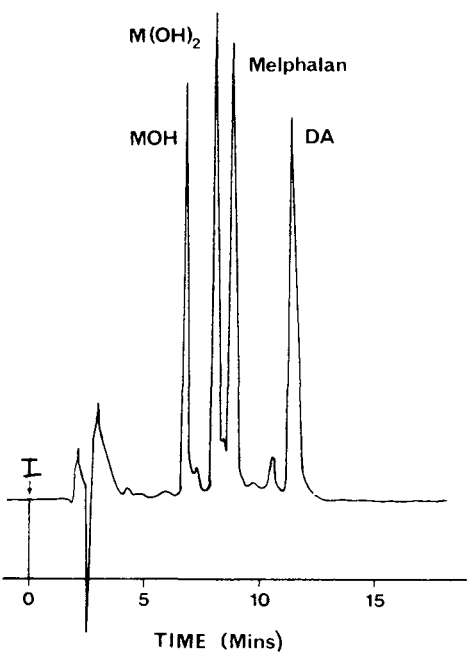

Fig. 2. Chromatogram showing the separation of approximately 400 ng DA, 200 ng melphalan, 400 ng $M(OH)_2$ and 200 ng MOH at 0.064 a.u.f.s.

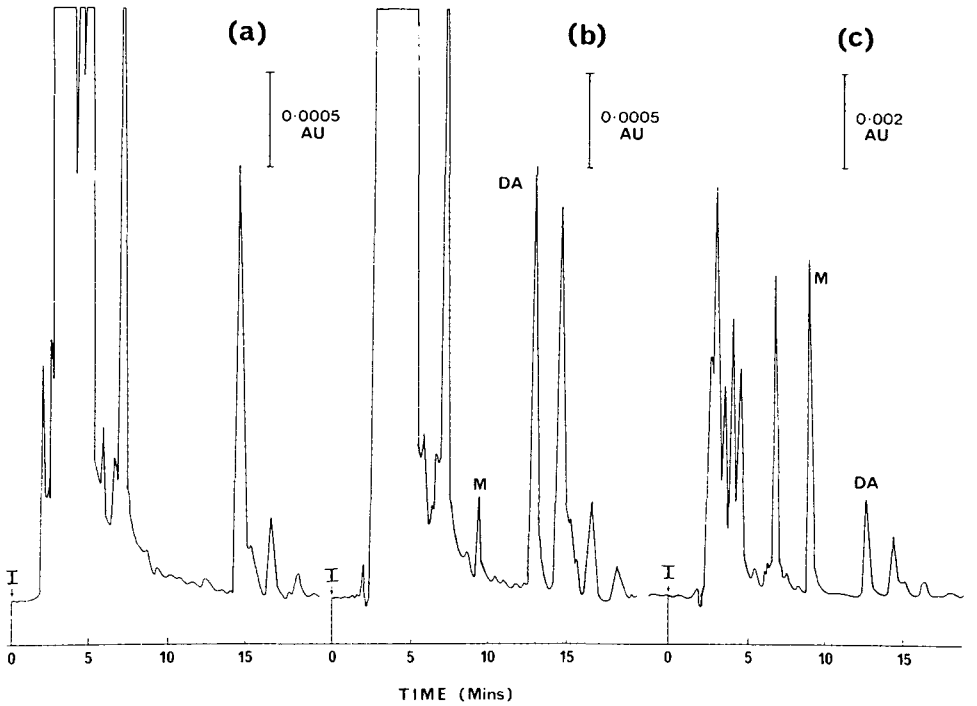

Fig. 3. Representative chromatograms of (a) a blank plasma extract at 0.002 a.u.f.s.; (b) an extract of plasma from a patient (adding DA at 500 ng per ml of plasma) 3 h 57 min after an intravenous dose of 25 mg melphalan (0.002 a.u.f.s.); and (c) identical extract of plasma taken 15 min after administration. Calculations from these chromatograms gave original plasma concentrations of melphalan of 35 and 661 ng/ml respectively. Peaks: M = melphalan; DA = internal standard; I = injection point.

melphalan and DA in mobile phase, and extracts of 1-ml blank plasma samples to which 6—1000 ng/ml melphalan had been added.

Linearity

The standard solutions of melphalan and DA gave excellent linearity with respect to peak height over 1—2000 ng per injection. A very good linearity was also seen between the melphalan/DA peak height ratio of extracted samples and melphalan concentration. The results of three separate experiments gave correlation coefficients of 1.000, 0.999 and 1.000.

Reproducibility

A reference mixture of melphalan (0.4 μg/ml) and DA (1.67 μg/ml) was always assayed, along with plasma extracts, to check the status of the HPLC system. Injections of 200 μl were made two to four times per day, and over a period of ten days the responses were for melphalan (measured as peak height) 14.73 ± 0.16 mm/ng at 0.002 a.u.f.s. [mean ± S.D.; $n = 9$; coefficient of variation (C.V.) = 1.07%], and for DA 5.43 ± 0.12 mm/ng (C.V. = 2.14%). The response ratio (DA/melphalan) calculated from these values was 0.3697 ± 0.0049 (C.V. = 1.32%).

Measurement of melphalan/DA ratios of five separate extractions on different days of 20 and 200 ng/ml plasma samples (adding 500 ng DA per ml of plasma) gave values of 0.167 ± 0.011 (C.V. = 6.2%) and 1.71 ± 0.11 (C.V. = 6.3%) respectively. The extraction efficiency for melphalan averaged 52.8%.

Sensitivity

The noise level of the high-performance liquid chromatograph, when pumping mobile phase and using the 5-sec time constant, was routinely about 10^{-5} a.u. However, this usually increased to two or three times this value around the area of the melphalan peak of a blank plasma extract (see Fig. 4a). Fig. 4b shows a melphalan peak just larger than the limits of detection, and from this the original plasma was calculated to contain 6 ng/ml melphalan. Thus the limit of detection is about 5 ng melphalan per ml of plasma, that is 5 ppb or $16 \cdot 10^{-9}$ *M* melphalan. The increased sensitivity of this method over other HPLC methods is partly due to an increased number of theoretical plates of the system, probably caused by the inclusion of an ion-pair reagent in the mobile phase. Figs. 2 and 3 show melphalan peaks with 4000—5000 plates ($N = 5.54 \cdot (d/w_{\frac{1}{2}})^2$ where N is the theoretical number of plates, d is the distance from the point of injection to the peak, and $w_{\frac{1}{2}}$ is the width of the peak at its half-height) and occasionally 8000—9000 plates are obtained. This compares favourably with the 1500—3500 plates of other published chromatograms [8—10].

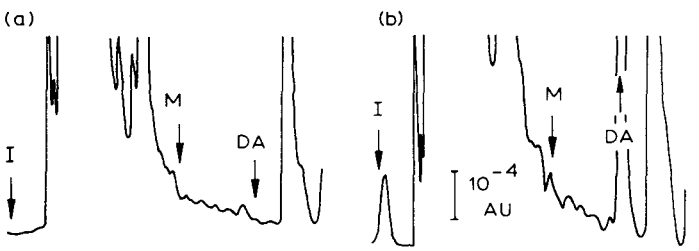

Fig. 4. Traces of (a) a blank plasma extract, and (b) a plasma sample that contained 6 ng melphalan per ml of plasma before extraction. These show the sensitivity that can be achieved with this method. Peaks: M = melphalan; DA = internal standard.

Stability of extracts

Plasma extracts were routinely kept at −40°C for up to 48 h before analysis by HPLC. Fifteen samples kept at −40°C for a further three months and analysed by HPLC a second time showed only minor differences when compared to the original chromatograms.

Melphalan concentrations in a patient

A patient who had been newly diagnosed as having multiple myeloma and had not received any prior chemotherapy was given 10 mg melphalan per day for a four-day course. On day 1 and day 4 he had a small breakfast of grapefruit, cereal and a drink before administration of melphalan. Blood

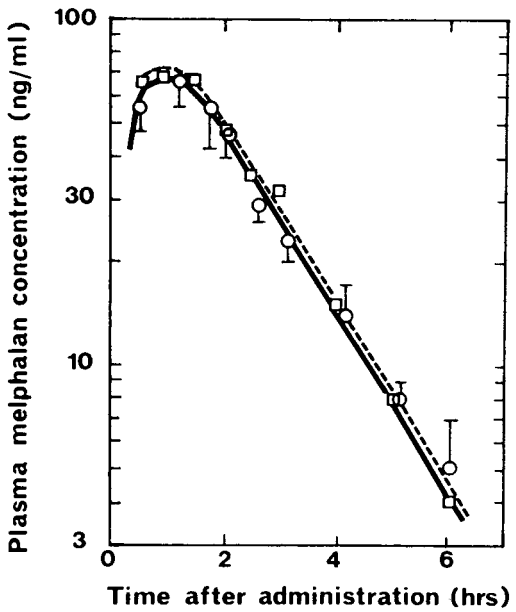

Fig. 5. Concentration of melphalan in the plasma of a patient after 10 mg melphalan given orally measured on day 1 (○—○) and day 4 (□— — —□) of a four-day course. The error bars on the day 1 points represent the S.D. of three separate determinations of melphalan concentration using the same original material.

samples were collected during day 1 and day 4, and Fig. 5 shows the concentration of melphalan in these samples. The error bars on the day 1 curve (continuous line) show the mean ± S.D. of three separate extractions of the same original material — the spread of results was probably due to a small percentage of the melphalan being converted to $M(OH)_2$ on each thawing of the plasma. The curves drawn through the points are the lines of best fit calculated by computer.

Table I gives the parameters calculated from the data in Fig. 5. The differences between the day 1 and day 4 values of t_0, B and $t_{\frac{1}{2}}ka$ are mainly due to

TABLE I

PARAMETERS CALCULATED FROM CURVES IN FIG. 5

The melphalan concentrations in the patient on day 1 (three separate extractions) and day 4 were fitted to biexponential curves. The parameters are calculated from the equations of these curves. The parameters for day 1 were determined for each extraction before a mean and S.D. were calculated.

	Units	Day 1	Day 4
Delay (t_0)	min	13.4 ± 0.7	9.2
Extrapolated intercept with Y axis (B)	ng/ml	130 ± 24	163
Half-life of absorption ($t_{\frac{1}{2}}ka$)	min	13.3 ± 0.6	19.4
Terminal half-life ($t_{\frac{1}{2}}\beta$)	min	70.7 ± 2.1	68.2
Area under the curve (AUC)	min·μg/ml·(mg dose)$^{-1}$	1.08 ± 0.15	1.15

the difficulty of fitting an equation to the absorption phase because the first sample was taken when the phase was nearly over. However, the terminal half-life is seen to be virtually identical on both days and the areas under the curves are not significantly different.

DISCUSSION

Recently, several groups have devised methods for the measurement of melphalan in biological samples [5—10]. However, none of the HPLC methods has the sensitivity to measure levels in human plasma samples after normal therapeutic doses of melphalan have been administered orally [8—10].

In this work we have been able to combine the sensitivity required with a simple isocratic HPLC system. The result is a method for the routine analysis of plasma melphalan that can be undertaken with equipment of relatively low cost. It has often been used in our laboratory to measure melphalan levels in solutions, and has also been used to follow the conversion of melphalan to MOH and to $M(OH)_2$ (data not shown). It has also been used to determine melphalan levels in cells in vitro at the end of drug uptake studies [18].

The sensitivity of this system has allowed us to obtain much useful information about melphalan levels in patients after they have taken normal therapeutic doses of oral melphalan (sometimes as little as 5 mg), as well as being able to follow the decay of the drug after intravenous administration [11]. The results from the patient in this study suggest that the administration of 10 mg melphalan over three days has not impaired the absorption of the drug on day 4 despite the sensitivity of the gastrointestinal tract to alkylating agents.

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DETERMINATION OF MITOMYCIN C IN PLASMA, SERUM AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET AND ELECTROCHEMICAL DETECTION

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SUMMARY

The performance of a number of normal phase and reversed-phase systems, with ultraviolet detection at 360 nm, has been investigated with respect to their applicability to pharmacokinetic studies of mitomycin C (MMC). The reversed-phase system developed was also combined with a polarographic detector in order to compare the sensitivity and selectivity of ultraviolet and electrochemical detection.

A simple isolation procedure, based on the adsorption of MMC on a non-ionogenic resin, has been developed. The developed assay is applied to a pharmacokinetic study from which some examples are given.

INTRODUCTION

The mitomycin antibiotics, which are derived from *Streptomyces caespitosus*, were discovered in 1956 [1]. Mitomycin C (MMC), having anti-tumor properties, was isolated in 1958 [2] and has the structure shown below. MMC is currently used in the treatment of various tumours. In order to obtain an insight into its pharmacokinetics and to relate its concentration in blood with therapeutic effectiviness, in order to optimize the schedule of administration,

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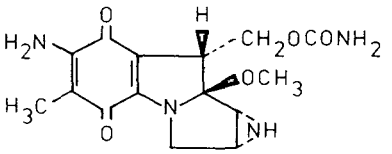

mitomycin C (MMC)

the determination of MMC and its possible metabolite(s) is of great importance.

For the determination of MMC in body fluids there are several analytical techniques, such as ultraviolet (UV) spectrophotometry [3], fluorometry, polarography [4—6], liquid chromatography [7—9] and bacterial growth inhibition [3]. UV spectrophotometry and bacterial growth inhibition were applied to the analysis of samples from dogs and rats in a pharmacokinetic study [3]. However, a detailed description of the pharmacokinetic parameters was not possible, due to the lack of sensitivity and selectivity of the method. Polarography, which is a fairly sensitive method, can only be applied if there are no interfering compounds, i.e. compounds with the same half-wave potential(s). Although to our knowledge no active metabolites have been reported so far, it should be noted that MMC decomposes rapidly under acidic and alkaline conditions. This implies that, prior to a polarographic technique, a separation of MMC from its (electrochemically active) degradation products might be necessary.

A few papers dealing with the high-performance liquid chromatographic determination of MMC have recently been published [7—10]. MMC can be chromatographed in the normal phase as well as in the reversed-phase mode. In order to be able to observe any degradation products that could be metabolites, we developed a normal phase system. The main degradation product of MMC is formed by cleaving off the carbamate moiety of the molecule. The resulting molecule is much less polar than MMC itself. Expecting low concentrations of this compound, one should apply normal phase chromatography. In such a phase system the degradation product will be eluted before MMC, which means that the detectability is better than in a reversed-phase system, in which MMC is eluted first.

For the quantitative determination of MMC in plasma, serum and urine, we developed a reversed-phase system, that is readily applicable to routine analysis. Moreover, reversed-phase systems are more suitable for electrochemical detection than normal phase systems. After slow infusion of MMC one can expect very low concentrations. Electrochemical detection might fulfil the demands for low limits of detection. In general, liquid adsorption systems do not allow direct injection of biological samples. We therefore developed a relatively simple isolation procedure, based on adsorption on Amberlite XAD-2 resin.

EXPERIMENTAL

Apparatus

Two liquid chromatographs were used, which were constructed from commercially available and custom-made parts and consisted of a thermostatted

glass eluent reservoir, a high-pressure pump (solvent delivery system, Model 6000A, Waters Assoc., Milford, MA, U.S.A., and Model 740 B, Spectra Physics, Santa Clara, CA, U.S.A.), a thermostatted column (stainless steel precision-bore tubing, 3.0 mm I.D., 6.35 mm O.D., length 100 mm), an injection device (Model U6K, Waters Assoc., and Model 7125, Rheodyne, Riviera Beach, FL, U.S.A.), a variable wavelength detector (LC-UV 3, Pye-Unicam, Cambridge, Great Britain) operating at 360 nm, a flat-bed recorder (BD 8, Kipp & Zoon, Delft, The Netherlands) and a computer integrator (Autolab, System I, Spectra Physics). Columns were packed by means of an air amplifier booster pump (DSTV-122, Haskel Inc., Burbank, CA, U.S.A.) as described elsewhere [11]. For the electrochemical experiments a polarographic detection system consisting of a polarographic cell (PAR 310, E.G. & G. Instruments, Princeton, NJ, U.S.A.), an interface (Bruker SMDE-Interface, Bruker, Brussels, Belgium) and a polarograph (Model E 100, Bruker) was used.

Chemicals and materials

MMC was obtained either as Mutamycin® from Bristol-Myers B.V. (Weesp, The Netherlands) or as Mitomycine from Kyowa (Tokyo, Japan). Porfiromycin was kindly donated by Upjohn (Ede, The Netherlands).

All organic solvents were of analytical grade (Merck, Darmstadt, G.F.R.) and water was obtained from a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). Hypersil-MOS (5 μm) was purchased from Ahrin (The Hague, The Netherlands), Servachrom Amberlite XAD-2 (100—200 μm) from Brunschwig Chemie, B.V. (Amsterdam, The Netherlands). The normal-phase support material (silica SI-60) was prepared by grinding silica SI-60 (Merck) with a particle size range of 63—200 μm in a rotating mortar and classifying the ground material by means of an air classifier (Alpine MZR, Augsburg, G.F.R.).

Chromatography

The capacity ratios were calculated from the retention times of MMC and of an unretained compound, for which toluene was used in the normal-phase systems and potassium periodate in reversed-phase systems. The theoretical plate height was determined from its retention time and half the peak width at 0.6 of the peak height.

Polarographic detection

Oxygen was removed from the mobile phase and the samples by bubbling through oxygen-free nitrogen, which was presaturated with the mobile phase.

Preparation of the biological samples

Plasma samples. During 24 h blood samples were taken before and after administration (intravenous and intra-arterial) of MMC. They were collected in heparinized tubes and centrifuged at 1000 *g* for 5 min. The plasma samples were stored at 243°K until analysis.

Serum samples. After clotting of the blood samples, the serum was decanted, centrifuged at 1000 *g* for 5 min and stored at 243°K until analysis.

Urine samples. The urine was collected during 24 h after administration and stored at 243°K until analysis.

Isolation

MMC was isolated from plasma, serum and urine by means of an Amberlite XAD-2 resin, of which 100 mg (100—200 μm) were transferred to a Pasteur pipette in which a plug of cotton wool had been inserted. The resin was pretreated with methanol (three aliquots of 3 ml) and water (10 ml). Then 2 ml of the sample were brought onto the resin. The XAD-2 was washed with water (three aliquots of 2 ml), after which MMC was eluted with methanol (three aliquots of 2 ml). The methanol fractions were collected in a conical flask and the methanol was evaporated at reduced pressure at a temperature of about 330°K. The residue was dissolved in 200 μl of mobile phase by thoroughly mixing it on a whirlmixer for 2 min. Aliquots of 10—100 μl were injected onto the column.

If an internal standard was used, porfiromycin was added to the sample as an aqueous solution (100 μl of a solution of about 1 μg/ml).

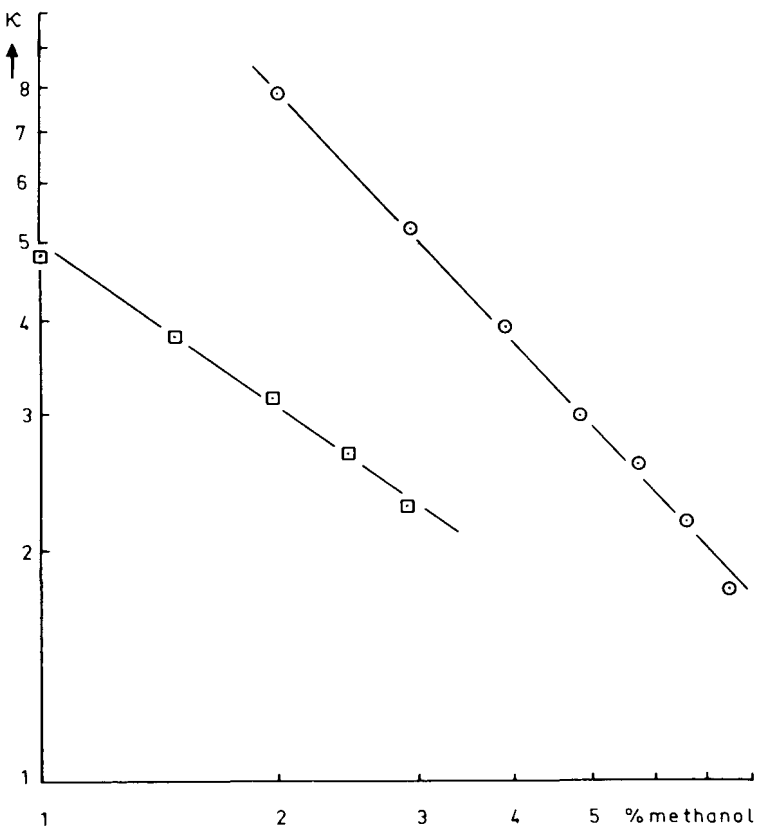

Fig. 1. Log—log plot of the relationship between the capacity ratio of MMC and the methanol content of the mobile phase in normal phase chromatography. (○), Ethyl acetate; (□), acetonitrile.

RESULTS AND DISCUSSION

Normal-phase system

The influence of the methanol (MeOH) content of the mobile phase (ethyl acetate and acetonitrile, respectively) on the capacity ratio of MMC was investigated first. In Fig. 1 the logarithm of the capacity ratio is plotted against the logarithm of the methanol content of the mobile phase. There is a remarkable linear relationship between the logarithms of these quantities, both with ethyl acetate (EtAc) and acetonitrile (ACN). The dependence of the capacity ratio, κ_{MMC}, on the methanol content, C_{MeOH}, as a percentage, is given by the following equations:

$$\log \kappa_{MMC}\,(\text{EtAc}) = -1.098 \log C_{MeOH} + 1.234 \quad (1)$$

$$\log \kappa_{MMC}\,(\text{ACN}) = -0.682 \log C_{MeOH} + 0.689 \quad (2)$$

The percentage goodness of fit is 99.8 and 99.6, respectively. It should be noted that eqns. 1 and 2 are only valid for a limited range of methanol content of the mobile phase.

The phase system was also optimized with respect to column efficiency. Although phase systems based on ethyl acetate and methanol showed better efficiencies than with acetonitrile and methanol, the MMC peak shape was very asymmetric. By adding water to the mobile phase to deactivate the silica and to obtain a more stable phase system, the peak symmetry improved. By adding dichloromethane next to water we obtained the best efficiencies. The methanol content was diminished to keep sufficient retention. In Table I some theoretical plate numbers are given, showing the effect of adding water and dichloromethane on column efficiency.

TABLE I

COLUMN EFFICIENCIES

$\langle u \rangle$ = 1.5 mm/sec, length 10 cm.

Mobile phase*		κ_{MMC}	N_{MMC}
EtAc—MeOH	(97 : 3)	5.2	520
EtAc—MeOH—H_2O	(97 : 3 : 1)	4.1	1640
EtAc—MeOH—H_2O	(97 : 3 : 2)	2.8	1830
EtAc—MeOH—H_2O—DCM	(97 : 2 : 1 : 1)	5.3	3280

*EtAc = ethyl acetate, MeOH = methanol, DCM = dichloromethane.

Reversed-phase system

The capacity ratio of MMC was measured as a function of the pH and the acetonitrile content of the mobile phase. In the pH range 5—8 the capacity ratio was constant, while at lower pH values it increased. However, at these pH values MMC decomposes rapidly, which means that in practice the capacity ratio cannot be affected by the pH of the mobile phase.

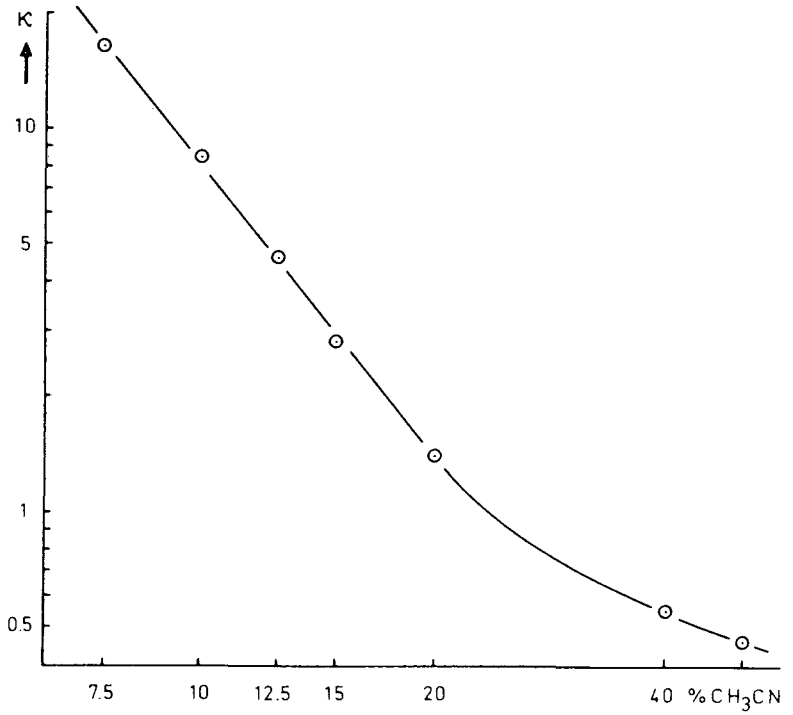

Fig. 2. Log—log plot of the relationship between the capacity ratio of MMC and the acetonitrile content of the mobile phase in reversed-phase chromatography.

In Fig. 2 the influence of the acetonitrile content of the mobile phase on the capacity ratio of MMC is shown by plotting $\log \kappa_{MMC}$ against the logarithm of the acetonitrile content. Between 7.5 and 20% acetonitrile $\log \kappa_{MMC}$ is, just as in the normal-phase systems, proportional to the logarithm of the acetonitrile content, C_{ACN}. In this range eqn. 3 is valid:

$$\log \kappa_{MMC} = -2.533 \log C_{ACN} + 3.445 \quad (3)$$

The percentage goodness of fit is 99.95%.

Although the efficiency of the reversed-phase system is slightly lower than the normal-phase system, the former allows electrochemical detection, is more stable and is less expensive than the latter. For these reasons we applied the normal phase system only to qualitative investigations in view of the possible occurrence of metabolites or degradation products. The reversed-phase system was used for the routine analysis of MMC.

Polarographic detection

It is known from the literature [4, 5] that MMC is polarographically active. This can also be concluded from its molecular structure: the quinone group can be reduced electrochemically to the corresponding hydroquinone.

The polarographic detection system used can be applied in the hanging mercury drop electrode (HMDE) mode as well as in the static mercury drop electrode (SMDE) mode. In the HMDE mode only one mercury drop is used as electrode during one chromatographic run, while in the SMDE mode the electrode is refreshed frequently, whereby the drop is forced to grow rapidly and reaches its maximum size long before the drop is knocked off and the current is sampled. So, at the moment of sampling, the drop size is constant and is said to be static. This results in a negligible charging current, normally caused by the increase of the drop size area.

For the detection of MMC, SMDE is expected to be more favourable than HMDE, because of the adsorption of MMC to the mercury surface. This adsorption might cause a decrease in the effective surface area of the mercury drop, resulting in a lowered sensitivity for the HMDE mode, while in the SMDE mode this effect can be neglected by the frequently refreshed electrode. Nevertheless, HMDE is observed to offer much better signal-to-noise ratios than SMDE, resulting in detection limits of about 250 pg and 15 ng, respectively. This unexpected result is probably caused by the increased noise level in the SMDE mode, due to fluctuations in the drop size areas as obtained with the applied detection systems.

With the detection system three different drop sizes have been used — small, medium and large, corresponding to about 50, 100 and 200 mg of mercury per drop. Due to the fact that the noise level is more or less proportional to the

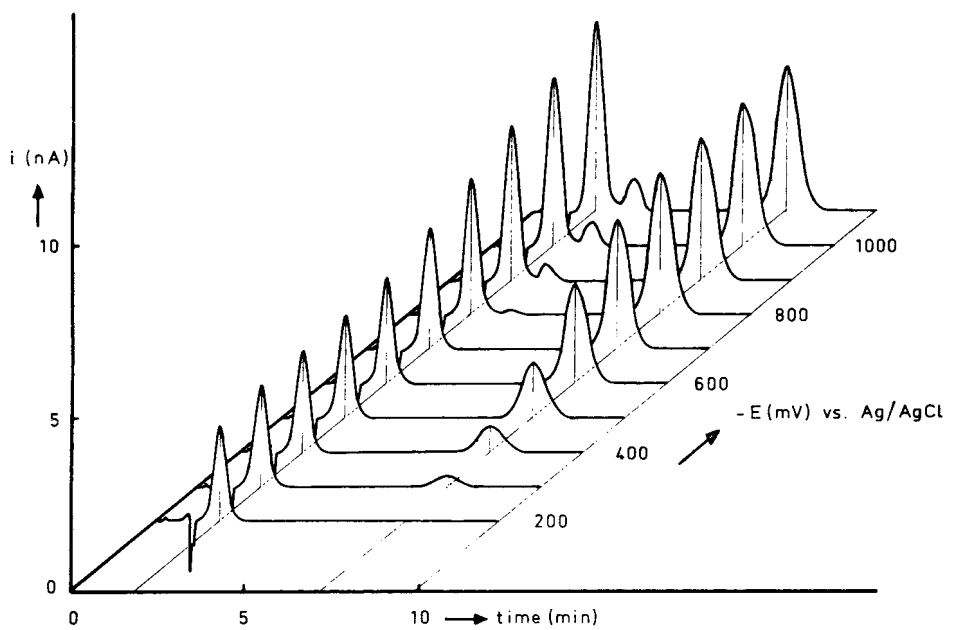

Fig. 3. Chromatoamperogram of MMC. Oxygen, about 2 min; MMC, about 7 min. Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 12% acetonitrile in phosphate buffer (0.05 M, pH = 7); flow-rate, 0.5 ml/min.

mercury surface area [12], while only a small part of the drop is used as reduction electrode, the signal-to-noise ratio of the smallest drop size is most favourable.

In Fig. 3 the relation is given between the reduction current, the time of analysis and the potential applied to the mercury electrode. It can be seen that at a potential of about —600 mV versus the silver/silver chloride reference electrode an adequate signal for MMC is obtained. Note the oxygen peak in the chromatograms at about 2 min, which is caused by the presence of remaining oxygen in the sample. Fig. 4 shows a chromatogram of an extract from plasma spiked with MMC. Porfiromycin was used as internal standard.

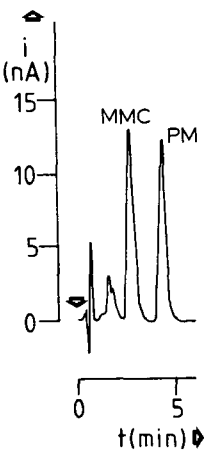

Fig. 4. Chromatogram of an extract of plasma, spiked with mitomycin C (MMC) and porfiromycin (PM) (concentration about 100 ng/ml). Detection: hanging mercury drop electrode (small size) (see text). Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 10% acetonitrile in phosphate buffer (0.05 *M*, pH = 7); flow-rate, 2.0 ml/min.

Isolation

The isolation of MMC from a biological matrix by means of the procedure described above enables one to concentrate the compound tenfold. By dissolving the residue in less than 200 μl of the mobile phase this concentrating can be enhanced, although there is loss of reproducibility. The recovery of MMC isolated from plasma, serum and urine was determined by treating samples spiked with amounts varying from 5 to 500 ng/ml. The peak areas were compared with those obtained by direct injection of the same amounts dis-

TABLE II

RECOVERY OF MMC (5—1000 ng/ml)

Matrix	Recovery (%)	C.V. (%, *n*=5) (5—1000 ng/ml)
Water	97.4	2.0—0.4
Plasma	84.8	2.1—0.6
Serum	84.3	2.5—0.6
Urine	86.4	4.3—1.1

solved in the mobile phase. In Table II the recoveries are given for MMC isolated from different biological samples. From the table it can be seen that even without an internal standard highly reproducible results can be obtained. In practice, porfiromycin appeared to be a suitable internal standard for UV detection as well as for polarographic detection.

Precision and linearity

The precision and linearity of the determination of MMC by HPLC, preceded by adsorption on XAD-2, were investigated by determining biological samples spiked with amounts of MMC varying from 5 to 1000 ng/ml for plasma and serum and from 50 to 1000 ng/ml for urine with UV detection as well as with electrochemical detection (ElCD). In Table III the equations for the calibration curves and their corresponding correlation coefficients are summarized for UV detection as well as for ElCD. The limits of detection, based on a signal-to-noise ratio of 3, are about 150 pg and 250 pg for HPLC—UV and HPLC—ElCD, respectively.

TABLE III

PRECISION AND LINEARITY

Matrix	Concentration (ng/ml)	Detection	Slope	Intercept	Correlation coefficient
Water	5—1000	UV*	18.7 mV·sec/ng	2.3 mV·sec	0.9999
Plasma	5—1000	UV	15.9	5.1	0.9981
Serum	5—1000	UV	16.0	3.0	0.9962
Urine	50—1000	UV	16.2	1.8	0.9934
Water	5—1000	ElCD**	1.15 nC/ng	0.26 nC	0.9987
Plasma	5—1000	ElCD	0.98	5.2	0.9931
Serum	5—1000	ElCD	0.99	3.7	0.9942
Urine	50—1000	ElCD	1.00	8.7	0.9914

*UV detection at 360 nm.

**Electrochemical detection at —700 mV vs. Ag/AgCl.

Application to biological specimens

In order to demonstrate the usefulness of the method two elimination curves and a cumulative excretion curve for MMC after a single dose are given. For the pharmacokinetic studies UV detection is applied because this detection is more suited for routine analysis.

Fig. 5 shows the chromatogram of an extract of plasma from a patient who received 20 mg of MMC intravenously. The blood sample was taken 2 h after administration and treated as described above. Fig. 6 gives the plasma concentration—time course for this patient. Fig. 7 shows the chromatogram of an extract of urine from this patient, collected during 24 h. Although there are a number of urine peaks in the chromatogram, it can be seen that only small interfering compounds are present in the chromatogram and that, due to the relatively high MMC concentrations occurring in urine it can be determined by applying the same isolation procedure. Moreover, it appears that porfiromycin

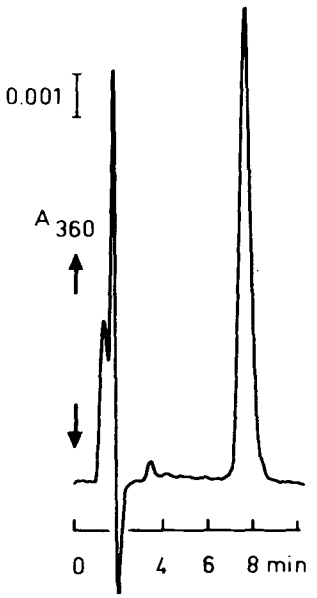

Fig. 5. Chromatogram of an extract of plasma from a patient who received 20 mg of MMC intravenously. MMC peak corresponds to 25 ng. Conditions: stationary phase, Hypersil—MOS (5 μm); mobile phase, 10% acetonitrile in water; flow-rate, 0.5 ml/min.

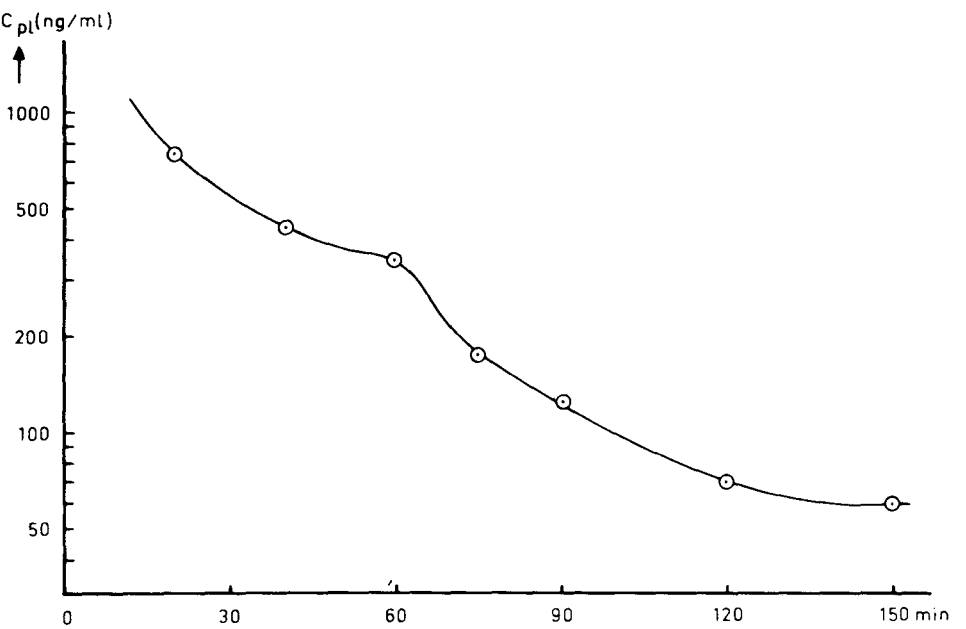

Fig. 6. Plasma concentration—time course for a patient receiving 20 mg of MMC intravenously.

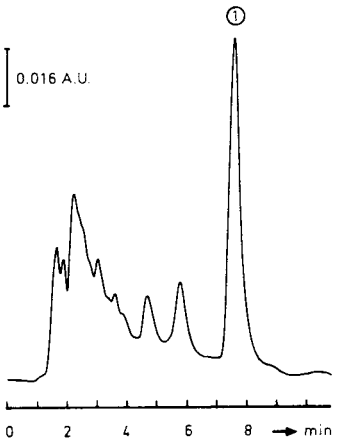

Fig. 7. Chromatogram of an extract of urine from a patient receiving 20 mg of MMC intravenously. Urine was collected during 24 h. MMC peak corresponds to 400 ng. Conditions: see Fig. 5.

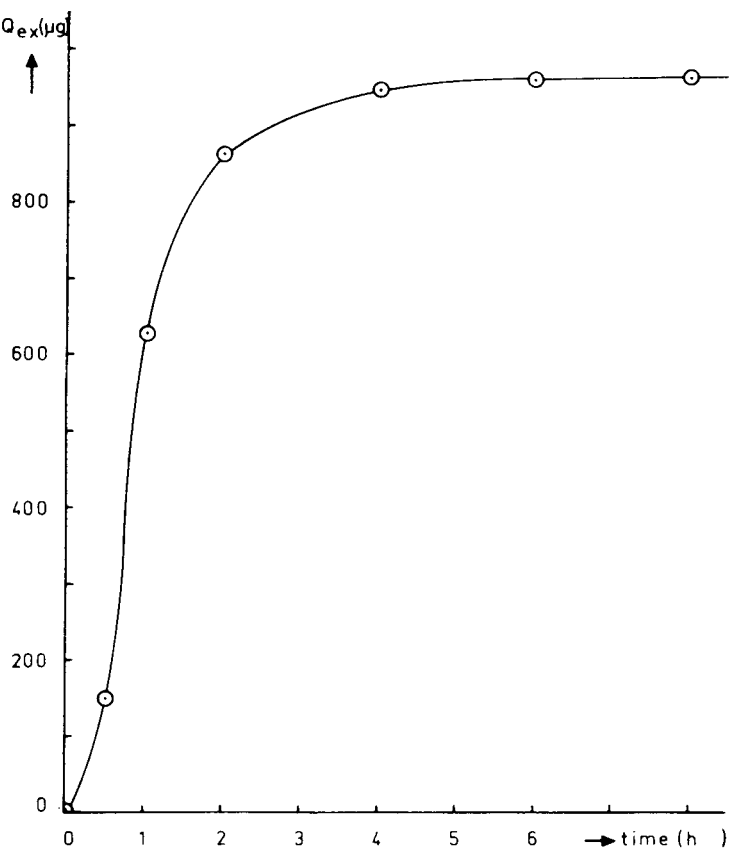

Fig. 8. Cumulative excretion curve for a patient receiving 20 mg of MMC intravenously.

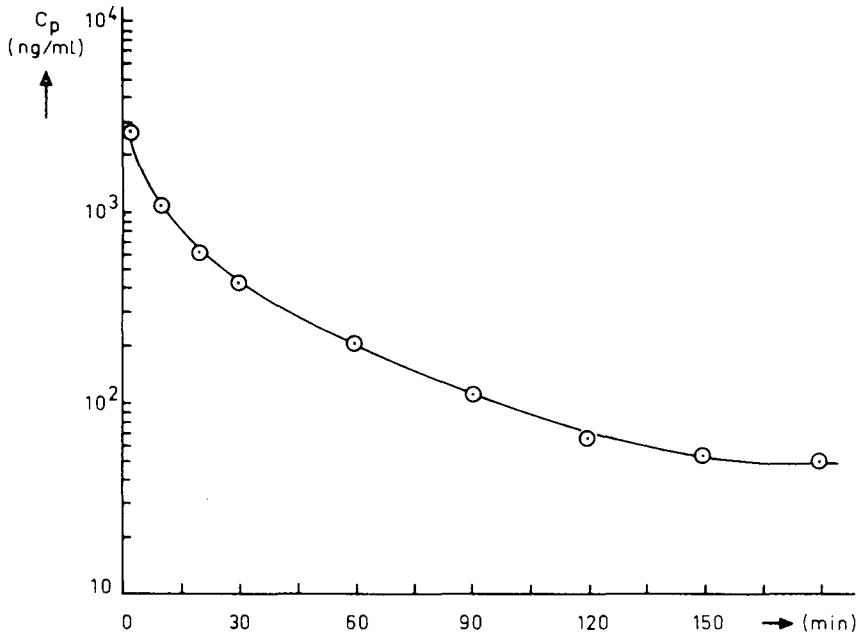

Fig. 9. Plasma concentration—time course for a rat receiving 100 μg of MMC intravenously.

is a good internal standard, because it is eluted after MMC and thus after the interfering urine compounds.

Fig. 8 gives the cumulative excretion curve for a patient who received 20 mg of MMC intravenously. It appears that only a small amount of the administered drug is recovered in the urine collected during the first 8 h after administration.

In Fig. 9 the plasma concentration—time course for a rat receiving 100 μg of MMC intravenously (jugularis externa) is shown. Blood samples were taken by means of a cannula that was inserted into the arteria carotis. In this experiment the maximum sample volume was only 300 μl, demonstrating the possibility of handling small sample volumes with the described method.

From both Fig. 6 and Fig. 9 it can be seen that the pharmacokinetics of MMC are rather complicated and cannot be described by a simple one- or two-compartment model.

CONCLUSIONS

It can be concluded that HPLC with UV detection as well as with polarographic detection can be applied to the determination of MMC in plasma, serum and urine. Because of the extensive provisions needed for polarographic detection (removal of oxygen), UV detection is more suited for routine analysis.

Until now no metabolites or degradation products have been determined in biological specimens.

The pharmacokinetic study of MMC is being continued and will be reported in due course.

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DETERMINATION OF RIFAMPICIN, DESACETYLRIFAMPICIN, ISONIAZID AND ACETYLISONIAZID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATION TO HUMAN SERUM EXTRACTS, POLYMORPHONUCLEOCYTES AND ALVEOLAR MACROPHAGES

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SUMMARY

A method for the determination of rifampicin, desacetylrifampicin, isoniazid, and acetylisoniazid by high-performance liquid chromatography and using the same extract of the same sample is reported. After protein precipitation and extraction of these antituberculous drugs, two reversed-phase chromatographies were necessary. The technique was applied to serum extracts, polymorphonucleocytes and alveolar macrophages from patients treated for tuberculosis.

INTRODUCTION

Rifampicin (RFP) and isoniazid (INH), two of the most widely used antituberculous drugs, present very interesting pharmacologic properties: on the one hand liver degradation of INH into inactive acetylisoniazid (AC-INH) by an acetyltransferase whose activity is genetically determined; on the other hand a powerful induction of RFP on liver metabolism occurs; the interaction of both mechanisms is responsible for actual toxicity [1].

There has been increasing interest in the determination of serum levels of the main antituberculous drugs but, as little knowledge of the action of these drugs in the organs involved has yet been attained, we sought to develop a method of

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assay capable of meeting the following criteria: the development of a technique sensitive enough to allow intracellular assays, notably in the case of polymorphonuclear cells and alveolar macrophages; the simultaneous determination of RFP and INH and their corresponding metabolites desacetylrifampicin (DS-RFP) and AC-INH.

The assay of the two antituberculous drugs in biological fluids can be effected according to various methods. The usual methods for RFP assay are colorimetry, microbiology and high-performance liquid chromatography (HPLC) [2—4]. The methods for INH assay are colorimetry [5, 6], spectroscopy [7], fluorometry [8] and HPLC [9]. Our objective was to use reversed-phase HPLC to assay both antituberculous drugs from one single sample. The determination of serum and intracellular levels of the drugs and their metabolites should help understand the way they diffuse through the body. Also, one single extraction allows the use of smaller sample amounts, which is an invaluable advantage.

MATERIAL AND METHODS

INH and AC-INH were kindly offered by Laboratoires Roche (France). RFP and DS-RFP were kindly offered by Laboratoires Lepetit (Milan, Italy). Evaporation was performed in a Brinkmann SC 48R evaporator.

A Waters HPLC chromatograph was used, equipped with a pump Model 6000 A, a universal injector Model U6K and a two wavelength ultraviolet (UV) detector Model 440 (254—280 nm) coupled with a two-channel Houston Omniscribe recorder.

A stainless-steel column was used (30 cm × 3.9 mm) filled with Waters μBondapak C_{18} (particle size 10 μm) non-polar phase. The top of the column was protected by a Waters prefilter No. 84560 to prevent obstruction by minute particles.

RFP and DS-RFP were eluted by methanol—0.05 *M* ammonium formate (65:35); the pH was adjusted to 7.3; the flow-rate was 2 ml/min (mobile phase I). Samples (20 μl) were injected on top of the column and detection was read at 254 nm.

INH and AC-INH were eluted by methanol—water (5:95) containing 5 mmoles/l *n*-heptanesulfonic acid (Pic-Reagent B7, Waters Associates); the flow-rate was 2 ml/min (mobile phase II). Samples (90 μl) were injected and detection was read at 280 nm.

Sample preparation

Standard solution. A 10 mg/ml stock solution was prepared by dissolving RFP and DS-RFP in methanol. The two stock solutions obtained were diluted at a concentration of 1/1000 in mobile phase I and made to form one single 10 μg/ml solution. The standard solution of INH and AC-INH was prepared in the same manner and the solutions were stored at 4°C.

Serum. Sampling of the sera required dry tubes. The sera were obtained from patients treated for pulmonary tuberculosis by a combination of INH and RFP. Assays were performed 3 h after oral administration of the usually prescribed doses of INH (250—300 mg) and RFP (600 mg).

Polymorphonuclear cells. Separation of polymorphonucleocytes was performed according to the method of Losito and Lorusso [10]. Polymorphonucleocytes were counted and placed in suspension in 1 ml of a 9 g/l sodium chloride solution.

Alveolar macrophages. These were obtained after centrifugation of the broncho-alveolar wash fluid. The cells were counted and placed in suspension in saline.

Cell lysis

The cells in suspension in saline (polymorphonucleocytes and alveolar macrophages) were treated for 1 min by an ultrasonic cell disintegrator Sonifier RB 30. A standard microprobe was used, screwed onto a disruptive sonotrode.

Extraction of antituberculous drugs

Mix 1 ml of sample and 2.5 ml of acetone in a vortex mixer. After centrifuging for 10 min at 200 *g*, 2 ml of the supernatant are transferred to a conical glass tube and evaporated at 45°C. The residue is taken up in 0.5 ml of mobile phase I and 1.5 ml of butanol—chloroform (30:70) are added. Mix and centrifuge for 5 min at 200 *g* 0.5 ml of organic phase and evaporate at 45°C in two separate tubes. The residue of the first tube is taken up in 0.2 ml of mobile phase I. The residue of the second tube is taken up in 0.2 ml of mobile phase II. Both tubes are then mixed in the vortex mixer and centrifuged for 5 min at 200 *g*.

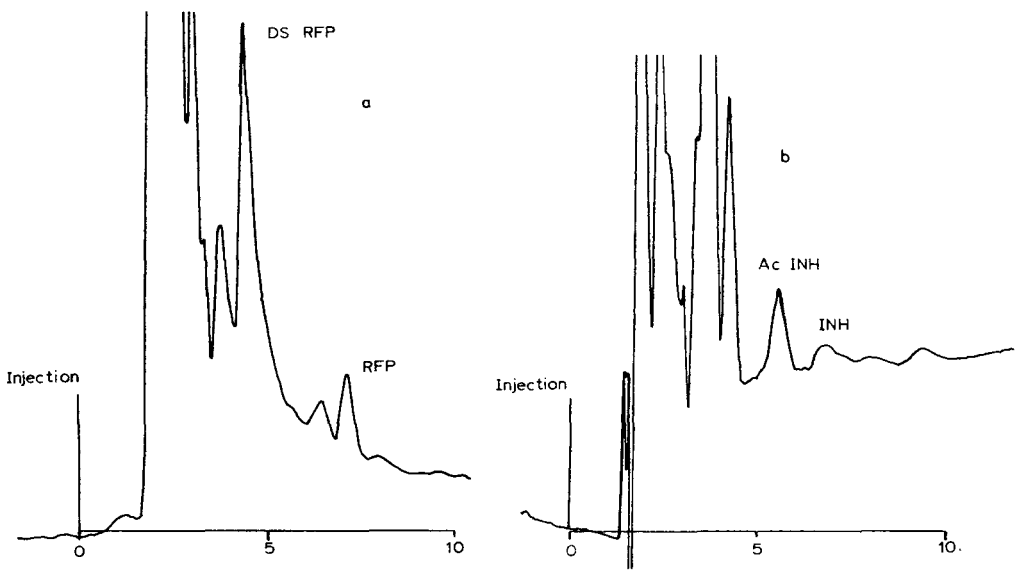

Fig. 1. (a) Chromatogram of a serum extract. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol—0.05 *M* ammonium formate (65 : 35, v/v), pH 7.30. Flow-rate: 2 ml/min. Injection volume: 20 μl. UV detector: 254 nm, 0.02 a.u.f.s. Chart speed: 1 cm/min. (b) Chromatogram of a serum extract. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol Pic B_7—water Pic B_7 (5 : 95, v/v). Flow-rate: 2 ml/min. Injection volume: 90 μl. Detector: 280 nm, 0.01 a.u.f.s. Chart speed: 1 cm/min.

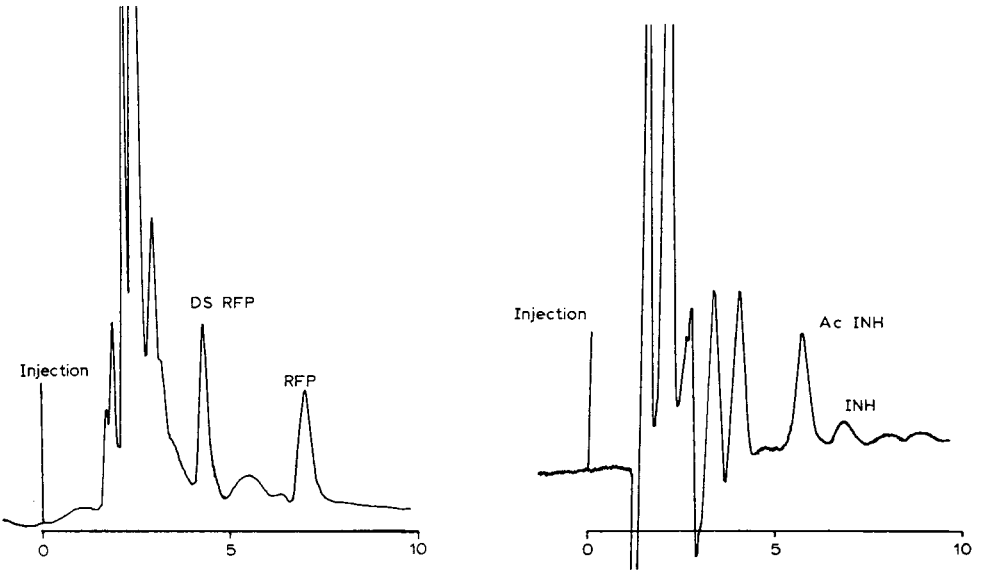

Fig. 2. (a) Chromatogram of an extract of polymorphonuclear cells. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol—0.05 *M* ammonium formate (65 : 35, v/v), pH 7.30. Flow-rate: 1 ml/min. Injection volume: 40 μl. UV detector: 254 nm, 0.02 a.u.f.s. Chart speed: 1 cm/min. (b) Chromatogram of an extract of polymorphonuclear cells. Column: 30 × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol Pic B_7—water Pic B_7 (5 : 95, v/v). Flow-rate: 2 ml/min. Injection volume: 90 μl. UV detector: 280 nm, 0.05 a.u.f.s. Chart speed: 1 cm/min.

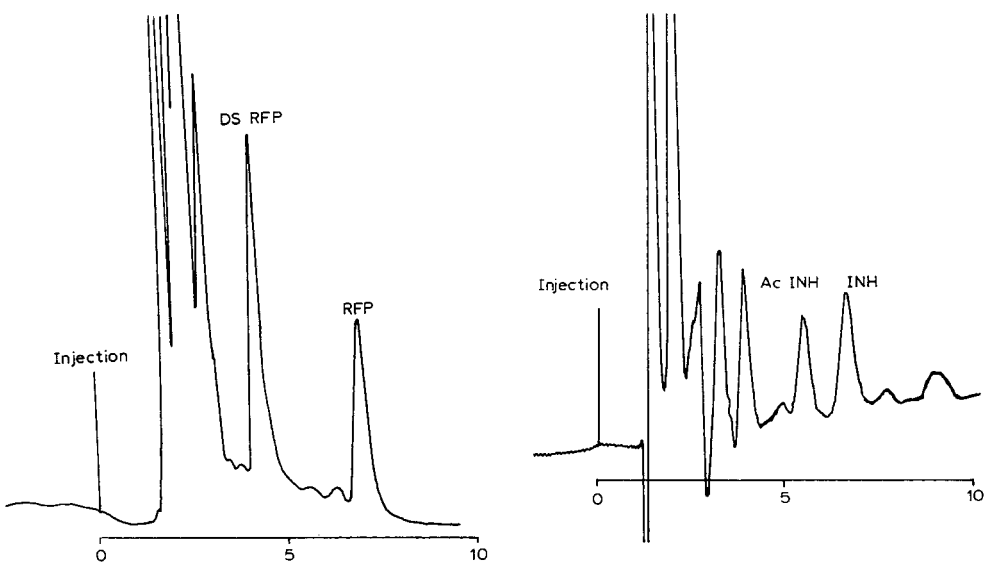

Fig. 3. (a) Chromatogram of an extract of alveolar macrophages. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol—0.05 *M* ammonium formate (65 : 35, v/v), pH 7.30. Flow-rate: 2 ml/min. Injection volume: 40 μl. UV detector: 254 nm, 0.02 a.u.f.s. Chart speed: 1 cm/min. (b) Chromatogram of an extract of alveolar macrophages. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol Pic B_7—water Pic B_7 (5 : 95, v/v). Flow-rate: 2 ml/min. Injection volume: 90 μl. UV detector: 280 nm, 0.005 a.u.f.s. Chart speed: 1 cm/min.

RESULTS

During the chromatography using mobile phase I, DS-RFP and RFP were eluted at retention times of 4.20 and 7 min, respectively. When using mobile phase II, retention times of AC-INH and INH were 5.20 and 6.30 min, respectively.

Fig. 1a and b represent the chromatograms obtained with mobile phases I and II, respectively, from the serum extract of a treated patient. Fig. 2a and b represent the same chromatograms obtained from a polymorphonuclear extract of the same patient.

Fig. 3a and b represent the chromatograms obtained from an extract of alveolar macrophages.

On each of these chromatograms the peaks of RFP, DS-RFP, INH and AC-INH are all sharply distinguished, which allowed their identification and their quantitative determination.

Linearity of response

Sample concentrations were calculated by measuring the peak heights with reference to the peak heights of increasing amounts of the standard solution. We found a linear relationship between peak heights and the concentrations: rifampicin, $r = 0.9994$; desacetylrifampicin, $r = 0.9847$; isoniazid, $r = 0.9403$; acetylisoniazid, $r = 0.9996$.

Overloading tests

These tests were performed by adding known amounts of antituberculous drugs to the serum of an untreated person. After extraction of the molecules, the concentrations and the recoveries were calculated (Table I).

Lower detection limits

In the operating conditions described above and for 1 ml of serum sample, the detection limits were: rifampicin 17 μg/l, desacetylrifampicin 10 μg/l, isoniazid 95 μg/l, acetylisoniazid 85 μg/l.

For polymorphonucleocytes and macrophages, the detection limits were found to be variable, depending on the number of cells contained in the original (initial) sample. In order to obtain reproducible results it is preferable to work with samples containing a constant number of cells. With a cell concentration of $5 \cdot 10^6$ cells/ml the detection limits were: rifampicin 3.4 ng per 10^6 cells, desacetylrifampicin 2 ng per 10^6 cells, isoniazid 19 ng per 10^6 cells, acetylisoniazid 17 ng per 10^6 cells.

DISCUSSION

Initially, our aim was to determine the serum or intracellular levels of the two antituberculous drugs by using one single chromatographic procedure.

The chromatography using mobile phase I does not allow the separation of INH from AC-INH without interference. The separation could be achieved theoretically by increasing the polarity of the mobile phase, but the retention of RFP and DS-RFP was enhanced and the separation of INH from AC-INH re-

TABLE I

EXTRACTION RECOVERIES

DS-RFP			RFP			AC-INH			INH		
Added (μg)	Measured (μg)	Recovery (%)	Added (μg)	Measured (μg)	Recovery (%)	Added (μg)	Measured (μg)	Recovery (%)	Added (μg)	Measured (μg)	Recovery (%)
0.52	0.47	92	0.52	0.48	93	0.61	0.57	93	0.61	0.51	94
1.04	1.03	98	1.04	0.93	92	1.22	1.09	90	1.22	1.04	86
2.08	1.99	96	2.08	1.89	90	2.44	2.26	92	2.44	2.10	86
5.20	5.13	99	5.20	5.14	99	6.10	5.08	83	6.10	5.11	84
10.40	9.86	95	10.40	9.38	90	12.20	11.24	92	12.20	10.76	88
Mean		96			93			90			88
Standard deviation		2.45			3.70			4.06			3.85

mained unsatisfactory. Therefore, we kept mobile phase I for assay and identification of RFP and DS-RFP, while for INH and AC-INH we had to revert to another chromatographic technique using ion-pair chromatography.

In acid medium INH and AC-INH can become ionized and can then be submitted to ion-pair chromatography with *n*-heptanesulfonic acid. The then nonpolar ion—counterion complex can be eluted by reversed-phase liquid chromattography. The use of mobile phase II allowed satisfactory separation of INH from AC-INH (Figs. 1b, 2b and 3b).

However, in order to assay the drugs and their metabolites, two successive chromatographic procedures were necessary. This is due to the basic difference in chemical structure of the four antituberculous drugs. Nevertheless, the advantage of one single extraction must be retained, as extraction represents the longest procedure of analysis. Besides, as pointed out before, the sample volume is reduced, which is quite an advantage when cells are to be obtained, particularly polymorphonucleocytes, as only 20 ml of blood are required for this operation.

The high sensitivity of the method allowed the determination of residual levels of antituberculous drugs: the comparison between those daily levels is more reliable than the comparison between maximum levels observed shortly after absorption of those drugs.

Being more commonly used, reversed-phase C_{18} columns, such as were used by Saxena et al. for isoniazid determination [9], prove to be more reliable than silica columns [3].

CONCLUSION

The development of the technique described allows the assay of the two antituberculous drugs and their metabolites when using one single test sample. The technique is also sensitive enough to ensure the determination of intracellular levels.

The use of the technique would therefore allow study of the comparison between levels in sera and in phagocytic cells (polymorphonucleocytes and macrophages), thus allowing a better understanding of the diffusion and mechanisms of action of those antituberculous drugs.

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CHROMBIO. 1373

QUANTITATION OF DAUNORUBICIN AND ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A selective and sensitive high-performance liquid chromatographic method was developed for the separation and quantitation of daunorubicin and its metabolites in serum, plasma, and other biological fluids. Daunorubicin and metabolites in human plasma were injected directly into the high-performance liquid chromatography system via a loop-column to pre-extract the drugs from the plasma, and quantitated against a multilevel calibration curve with adriamycin as the internal standard. The column effluent was monitored with an electrochemical detector at an applied oxidative potential of 0.65 V and by fluorescence. Daunorubicin and four metabolites were separated and characterized by this method. In a blinded evaluation of accuracy and precision, the mean coefficients of variation were 3.8, 3.6 and 9.8% at concentrations of 150, 75 and 15 ng/ml, respectively, and blank samples gave negligible readings. The amperometric sensitivity was greater than achieved by fluorescence detection, and offers an alternative method for quantitation of these compounds. The new method has a limit of detection of less than 2 ng on column, allowing quantitation of < 10 ng/ml in plasma samples without organic extraction prior to chromatographic analysis.

INTRODUCTION

Daunorubicin is an anthracycline antibiotic which has been under clinical investigation since 1965, and has proven to be an effective antitumor agent [1, 2]. It is generally accepted that daunorubicin exerts its effects by intercalaction between the base pairs in the DNA helix in the cell nucleus, thereby preventing their replication [3, 4], although there is still uncertainty concerning the precise mode of action of the drug, its distribution and metabolism in plasma, tissue, and leukemia cells, and the possible cytotoxic role of

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intermediate metabolites. Since the majority of human pharmacokinetic studies have used assays which measure the fluorescence of the parent drug and metabolites, detection of potentially non-fluorescent metabolites was not possible, while the potential for non-fluorescent human metabolites of anthracyclines has previously been suggested [5, 6]. Therefore, the development of a sensitive and selective alternative analytical method should facilitate future investigations of anthracycline pharmacokinetics. Also, previous high-performance liquid chromatographic (HPLC) methods have generally required organic extraction of these drugs and metabolites from biological fluids by methods which are frequently inefficient (<60%) [7—11]. The present study describes a selective and sensitive isocratic HPLC method for the separation and electrochemical quantitation of daunorubicin and four potential metabolites. The new method is simple, rapid, requires no organic extraction of plasma or serum and has more sensitivity than HPLC with fluorescence methods.

MATERIALS AND METHODS

Chemicals and reagents

Daunorubicin (DNR), daunorubicinol (DOL) and daunorubicin-aglycone (DNR-AGLY) were obtained from Dr. A. Goodman, Ives Laboratories (New York, NY, U.S.A.). 7-Deoxydaunorubicinol-aglycone (7-d-DOL-AGLY) and 7-deoxydaunorubicin-aglycone (7-d-DNR-AGLY) were obtained from Drs. Nahed Ahmed and N.R. Bachur. Adriamycin (doxorubicin hydrochloride, ADR) was obtained from Adria Labs. (Columbus, OH, U.S.A.).

Acetonitrile was chromatographic grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and sodium acetate was analytical grade from Fisher Scientific (Pittsburgh, PA, U.S.A.).

High-performance liquid chromatography

Plasma samples (usually 250 μl) spiked with 25 ng of adriamycin (internal standard) were injected directly into a loop-column (3.9 × 2.3 mm) connected to the injector (Rheodyne 7125) of the LDC Model III high-performance liquid chromatograph, as previously described by Riley and Evans [12] for the analysis of doxorubicin. The analytical column was attached at port 2 rather than port 3 and the pump was connected at port 3 instead of port 2, providing a one-way flow of mobile phase through the injection loop-column. After the plasma sample was loaded onto the loop-column, potentially interfering substances (polar compounds, proteins, etc.) were removed by washing the loop-column (still in "load" position) with 1.0 ml of deionized water. The compounds of interest were then eluted from the loop-column and subsequently the analytical column by the mobile phase, after the injector was put in the "inject" position. After 1 min the injector was returned to the load position and the loop-column prepared to receive another sample by washing with 1—2 ml of filtered deionized water. The analytical column was a 30 cm × 3.9 mm μBondapak C_{18} 10 μm column (Waters Assoc., Milford, MA, U.S.A.), which was eluted isocratically at room temperature using a mobile phase containing acetonitrile—water—acetic acid (28:71:1) adjusted to pH 4.0 with 20% sodium acetate. Flow-rates were adjusted to 2.0 ml/min and eluates were

detected with a Gilson fluorescence detector (Spectra/Glo, Model FL-IA) and an LC-3 amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) in series. The fluorometer was equipped with a 45-μl flow cell, a 480-nm excitation filter and a 560-nm emission filter (Doxorubicin Filters, Gilson). The electrochemical detector was used at an applied oxidative potential of 0.65 V. Chromatograms were traced on a Fisher Series 5000 dual-pen chart recorder at a speed of 0.25 cm/min and at a setting of 1.0 and 0.01 V for the electrochemical and fluorescence detectors, respectively.

Precision and accuracy

To assess precision and accuracy of the new method, three 5-ml serum samples obtained from volunteers not receiving anthracyclines were spiked with 15, 75 and 150 ng/ml of DNR, DOL and DNR-AGLY. Each of these three samples were vortexed and then aliquoted into 8 equal volumes, coded, placed in random order, and frozen at −70°C, then subsequently analyzed by a single investigator not involved in the preparation of these samples. Three samples were analyzed, in duplicate, each day until all 24 samples had been analyzed. Mean values and coefficients of variation (C.V.) were computed after all samples had been analyzed. All analysis for the precision and accuracy studies were performed using electrochemical detection.

The extraction efficiency of the loop-column method was assessed by comparing peak heights of the compounds of interest injected onto the loop-column in 250 μl of plasma against the peak height of the identical amount injected directly onto the chromatographic column in methanol.

RESULTS

The new HPLC method, using a μBondapak C_{18} column eluted isocratically with a mobile phase consisting of acetonitrile—water—acetic acid (28:71:1) adjusted to pH 4.0 with sodium acetate, resolved DNR and all metabolites (Fig. 1). ADR was also resolved from all daunorubicin metabolites permitting its use as an internal standard for quantitation.

Linearity and sensitivity of the electrochemical detector were determined from serial concentrations of parent drug and metabolites. Linear calibrations were obtained for DNR, DOL and DNR-AGLY in the concentration range of 15—150 ng/ml (Fig. 2). The lowest amount of DNR detectable, defined as 2.5 times the noise level, was 2 ng which compares very favorably to other published detection methods [13—15] and was achieved at 1/10 of the maximum detector sensitivity. This amount corresponds, for the 250 μl injected into the loop column, to a concentration of ca. 8 ng/ml. As can be seen in Fig. 1, the electrochemical detector signal (10-fold lower than maximum detector settings) was considerably greater than the fluorescence signal (detector at maximum sensitivity).

Fig. 1. also demonstrates the resolving power of the system used; the following four DNR derivatives were resolved with retention times (at a flow-rate of 2.0 ml/min) of 4.8 min for DOL, 6.5 min for 2-d-DOL-AGLY, 8.0 min for DNR, 12.6 min for DNR-AGLY, and 22.3 min for 7-d-DNR-AGLY. The corresponding capacity factors (k') were 2.8, 3.8, 4.7, 7.4 and 13.1, respectively (Table I).

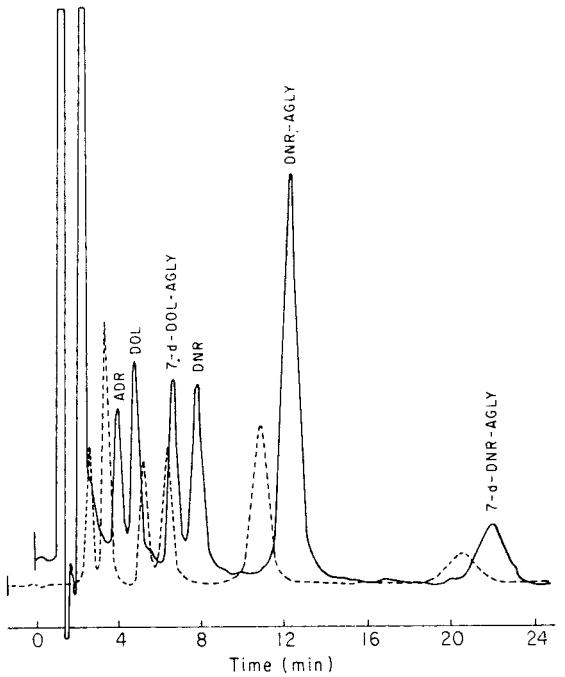

Fig. 1. Separation of a mixture of daunorubicin and metabolites in plasma by HPLC, with quantitation by fluorescence (— — —) and electrochemical (——) methods. The column was a μBondapak C_{18} (30 cm × 3.9 mm) and the solvent contained acetonitrile—water—acetic acid (28:71:1) adjusted to pH 4.0. Mobile phase flow-rate was 2.0 ml/min. The sample was a mixture of ADR (3.8 min), DOL (4.8 min), 7-d-DOL-AGLY (6.5 min), DNR (8.0 min), DNR-AGLY (12.6 min) and 7-d-DNR-AGLY (22.3 min).

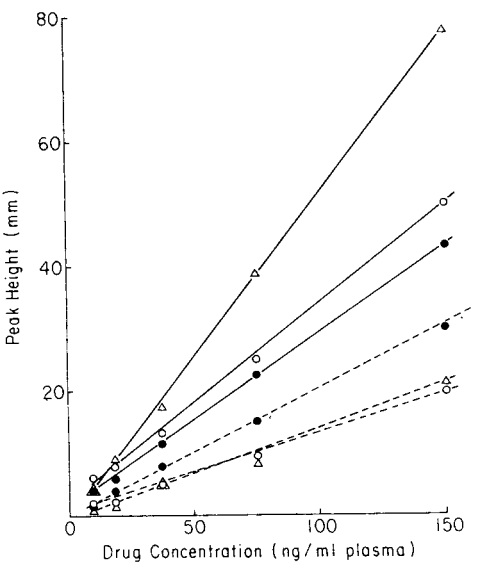

Fig. 2. Calibration graphs of peak height versus concentration for daunorubicin and metabolites in human plasma. Standard amounts of DNR (○), DOL (●), DNR-AGLY (△) were added to plasma in order to obtain drug concentrations of 4.69, 9.37, 19.75, 37.5, 75.0 and 150 ng/ml. Electrochemical (——) and fluorescence (— — —) detection.

TABLE I

DAUNORUBICIN AND METABOLITES SEPARATED BY REVERSED-PHASE HPLC

Compound	Abbreviation	Retention time (min)	k'
Adriamycin (internal standard)	ADR	3.8	2.2
Daunorubicinol	DOL	4.8	2.8
7-Deoxy-daunorubicinol-aglycone	7-d-DOL-AGLY	6.5	3.8
Daunorubicin	DNR	8.0	4.7
Daunorubicin-aglycone	DNR-AGLY	12.6	7.4
7-deoxydaunorubicin-aglycone	7-d-DNR-AGLY	22.3	13.1

TABLE II

SUMMARY OF PRECISION AND ACCURACY EVALUATION (n = 16) OF THE NEW HPLC—ELECTROCHEMICAL DETECTION METHOD FOR DAUNORUBICIN AND METABOLITES

Spiked concentration (ng/ml)	Internal standard method			Without internal standard		
	Measured (mean, ng/ml)	Accuracy (%)	Precision (C.V., %)	Measured (mean, ng/ml)	Accuracy (%)	Precision (C.V., %)
Daunorubicin						
150	151.0	100.7	3.9	151.4	100.9	3.6
75	75.1	100.1	3.6	75.6	100.8	4.6
15	16.5	110.0	12.2	16.2	108.0	11.4
Daurorubicinol						
150	152.3	101.5	4.6	148.8	99.2	2.9
75	76.0	101.3	3.4	75.1	100.1	3.4
15	16.5	110.0	7.1	16.8	112.0	18.3
DNR-aglycone						
150	149.1	99.4	3.0	148.3	98.9	1.7
75	75.8	101.1	3.7	74.9	99.9	2.4
15	16.0	106.6	10.0	16.5	110.0	13.4

A summary of the precision and accuracy studies is presented in Table II. Since an internal standard may not necessarily improve the precision or accuracy of an HPLC method [16], the necessity of the internal standard in our new method was assessed by independently computing concentrations against two separate sets of calibration curves using either peak heights (Fig. 2) or peak height ratio of compound of interest/internal standard. Both calibration curves were linear over the concentration ranges evaluated. The lowest coefficients of variation and greatest accuracy were achieved using the internal standard method (i.e., peak height ratio). The coefficients of variation were highest at the lowest concentration (7.1—12.2%), and declined to less than 5% (3.0—4.6%) at the higher concentrations. All results were within 10% of the spiked values, with the greatest deviation (106—110%) at the lowest concentrations.

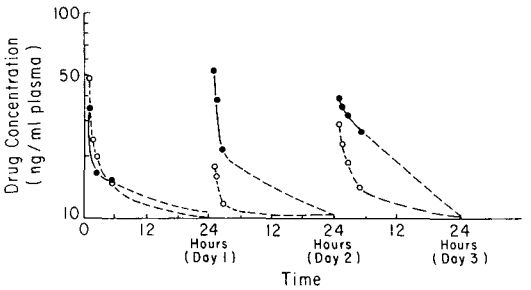

Fig. 3. Semilogarithmic plots of daunorubicin (○) and daunorubicinol (●) serum concentration versus time in a patient's plasma following 45 mg/m² daunorubicin administered intravenously on three consecutive days.

The extraction efficiency of daunorubicin and metabolites averaged 80 ± 6.2% over the concentration range evaluated.

Fig. 3 shows the plasma concentration—time profile of DNR and DOL measured by the new HPLC—electrochemical detection method in a patient with acute non-lymphocytic leukemia. Daunorubicin aglycones were not detectable in this patient's plasma.

DISCUSSION

The current study demonstrates that daunorubicin and four of its metabolites can be simultaneously separated and identified by a new HPLC technique using electrochemical detection. The procedure is simple and easy to perform. The use of the loop-column permits direct injection of plasma samples and overcomes the variability and inefficiencies of organic extraction procedures [7—11]. The electrochemical detector displayed high specificity and sensitivity, indicating the feasibility of determining anthracycline antineoplastic agents by nonfluorescent HPLC methods. The assay satisfied accepted analytical performance criteria and has been further validated by reference to a standard fluorescence detection method.

The chromatographic data from the patient's plasma samples following 45 mg/m² intravenous infusion indicate the extensive metabolism of daunorubicin following intravenous infusion, which could result in significant differences in plasma and leukemic cell exposure to parent drug and its principal cytotoxic metabolite (DOL). More extensive studies to establish the pattern of accumulation of DNR and metabolites in plasma and leukemia cells are needed, since previous work suggest that DNR metabolism may relate to clinical efficacy [17]. Previously described HPLC—fluorescence detection methods are affected by alterations of the anthracycline chromophore or changes in the quantum efficiency of chromophore fluorescence during human disposition. The new HPLC—electrochemical detection method offers a sensitive and specific alternative which is not dependent on fluorescence of parent drugs or potential metabolites.

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RAPID DETERMINATION OF TETRACYCLINE AND LUMECYCLINE IN HUMAN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and accurate method for the determination of tetracycline in human plasma and urine is presented. Determination of tetracycline in plasma is based on precipitation of plasma proteins with trifluoroacetic acid, followed by injection of the centrifuged plasma sample onto a μBondapak C_{18} column. Acetonitrile in phosphate buffer pH 2.2 is used as mobile phase. Only tetracycline, and no trace of lumecycline can be detected in plasma and urine after administration of lumecycline, indicating that lumecycline is completely degraded to tetracycline, lysine and formaldehyde in the gastrointestinal tract prior to absorption.

Determination of tetracycline in urine was performed by injection of urine diluted with phosphoric acid onto a μBondapak Phenyl column. The precision of determination of tetracycline in plasma, expressed as the relative standard deviation, was < 3% at tetracycline concentrations of 0.05 and 3.7 μg/ml. Urine determinations were made with a precision of < 1.5% at tetracycline concentrations of 0.5 and 6.7 μg/ml.

INTRODUCTION

Different techniques such as microbiological [1, 2] and fluorimetric methods [3—5] have been applied for the analysis of tetracyclines. However, these methods suffer from a lack of selectivity. By use of a chromatographic method, like high-performance liquid chromatography (HPLC), this problem can be overcome. During the last few years numerous papers have been published on the chromatography of the tetracyclines and their potential impurities [6—12].

A few papers, which deal with the determination of tetracycline in biological material using HPLC, have also been published [13—16]. The determination of tetracycline in plasma is based on extraction of tetracycline as a complex with calcium [13—15] or as an ion pair [16] into an organic solvent, followed by

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extraction into an acidic water phase prior to its chromatographic isolation. Determination of tetracycline in urine has been performed by injection of a urine extract [13—15], or by injection of diluted urine onto the column [16]. To obtain a sufficiently high detection selectivity for tetracycline in plasma and urine from cattle, sheep and swine, Sharma and co-workers [13, 14] monitored the eluate at 355 nm. Detection of tetracycline in this range (357 nm) was later applied for the analysis of tetracycline in human plasma and urine [16].

The present paper describes a rapid method for the determination of tetracycline and lumecycline in human plasma and urine. The time-consuming extractions of tetracycline from plasma are replaced by a rapid isolation step. The method is based on precipitation of the plasma proteins with trifluoroacetic acid, followed by injection of the centrifuged plasma sample onto the column. The eluate is monitored at 357 nm. The aim of this study was to design a rapid and accurate method suitable for use in a comparative study of the bioavailability of tetracycline and lumecycline in man.

EXPERIMENTAL

Apparatus

The pump was an Altex Model 100 solvent delivery system and the injector a Waters Model U6K. A Spectra-Physics Model 770 UV detector with variable wavelength was used. The detector was operated at 357 nm. Peak areas were calculated using a Hewlett-Packard 3370 B integrator. pH was measured with an Orion Research Model 701 A digital pH-meter, with an Ingold Type 401 combined electrode.

Chemicals

Tetracycline hydrochloride was kindly supplied by ACO Läkemedel AB (Solna, Sweden) and lumecycline was obtained from Carlo Erba (Milan, Italy). Capsules containing tetracycline hydrochloride or lumecycline corresponding to 300 mg of tetracycline as base were prepared in a Swedish pharmacy. The solid phases μBondapak C_{18} (10 μm) and μBondapak Phenyl (10 μm) were obtained from Waters Assoc. (Milford, MA, U.S.A.). The acetonitrile was of grade S quality and purchased from Rathburn Chemicals (Walkerburn, Great Britain). Trifluoroacetic acid (TFA) für die Spectroskopie was obtained from E. Merck (Darmstadt, G.F.R.). All other chemicals used were of analytical grade and used without further purification.

Column preparation

The columns 100 × 3.2 mm (for plasma samples) or 150 × 3.2 mm (for urine samples) were made of 316 stainless steel with a polished inner surface, equipped with modified Swagelok connections and Altex stainless-steel frits (2 μm).

The columns were packed by a modification of the balanced density slurry technique described previously [17]. The support was suspended in chloroform (10 ml chloroform per solid phase) 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 6.2 MPa. After packing, the columns were washed by

pumping 100 ml of acetonitrile through the columns followed by 100 ml of acetonitrile—water (1:1).

Plasma and urine samples

Blood samples were collected in 10-ml heparinized Venoject tubes according to a protocol approved by our Ethical Committee. Plasma was separated immediately after collection and frozen at −20°C until analyzed. Urine samples were stored at −20°C until analyzed.

Standard curves

The standard samples were prepared by spiking drug-free plasma and urine with tetracycline. Tetracycline HCl was dissolved in 0.1 *M* phosphoric acid.

Determination of tetracycline in biological material

Plasma. To 0.5 ml of plasma 65 μl of TFA were added. The tube was agitated in a Whirl mixer for 0.5 min and centrifuged for 5 min at 5400 *g*. Two hundred and fifty microlitres were injected onto the μBondapak C_{18} column. The column was eluted with phosphate buffer pH 2.2 (μ = 0.1) containing 16% (v/v) acetonitrile. The flow-rate was 0.5 ml/min.

Urine. The urine was diluted with 0.1 *M* phosphoric acid (1:1) and 25 μl (or more if needed) were injected onto the μBondapak Phenyl column. The mobile phase and the flow-rate were the same as above.

RESULTS AND DISCUSSION

Chromatographic retention, selectivity and peak symmetry

The retention of tetracycline and its most commonly found impurity, epitetracycline, is easily regulated by varying the acetonitrile concentration

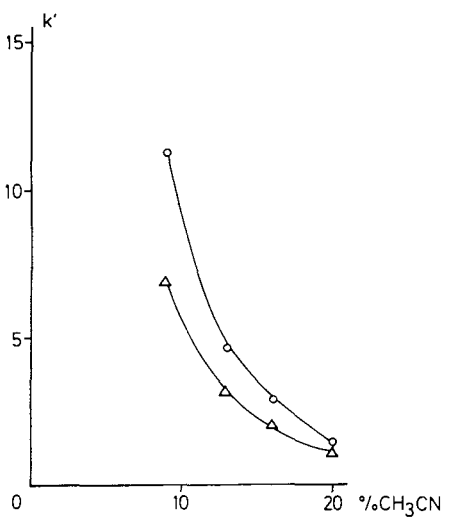

Fig. 1. Regulation of the capacity factor (k') with acetonitrile. Mobile phase: sodium phosphate buffer, pH 2.2, containing acetonitrile. Solid phase: μBondapak C_{18} (10 μm). Column: 100 × 3.2 mm. Samples: tetracycline (○), epitetracycline (△).

in the mobile phase as demonstrated in Fig. 1. The separation factor (α) decreases with increasing acetonitrile concentration. Baseline separation between tetracycline and epitetracycline was obtained on a 100 × 3.2 mm μBondapak C_{18} column within 7 min using a mobile phase of 16% acetonitrile in phosphate buffer pH 2.2. The separation factor was 1.42.

Wide variations in the chromatographic properties of tetracyclines have been observed using solid phases obtained from different manufacturers, despite the fact that the nominal alkyl chain lengths attached were identical [12]. By use of μBondapak C_{18} or μBondapak Phenyl as solid phase almost symmetrical peaks were obtained (asymmetry factor 1.2). This is a prerequisite for high sensitivity for the method. Tetracyclines have been isolated from plasma and urine using LiChrosorb RP-2 as solid phase and a mobile phase similar to the above [16]; however, there was a significant tailing of the tetracycline peak in this separation system. LiChrosorb RP-8 has also been used for chromatography of tetracyclines. It was, however, necessary to add tertiary amines to the mobile phase [10] or to pretreat the solid phase [18] in order to obtain good chromatographic conditions for tetracyclines using this solid phase. Sharma and Bevill [14] reported that new μBondapak C_{18} columns must be conditioned before use, in order to obtain reproducible results in chromatography of tetracyclines. These data disagree with the present findings and there is no obvious explanation for the discrepancy. However, a slightly higher asymmetry factor of the tetracycline peak was observed during the first injections on a new column.

Stability of tetracycline and lumecycline

Due to the sensitivity to epimerization, dehydration and oxidation of tetracyclines [19–21] the stability of tetracycline at different stages of the method was investigated. The stability of tetracycline in plasma stored at room temperature was investigated by repeated injection of a plasma sample containing 2.1 μg/ml tetracycline. No significant degradation was observed after 6 h.

Before injection of the plasma samples onto the column the plasma

TABLE I

STABILITY OF TETRACYCLINE IN PLASMA AFTER PRECIPITATION OF PROTEINS WITH TRIFLUOROACETIC ACID AND PERCHLORIC ACID

Trifluoroacetic acid		Perchloric acid	
Storage time (min)	Area (μV·sec)	Storage time (min)	Area (μV·sec)
0	9723	0	9100
30	9578	30	8864
37	9401	60	8605
60	9365	67	8452
68	9278	120	8039
120	8998	129	8168
126	8840		

proteins were precipitated by addition of an acid. Two different acids were tested, perchloric acid (60 μl/ml of plasma) and TFA (130 μl/ml of plasma). The stability of tetracycline in plasma after precipitation of proteins was investigated chromatographically and the results are demonstrated in Table I. There was a 12% decrease of the tetracycline concentration in plasma samples precipitated with perchloric acid after storage for 120 min at room temperature. Tetracycline was slightly more stable in plasma precipitated with TFA, and the tetracycline concentration decreased 8% under the same conditions. TFA was therefore preferred for the bioanalytical method. To avoid problems with degradation of tetracycline the plasma samples were injected onto the column directly after precipitation and centrifugation.

In a recent publication lumecycline was shown to be very sensitive to degradation in water solutions of pH 2.2 and 7.5 [10]. Under these conditions lumecycline was degraded very quickly to tetracycline, lysine and formaldehyde. Healthy volunteers were administered a lumecycline capsule containing lumecycline corresponding to 300 mg of tetracycline. Only tetracycline and no trace of lumecycline could be detected in their plasma 0.5, 1 and 3 h after administration of the capsule using the method described under Experimental for plasma analysis, with the exception that the column was eluted with 9% acetonitrile in phosphate buffer pH 2.2. This suggests that lumecycline is completely degraded to tetracycline in the gastrointestinal tract prior to absorption. Blank plasma spiked with lumecycline was also analysed to ensure that lumecycline can be detected in original plasma samples after treatment with TFA. Two peaks were obtained in the chromatogram, corresponding to lumecycline and tetracycline, respectively. From the above results it is obvious that lumecycline is completely degraded by the time it reaches the systemic circulation and can be analysed as tetracycline.

The stability of tetracycline in urine (pH 6.0) at room temperature was also determined by repeated injection of a urine sample containing 6.7 μg/ml tetracycline. A 5.3% decrease in the tetracycline concentration was observed after 22 h.

Handling of plasma samples before chromatography

Tetracyclines have previously been determined in plasma using extraction of tetracycline into an organic phase as a complex with calcium [13,14] or as an ion pair [16], followed by extraction into an acidic water phase and chromatographic isolation. The extraction procedures are very time-consuming, and help to decrease the precision of the determinations. Therefore precipitation of the plasma proteins is preferable in the isolation of tetracycline prior to the chromatographic step. The plasma proteins were precipitated in three different ways, using either acetonitrile, perchloric acid or TFA.

To precipitate the plasma proteins in 1.0 ml of plasma, 1.0 ml of acetonitrile, 60 μl of perchloric acid or 130 μl of TFA were required. Chromatography of an acetonitrile-precipitated plasma sample containing tetracycline resulted in elution of tetracycline in the front, using the separation system described under Experimental. This is the result of the high acetonitrile con-

centration of the plasma sample (50%, v/v) compared with that in the mobile phase (16%, v/v), and the pH of the sample (7—7.5) under which conditions the tetracycline exists in zwitterionic form [7]. Better results were obtained by chromatography of plasma samples precipitated with perchloric acid or TFA. This is probably caused by the lack of acetonitrile in the injected sample and the low pH of the acid-precipitated sample, where tetracycline exists as a singly charged cation. No significant differences in the chromatographic properties of tetracycline were observed by injection of a plasma sample precipitated with perchloric acid or TFA. However, TFA was preferred because of the slightly higher stability of tetracycline in TFA than in perchloric acid solutions (see above).

Recovery and precision

The recovery of tetracycline from plasma was studied at two different concentrations, 0.12 and 3.50 μg/ml. Drug-free plasma and phosphate buffer pH 7.5 ($\mu = 0.1$) were spiked with tetracycline and analysed according to the method described under Experimental. The recovery of tetracycline was calculated by comparing the data from the two series and the results are summarized in Table II.

The precision of the determination of tetracycline in plasma was determined by analysing original plasma samples containing three different concentrations of tetracycline, 0.06, 0.73 and 3.70 μg/ml. The precision of analysis of tetracycline in urine containing 0.5 and 6.6 μg/ml was determined using the described procedure. The relative standard deviations were calculated and the results are summarized in Table III.

TABLE II

RECOVERY OF TETRACYCLINE FROM PLASMA

Tetracycline conc. (μg/ml)	Recovery* (%)
0.12	68.7 ± 2.3
3.50	72.8 ± 1.1

*Recovery ± relative standard deviation ($n = 5$).

TABLE III

PRECISION OF PLASMA AND URINE DETERMINATIONS

Sample	Tetracycline conc. (μg/ml)	Relative S.D.* (%)
Plasma	0.05	2.66
Plasma	0.73	1.31
Plasma	3.70	2.69
Urine	0.50	1.40
Urine	6.67	1.30

*Calculated for $n = 5$.

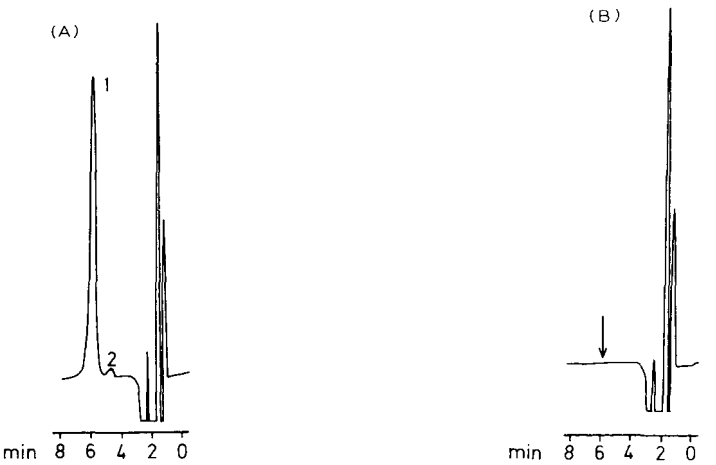

Fig. 2. (A) Chromatogram of a plasma sample containing 2.45 μg/ml of tetracycline (1) and epitetracycline (2). (B) Blank plasma chromatogram. Mobile phase: 16% acetonitrile in phosphate buffer, pH 2.2 (μ = 0.1). Column and solid phase as in Fig. 1.

Determination of tetracycline and lumecycline in plasma and urine

The plasma concentration of tetracycline and lumecycline (measured as tetracycline, see above) was determined as described under Experimental. Fig. 2A demonstrates the isolation of tetracycline from human plasma 4 h after administration of a capsule containing lumecycline corresponding to 300 mg of tetracycline. A blank plasma chromatogram is demonstrated in Fig. 2B. Using the described conditions, no interfering peaks were present in the chromatograms. This is largely the result of the high detection selectivity achieved at 357 nm [13, 16].

The quantitation of tetracycline was performed using external standards prepared as described under Experimental. Standard curves were prepared by plotting the peak areas against the sample concentration. The curves were linear in the studied concentration range, 0.1—4 μg/ml. Correlation coefficients were in all cases better than 0.9990.

With repeated injection of plasma samples precipitated as described under Experimental, there was a small tendency for pressure to increase, although no cavity was formed on the top of the column. This problem was overcome by changing the stainless steel frit on the column top after injection of about 125 plasma samples. The pressure increase is probably caused by accumulation of precipitated proteins on the frit.

The retention time of tetracycline and the resolution between tetracycline and epitetracycline decreases after repeated injections of plasma samples. This effect can be due to changes in the solid phase, due to the strongly acidic plasma samples injected, or to coating of the solid phase with components from the plasma samples. The column was repacked twice during the analysis of 750 plasma samples.

Figs. 3A and B demonstrate the plasma concentrations of tetracycline in two subjects who received a single oral dose of tetracycline (300 mg) and lumecycline (corresponding to 300 mg of tetracycline), respectively. The half-

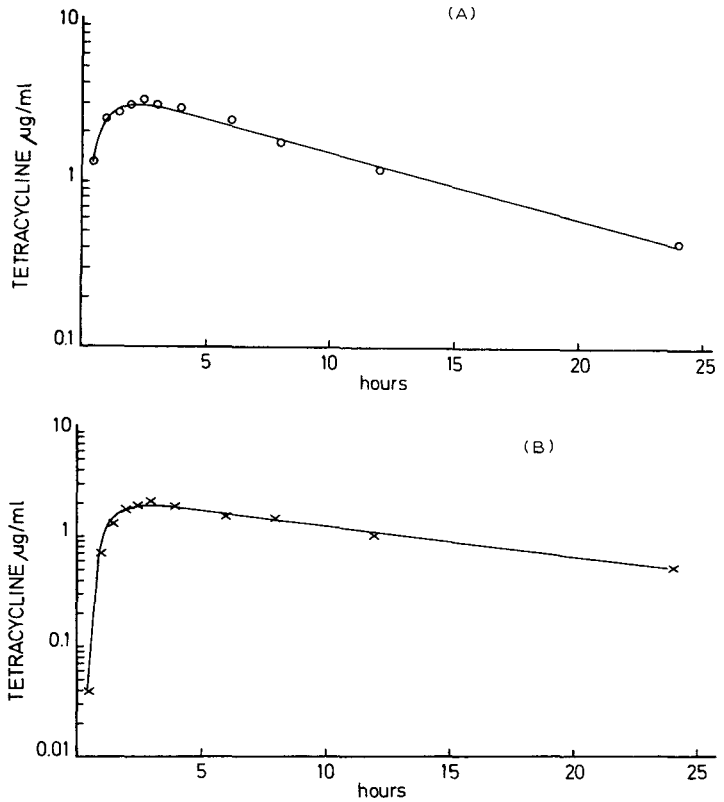

Fig. 3. Plasma concentrations in two subjects receiving 300 mg of tetracycline (A), and lumecycline (B) corresponding to 300 mg of tetracycline. (○) Tetracycline, (×) lumecycline (determined as tetracycline).

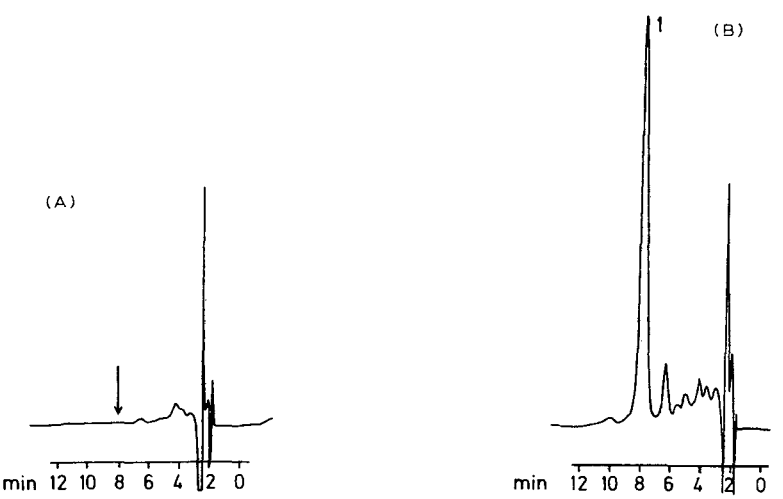

Fig. 4. Isolation of tetracycline from human urine. (A) Blank urine chromatogram. (B) Chromatogram of urine containing 6.6 µg/ml tetracycline. Solid phase: µBondapak Phenyl. Column: 150 × 3.2 mm. Mobile phase: as in Fig. 2.

lives of tetracycline were calculated from the curves and were found to be 7.4 and 10.5 h for tetracycline and lumecycline, respectively.

Urine samples containing tetracycline were analyzed as described under Experimental. Fig. 4A and B demonstrate a blank urine chromatogram and a chromatogram of urine from a subject 16 h after receiving an oral dose of lumecycline (corresponding to 300 mg of tetracycline). μBondapak Phenyl was preferred as solid phase for the urine analysis of tetracycline, because of the more selective isolation of tetracycline from endogenous compounds obtained on this solid phase compared with μBondapak C_{18}. No deterioration of the column has been observed after injection of urine onto the column. Quantitation of tetracycline in urine was performed as described for plasma samples (see above).

The described methods are now in use in a study for comparison of the bioavailability of tetracycline and lumecycline.

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Note

Prepurification and derivatization of α-keto acids using hydrazide gel

Application in gas chromatography and gas chromatography—mass spectrometry

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Several methods [1—8] have been reported for the analysis of α-keto acids by means of gas chromatography and/or gas chromatography—mass spectrometry using certain kinds of derivatization. The methods most widely employed have been those using 2,4-dinitrophenylhydrazine [9, 10]. However, with these methods chromatographic separations were complicated since the derivatives of α-keto acids exist as *syn—anti* isomers. Moreover, no effective clean-up methods have been reported. In a previous paper [11] we reported that the clean-up and derivatization of α-keto acids using hydrazide gel was very effective for the high-performance liquid chromatographic method. This present paper describes the application of this pretreatment to the gas chromatographic analysis of α-keto acids in biological samples.

MATERIALS AND METHODS

Apparatus

The gas chromatograph (Model 163, Hitachi, Tokyo, Japan) used was equipped with a flame ionization detector. The separation was carried out using a Dexsil 300GC capillary column (20 m × 0.25 mm I.D., support-coated,

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open-tube capillary column; Gasukuro Kogyo Co., Tokyo, Japan), of which the split ratio was 1:30. Chromatography was conducted at an initial temperature of 160°C with temperature programming at the rate of 2°C/min to a temperature of about 250°C, then isothermally at 310°C for 10 min. The injection port temperature was 320°C and the detector temperature was 300°C. The nitrogen carrier gas flow-rate was 1.2 ml/min. Gas chromatographic—mass spectrometric measurement was performed on a Hitachi Model M-80/M-003 using an electron-accelerating energy of 20 eV. Conditions for gas chromatography were as described above, with the following exceptions: the carrier gas (helium) flow-rate was 1.0 ml/min, and the temperature program was 5°C/min (160—260°C).

Reagents

Sodium pyruvate (PA), sodium α-ketoisovalerate (KIVA), sodium α-ketoisocaproate (KICA), sodium α-keto-β-methylvalerate (KMVA), sodium α-ketobutyrate (KBA), sodium α-ketovalerate (KVA), and phenylpyruvic acid (PPA) were obtained from Sigma (St. Louis, MO, U.S.A.). *o*-Phenylenediamine sulfate was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and used after recrystallization from a mixture of 1% aqueous sulfuric acid and ethanol (1:1). Bio-gel P-60 (100—200 mesh) was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Wako Pure Chemicals (Osaka, Japan). The other reagents and solvents were reagent grade.

The preparation of hydrazide gel was as follows. Dry polyacrylamide beads (15 g) were allowed to swell overnight in distilled water (200 ml) contained in a siliconized, conical glass-stoppered flask. The flask with the gel suspension and a glass-stoppered cylinder containing 98% hydrazine hydrate (120 ml) were immersed in a constant-temperature water bath (50°C). After 45 min the hydrazine hydrate was added to the gel, the flask was stoppered, and the mixture was stirred by an immersible magnetic stirrer for 6 h. At the end of the reaction period, the gel was washed with 0.1 *M* NaCl on a Buchner funnel until the washings were essentially free of hydrazine. The gel was stored under refrigeration suspended in an approximately pH 7.3 buffer of the following composition: 0.20 *M* NaCl, 0.02 *M* disodium EDTA, 0.10 *M* H_3BO_3, 0.005 *M* NaOH and $5 \cdot 10^{-6}$ *M* pentachlorophenol. The specific capacity of the gel was measured in the following manner. A certain volume of the gel was resuspended in 0.1 *M* NaCl, and a certain excess of pyruvate was added to the suspension and the solution was made moderately acidic with 0.1 *M* acetic acid. Then the gel was filtered and washed with 0.1 *M* NaCl. The filtrates and washings were combined. The amount of pyruvate in this solution was determined using the high-performance liquid chromatographic method. Next, pyruvate trapped in the gel was estimated.

Reagent preparation

All aqueous reagent solutions were prepared with redistilled water. The standard stock solutions of α-keto acids were prepared separately at the concentrations of 2 μmol/ml in water or 10% aqueous ethanol (PPA). A standard mixture of α-keto acids was prepared by mixing the standard stock solutions

and diluting with redistilled water to contain 400 nmol of each α-keto acid per milliliter. *o*-Phenylenediamine solution was prepared by dissolving 40 mg of *o*-phenylenediamine sulfate in 40 ml of 2 *N* HCl.

Prepurification and derivatization of α-keto acids in human urine

Three milliliters of 0.2 *M* aqueous acetic acid and 6 ml of 0.1 *M* NaCl solution were added to 3 ml of human urine. The mixture was poured into a siliconized glass column (150 mm × 8 mm I.D.) containing 1 ml of hydrazide gel. After the column was drained, the gel was washed with 20 ml of 0.1 *M* NaCl solution. The gel was then transferred to a test tube. Four milliliters of *o*-phenylenediamine solution were added to it and the mixture was warmed in a water bath at about 80°C for 2 h. At the end of the reaction period, the reaction mixture was diluted with 16 ml of a saturated sodium sulfate solution. The products were extracted into 20 ml of ethyl acetate by shaking for 5 min. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was redissolved in 10 μl of pyridine and reacted with 50 μl of BSTFA. About 0.1 μl of the reaction mixture was injected into the gas chromatograph.

Prepurification and derivatization of α-keto acids in human plasma

Ten milliliters of methanol were added to 3 ml of human plasma and shaken vigorously and centrifuged. The methanol in the supernatant was evaporated on a rotary evaporator. Three milliliters of 0.2 *M* acetic acid solution and 6 ml of 0.1 *M* NaCl solution were added to the residual solution and mixed. The solution was then treated in the same manner as with the human urine sample.

RESULTS AND DISCUSSION

Figs. 1 and 2 show a typical chromatogram and mass spectra, respectively, as obtained from a standard mixture of TMS-quinoxalinols derived from α-keto acids. The good separation, especially in the case of the derivatives of KIVA, KMVA and KICA, was the result of using a glass capillary column instead of a packed column. The mass spectra in Fig. 2 were measured at the top of each peak and corrected by the background.

Fig. 3A shows the gas chromatogram of a normal human urine sample. The components which gave the same retentions as those of PA, KMVA and KICA derivatives were identified by comparing each mass spectrum with that of the corresponding authentic derivatives. The gas chromatogram shown in Fig. 3B is from a urine sample of a phenylketonuria (PKU) patient. The peak components of corresponding to PA and PPA were also identified by comparing the mass spectra.

Fig. 4 shows chromatograms of plasma samples from a normal human and from a patient with maple syrup urine disease (MSUD). The gas chromatogram of plasma from the MSUD patient was dominated by the large peaks of KMVA, KIVA and KICA derivatives. Virtually no peaks were found on the chromatograms except those from α-keto acids. The data mentioned above seem to indicate that our prepurification and derivatization were very effective for gas chromatography of α-keto acids in biological samples.

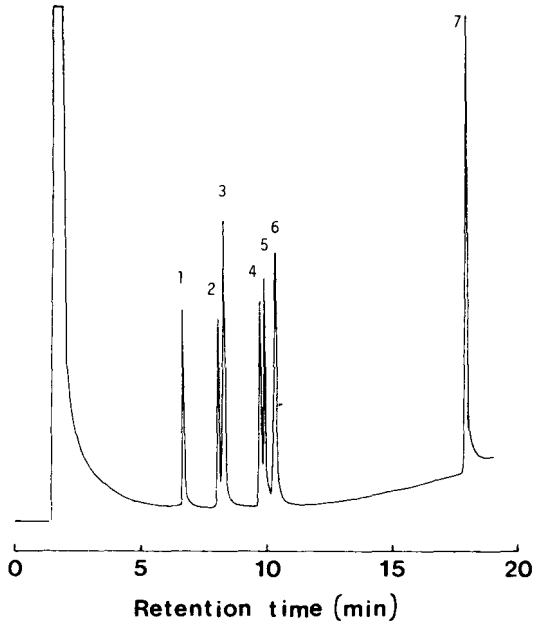

Fig. 1. Gas chromatogram of standard mixture of the TMS-quinoxalinol derivatives of the α-keto acids. Peaks: 1 = PA, 2 = KBA, 3 = KIVA, 4 = KVA, 5 = KMVA, 6 = KICA, 7 = PPA.

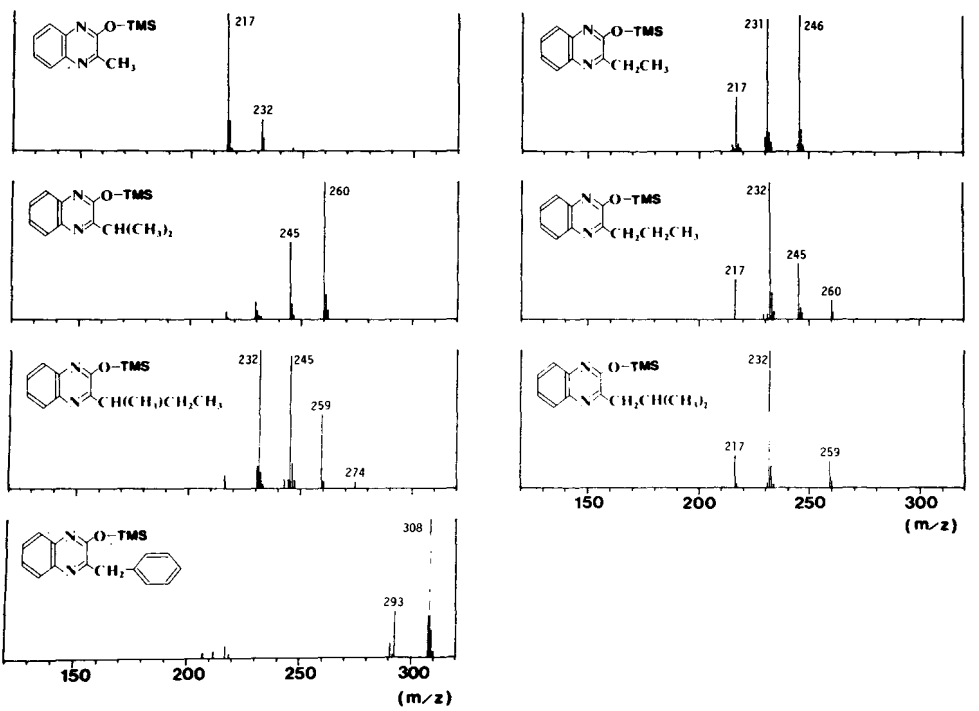

Fig. 2. Mass spectra of TMS-quinoxalinol derivatives of α-keto acids.

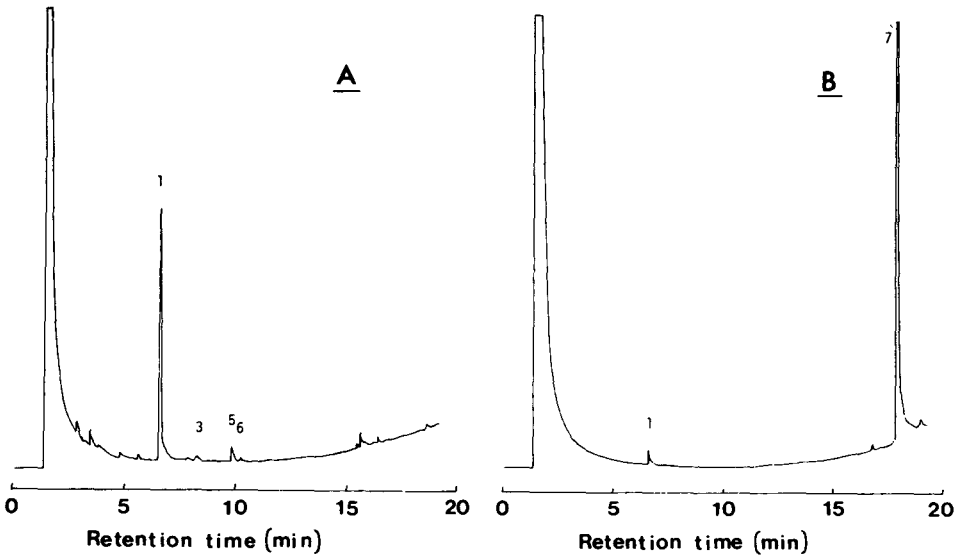

Fig. 3. Gas chromatograms obtained from urine samples of a normal subject (A), and of a PKU patient (B).

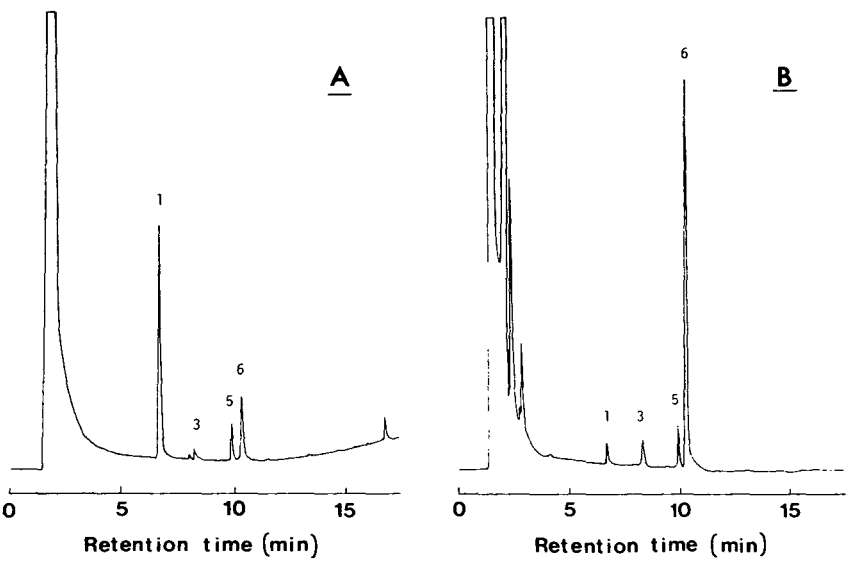

Fig. 4. Gas chromatograms obtained from plasma samples of a normal subject (A), and of a MSUD patient (B).

Most reported methods use extraction with some kind of organic solvent for prepurification and condensation after the reaction with reagent in an aqueous medium. Cree et al. [6] prepurified the branched-chain α-keto acids from physiological sources by deproteinization with acetone, and cation-exchange chromatography. However, these methods seem to be time consuming and not very effective.

The new prepurification and derivatization method which was confirmed to be very useful for the high-performance liquid chromatographic determination of α-keto acids in biological samples was also effective as a method of pretreatment for gas chromatography.

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Note

Simultaneous determination of methyl esters of α-hydroxy and nonhydroxy fatty acids from brain cerebroside by fused-silica capillary gas chromatography

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α-Hydroxy fatty acids are widely distributed in the complex lipids of biological materials. In particular, glycosphingolipids of nerve tissues contain most abundantly long-chain hydroxy fatty acids as well as straight-chain fatty acids [1]. Thus, when glycosphingolipids such as cerebroside and sulfatide from nerve tissue are methanolyzed with methanolic hydrochloride for the purpose of determining the fatty acid composition by gas—liquid chromatography (GLC), a mixture of methyl esters of hydroxy and nonhydroxy fatty acids is obtained, and they cannot be fully resolved on a packed column. Therefore, prior to GLC analysis, the methyl esters of hydroxy fatty acids have to be separated from those of nonhydroxy fatty acids with a column of Florisil [2] or silicic acid [3], or by preparative thin-layer chromatography (TLC) [4]. Furthermore, if packed columns are used for GLC, the determination of the fatty acid composition becomes time consuming because it is necessary to use two columns of different polarities to resolve fully the various classes of fatty acids. Recently, flexible fused-silica capillary columns have become available [5], which are highly efficient compared not only with packed columns but also with glass capillary columns, and they are easy to use [6, 7]. This report describes the determination of all classes of fatty acids of glycosphingolipids from nerve tissue in a single injection by the use of a fused-silica capillary column without prior separation or further derivatization.

MATERIALS AND METHODS

A Shimadzu GC-5A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a solventless injection system and a flame ionization detector was used,

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and modified to accept the fused-silica capillary column. The column was an OV-101 coated fused-silica capillary column, 25 m × 0.25 mm I.D., supplied by Shimadzu. The conditions of operation were: column temperature, 280°C (isothermal); carrier gas, nitrogen; flow-rate, 0.5 ml/min; split ratio, 1 : 8. Nitrogen gas was purified by passing it through stainless tubing packed with Molecular Sieve 5A. The chromatograms were recorded and the percentage composition of individual peaks calculated with a Chromatopack C-R1A (Shimadzu).

Cerebroside and sulfatide were isolated from crude lipid extracts of bovine brain white matter in our laboratory and proved to be pure enough for further analysis by TLC. Glycolipids were methanolyzed in 3% methanolic hydrochloride at 100°C for 3 h, and the methyl esters of fatty acids were extracted with petroleum ether (b.p. 30—60°C). An aliquot of the total fatty acid methyl esters thus obtained was separated into the hydroxy and nonhydroxy components on a silica gel H (Merck, Darmstadt, G.F.R.) plate which was prepared in our laboratory, using as solvent petroleum ether—diethyl ether (80:30, v/v) [4]. The two components were visualized with iodine vapor, scraped off separately from the plate, extracted and finally purified through a small column of silica beads. Standard samples of methyl esters of hydroxy and nonhydroxy fatty acids were products of Applied Science Labs. (State College, PA, U.S.A.).

RESULTS AND DISCUSSION

Fig. 1a clearly shows that baseline resolution was obtained for each of the 23 major fatty acids contained in brain cerebroside on a fused-silica capillary column. Fig. 1b and c are chromatograms of nonhydroxy and hydroxy fatty acid methyl esters, respectively, isolated by preparative TLC. Comparing Fig. 1a with Fig. 1b and c, it is evident that all of the nonhydroxy and hydroxy components from cerebroside were fully resolved on a single column. Fig. 2a, b and c also shows that total fatty acid methyl esters from sulfatide were well resolved on a single column as observed for those of cerebroside. This degree of separation of the methyl esters has not been attained with a packed column under any chromatographic conditions. In general, with a packed column of a nonpolar liquid phase such as SE-30 or OV-1, $C_{n:0}$ nonhydroxy and $C_{(n-1):1}$ hydroxy acid series, and $C_{n:1}$ nonhydroxy and $C_{(n-2):0}$ hydroxy acid series overlap or appear as one peak with leading or tailing shoulders*. For example, $C_{22h:0}$ and $C_{24:1}$, and $C_{25:0}$ and $C_{24h:1}$ which are contained in significant amounts in brain cerebroside were unresolved, respectively, on a packed column, so that proportions of total nonhydroxy and total hydroxy fatty acids could not be calculated by a single chromatography.

Table I compares percentage compositions of individual fatty acids calculated from chromatograms shown in Figs. 1 and 2. It is clear that values for a mixture of total fatty acids show good agreement with values from a separate chromatogram after preparative TLC. An earlier study [8] on the fatty acid composition of bovine brain lipids showed the relatively higher

*The numbers before and after the colon refer to the number of carbon atoms and double bonds, respectively, and h before the colon indicates hydroxy fatty acid.

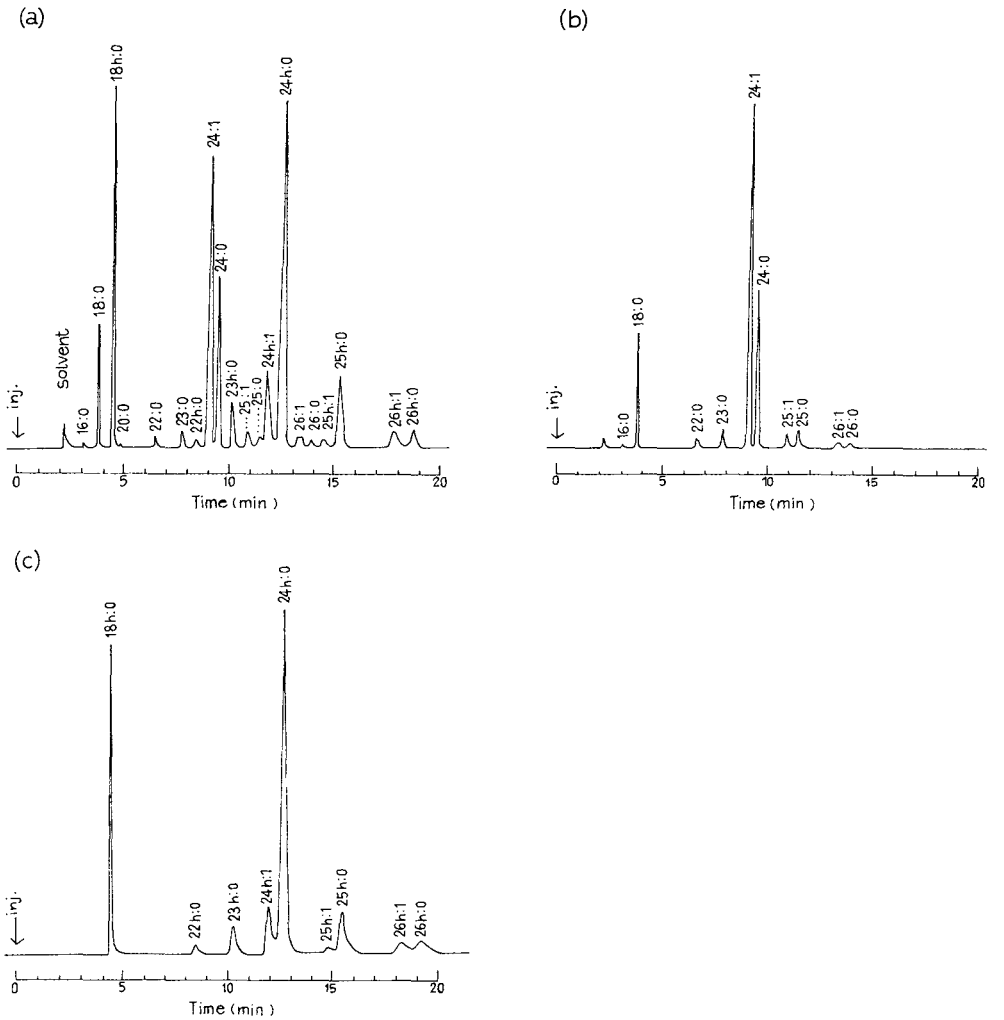

Fig. 1. Gas chromatograms of fatty acid methyl esters from bovine brain cerebroside on an OV-101 coated fused-silica capillary column. The analytical conditions were as described in Materials and methods. (a) Total fatty acid methyl esters from cerebroside; (b) nonhydroxy fatty acid methyl esters isolated by preparative TLC; (c) hydroxy fatty acid methyl esters isolated by preparative TLC. Individual esters were identified by comparison with standard samples and with the aid of a diagram of log retention times.

concentration of 16:0, 18:1, 22:0 and 24h:1 acids, and a lower concentration of 24:0 and 24:1 and 24h:0, compared with the present findings. This discrepancy may primarily be due to the difference in materials used, that is, whole brain or white matter.

For the analytical conditions used in the present study there are still some improvements to be made; the required operating temperature was too close to the maximum allowable temperature, and peak width became greater with longer chain length, particularly for hydroxy fatty acids. Besides, the life-time of the column has to be more carefully investigated, although the same column

TABLE I

PERCENTAGE DISTRIBUTION OF FATTY ACIDS OF BRAIN CEREBROSIDE AND SULFATIDE BASED ON CHROMATOGRAMS AFTER CHROMATOGRAPHY ON AN OV-101 COATED FUSED-SILICA CAPILLARY COLUMN

The column was run isothermally, 280°C, and percentages of individual peaks were calculated with a Chromatopack C-R1A. Values are averages of three determinations and expressed as mean ± S.D.

Fatty acids	Cerebroside		Sulfatide	
	Total*	Separated**	Total*	Separated**
16:0	0.6 ± 0.11	0.3 ± 0.09		
18:0	8.0 ± 0.28	9.1 ± 2.13	1.6 ± 0.22	1.3 ± 0.14
20:0	0.5 ± 0.17	0.2 ± 0.06		
22:0	2.0 ± 0.38	1.8 ± 0.34	0.6 ± 0.16	0.9 ± 0.42
23:0	3.6 ± 0.56	2.5 ± 0.33	1.6 ± 0.44	2.0 ± 0.66
24:0	24.2 ± 0.99	22.9 ± 1.44	24.7 ± 2.26	26.5 ± 3.82
24:1	47.2 ± 4.90	53.9 ± 0.77	62.5 ± 5.38	57.6 ± 1.88
25:0	4.2 ± 1.33	3.5 ± 0.40	3.1 ± 1.14	4.6 ± 1.18
25:1	4.9 ± 0.74	3.5 ± 0.38	3.3 ± 0.60	3.9 ± 1.59
26:0	2.3 ± 0.59	1.1 ± 0.27	1.2 ± 0.54	1.6 ± 0.72
26:1	2.5 ± 0.70	1.2 ± 0.19	1.4 ± 0.20	1.7 ± 0.97
18h:0	17.1 ± 1.79	20.2 ± 4.14	4.7 ± 0.70	3.2 ± 0.22
22h:0	1.7 ± 0.34	1.3 ± 0.49		0.7 ± 0.03
23h:0	5.6 ± 0.78	4.2 ± 1.10	4.7 ± 0.31	3.0 ± 0.12
24h:0	41.5 ± 2.92	45.0 ± 3.21	52.9 ± 3.98	60.3 ± 1.75
24h:1	11.4 ± 0.36	9.6 ± 0.99	12.0 ± 0.25	8.4 ± 0.40
25h:0	11.0 ± 0.70	10.0 ± 1.55	13.0 ± 2.04	14.0 ± 0.43
25h:1	2.3 ± 0.41	1.5 ± 0.23	2.7 ± 0.18	1.3 ± 0.08
26h:0	4.6 ± 0.64	4.1 ± 1.06	5.1 ± 1.19	5.3 ± 0.30
26h:1	4.8 ± 0.65	4.0 ± 0.47	4.9 ± 0.14	3.8 ± 0.31
OH%***	63.8%		17.8%	

*Values were obtained from chromatograms of total fatty acid methyl esters (Figs. 1a and 2a), and expressed as percentages of total nonhydroxy or total hydroxy fatty acids.

**Values were obtained from chromatograms of methyl esters of nonhydroxy (Figs. 1b and 2b) and hydroxy (Figs. 1c and 2c) fatty acids separated by preparative TLC, and expressed as percentages of individual components.

***Weight percentage of hydroxy fatty acids of total fatty acids from cerebroside or sulfatide, obtained from chromatograms in Fig. 1a or Fig. 2a, respectively.

had been used for almost daily analysis over a period of several months without serious loss of column efficiency. In practice, we checked correction factors with reference mixtures of nonhydroxy fatty acid series, and found that the variation of those values was so minor that it did not significantly affect the percentage composition of the fatty acids from biological materials. We think, therefore, that the life-time of a fused-silica capillary column is fairly satisfactory, although its precise comparison with a packed column was not done for an operation period of longer than a year. Previously, Ackman [9] and Slover and Lanza [10] intensively discussed the problems encountered in GLC analysis using a glass capillary column. Recently, the more efficient fused-silica column has become more generally available and many of the limitations dis-

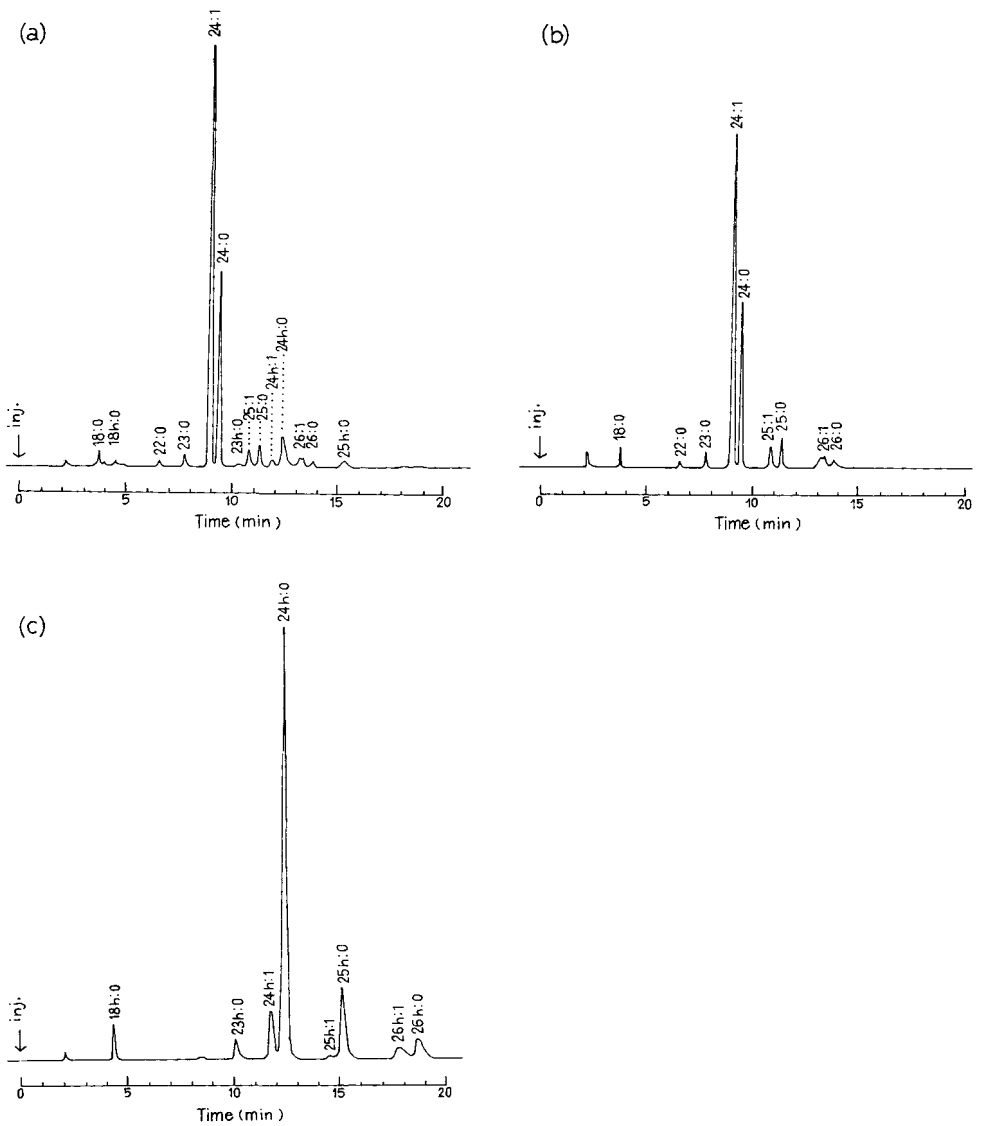

Fig. 2. Gas chromatograms of fatty acid methyl esters from bovine brain sulfatide on an OV-101 coated fused-silica capillary column. (a) Total fatty acid methyl esters from sulfatide; (b) nonhydroxy fatty acid methyl esters isolated by preparative TLC; (c) hydroxy fatty acid methyl esters isolated by preparative TLC. For other details see the legend to Fig. 1.

cussed above will probably be resolved in the near future [7]. Regardless of some limitations pointed out above, there are still some great advantages compared with a conventional method using a packed column: no pretreatment such as preparative TLC or derivatization is required and a fused-silica capillary column provides efficient and rapid separation of fatty acid methyl esters containing hydroxy and nonhydroxy components (within 20 min for the C_{26} hydroxy acid series). Previously, Tschöpe [11] reported the simultaneous

analysis of normal and α-hydroxy fatty acids on a packed column with ethylene glycol succinate. Prior to GLC, however, hydroxy fatty acids had to be converted to their acetyl derivatives, and, in addition, a considerably long time for chromatography was required (e.g. 30 min at least, for C_{22} hydroxy fatty acid). Recently, on a fused-silica capillary column a mixture of fatty acid methyl esters containing a few classes of hydroxy (chain length shorter than C_{16}) as well as nonhydroxy components has been separated [12]. However, this was a standard mixture, not biological material, and a fairly long analytical time was still required compared with that in the present study. To our knowledge, this is the first report of the simultaneous and full resolution of a mixture of hydroxy and nonhydroxy fatty acids from biological sources by GLC, and we believe that fused-silica capillary columns will become more widely used because of their high efficiency as well as the ease of handling.

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Note

Determination of prednisolone in plasma by high-performance liquid chromatography

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Recent clinical interest in the bioavailability of prednisolone (11β,17α,21-trihydroxy-$\Delta^{1,4}$-pregnadiene-3,20-dione) in paediatric applications indicates the need for a specific and sensitive assay. Unfortunately established radioimmunoassay techniques suffer from poor reproducibility [1] and poor specificity [2]. However, the introduction of high-performance liquid chromatography (HPLC) has led to the development of specific methods for the measurement of corticosteroids in biological fluids [3—8]. This paper describes an HPLC method which is both specific and sufficiently sensitive to allow the precise determination of prednisolone in small sample volumes usually available in paediatric practice. A mobile phase consisting of dichloromethane—ethanol—water—glacial acetic acid (500:30:30:1% total volume on separation, v/v) is used in conjunction with a 10-μm porous silica column. Operating at a flow-rate of 2 ml/min and employing ultraviolet (UV) detection at 254 nm permits the simultaneous determination of prednisolone, prednisone (17α,21-dihydroxy-$\Delta^{1,4}$-pregnadiene-3,11,20-trione) and cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione) concentrations in plasma.

EXPERIMENTAL

Chemicals

Ethanol (absolute alcohol AR grade) was obtained from James Burrough, London, Great Britain. Dichloromethane (general purpose reagent grade) and diethyl ether, glacial acetic acid, hydrochloric acid and sodium hydroxide ("Analar" quality) were all purchased from BDH, Poole, Great Britain. Prednisone, dexamethasone, cortisol and prednisolone were supplied by Sigma

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(London), Poole, Great Britain. [6,7(*n*)-^{3}H]prednisolone with a specific activity of 41 Ci/mmol was provided by the Radiochemical Centre, Amersham, Great Britain. Bovine serum albumin (30% solution) was acquired from Armour Pharmaceutical, Eastbourne, Great Britain.

General procedure

A 1-ml volume of heparinised plasma containing 150 ng of dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) as the internal standard, 1 ml of 0.1 *N* NaOH and 10 ml of ether—dichloromethane (60:40, v/v) were added to a 15-ml glass stoppered, round bottomed centrifuge tube and shaken for 10 min. After separation, achieved by centrifuging for 5 min at 1000 rpm (approx. 300 *g*), the organic phase was removed and washed by shaking with 1 ml of 0.1 *N* HCl for 5 min. Following centrifugation, as before, the organic layer was transferred to a 15-ml conical tube and evaporated to dryness under nitrogen at 55°C. The residue was reconstituted in 100 μl of the mobile phase and a 50-μl aliquot was injected onto the column.

Preparation of mobile phase

A 30-ml volume of water (double glass distilled), an equal volume of ethanol and 500 ml of dichloromethane were mixed for 1 h in a stoppered conical flask using a magnetic stirrer. Following separation in a glass separating funnel, the lower organic-rich layer was removed, and its volume determined. After the addition of glacial acetic acid (equivalent to 1% of the measured volume) and final mixing, the solvent was filtered through a 0.5-μm Millipore filter (type FH) using a Waters Assoc. Solvent Clarification Kit attached to a vacuum pump (approx. 15 inches of Hg).

Chromatography

The high-performance liquid chromatograph consisted of a Waters Assoc. Model 6000A constant volume pump, a U6K universal loop injector and a Model 440 UV detector. Absorbance was measured at 254 nm with an attenuation of 0.005 absorbance units full scale (a.u.f.s.). A standard 30 cm × 3.9 mm I.D. stainless steel μPorasil column (10 μm porous silica) was used in conjunction with a guard column packed with μBondapak C_{18}/Corasil. All of the chromatography equipment and columns were supplied by Waters Assoc., Hartford, Great Britain. The mobile phase, which was prepared fresh daily, was used at a flow rate of 2 ml/min (approximately 68 bars).

Preparation of steroid standards for calibration

Stock solutions of prednisolone and dexamethasone (100 μg/ml in ethanol) were diluted with ethanol to give standard solutions of 1 μg/ml and 10 μg/ml, respectively. From these, calibration standards were prepared in 1 ml of plasma or 3% bovine serum albumin so that each contained 25, 50, 100 or 150 ng of prednisolone and 150 ng of dexamethasone as the internal standard.

Extraction recovery experiment

Tritiated prednisolone (449,762 dpm) was added to 1-ml aliquots of plasma

TABLE I

EXTRACTION RECOVERIES OF [^{3}H]PREDNISOLONE FROM PLASMA

Prednisolone conc. (ng/ml)	[^{3}H]Prednisolone added (dpm)*	[^{3}H]Prednisolone recovered (dpm)**	[^{3}H]Prednisolone recovered corrected for volume losses (dpm)**	Percentage recovery**	Coefficient of variation** (%)
0	449 672.2	352 294.04	409 163.22	90.99	2.41
25	449 672.2	350 262.00	407 276.78	90.57	2.74
100	449 672.2	348 312.06	408 877.96	90.93	1.91

*Mean, $n = 4$.
**Mean, $n = 5$.

containing 0, 25 and 100 ng of prednisolone plus 150 ng of dexamethasone and extracted using the procedure described. The organic extract was transferred to a scintillation vial and evaporated to dryness. Ten millilitres of Lumagel scintillation cocktail (LKB-Wallac, Turku, Finland) were added and the samples counted in an LKB-Wallac Rack Beta Model 1215 liquid scintillation counter equipped with automatic quench calibration. Using the external standards ratio method of quench correction it was possible to determine counting efficiency and express all results as disintegrations per min.

RESULTS

It is readily apparent from Table I that the extraction efficiency (approximately 91%) and reproducibility (coefficient of variation < 2.74) are independent of prednisolone concentration. Calibration curves were obtained by comparing the peak height ratio (prednisolone/internal standard) with the actual concentration of prednisolone in spiked aliquots of plasma or 3% bovine serum albumin. A linear relationship exists over the concentration range 25—150 ng/ml, with the line passing through the origin. Slope values are 0.0052 and 0.0055 for 3% bovine serum albumin and plasma, respectively, and the correlation coefficient (r) is 0.999 in both cases.

The effects of sample storage on reproducibility of results were examined and the findings are listed in Table II. Analyses were carried out in duplicate, at weekly intervals, on ten replicate samples containing either 20 or 100 ng/ml prednisolone in 3% bovine serum albumin. These had been stored at −20° C for up to four weeks maximum. The mean recovery and coefficient of variation were 103.9% and 8.04% and 101.6% and 6.08% for prednisolone concentrations of 20 and 100 ng/ml, respectively. Clearly the method is valid and storage for one month at −20° C has no adverse effects.

TABLE II

DETERMINATION OF PREDNISOLONE IN 3% BOVINE SERUM ALBUMIN

Samples stored at −20°C and assayed in duplicate at weekly intervals over a period of four weeks.

Prednisolone added (ng/ml)	Prednisolone estimated* (ng/ml)	Recovery (%)	Coefficient of variation (%)
20	20.78	103.9	8.04
100	101.6	101.6	6.08

*Mean, $n = 10$.

The separation of prednisolone from other steroids was also examined. A representative chromatogram obtained following the injection of a mixture of prednisone, dexamethasone, cortisol and prednisolone is illustrated in Fig. 1. It can be seen that complete separation of these steroids is achieved by this method.

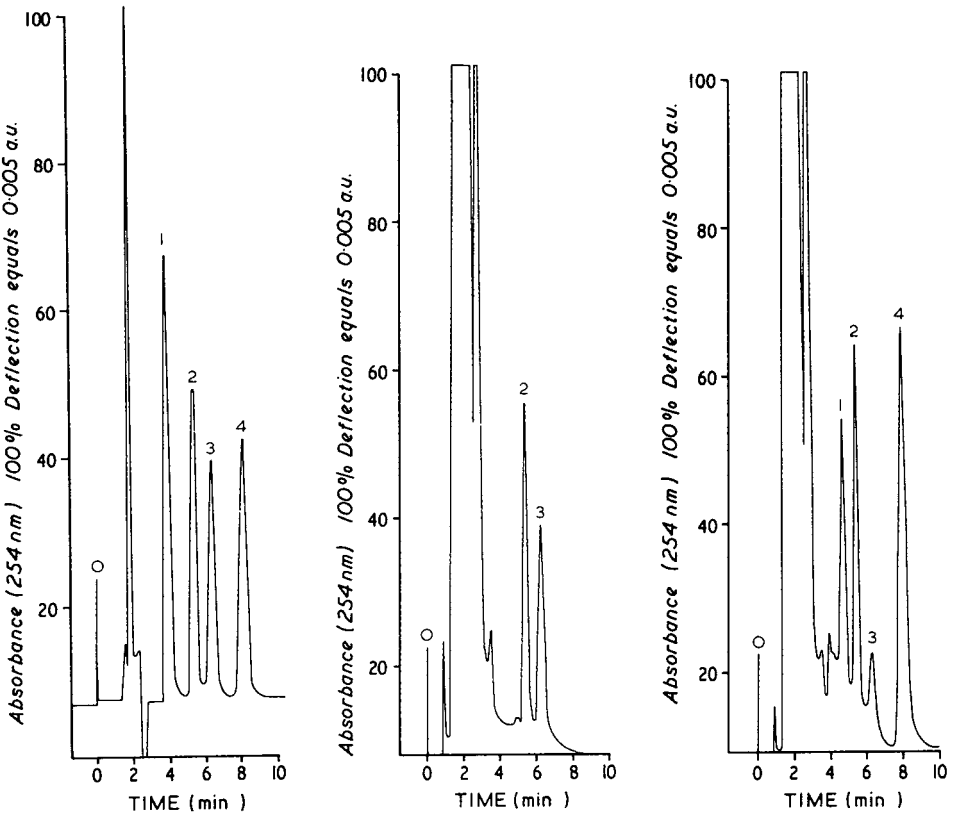

Fig. 1. Chromatogram showing separation of a standard steroid mixture. Peaks: 0 = injection, 1 = prednisone, 2 = dexamethasone, 3 = cortisol, 4 = prednisolone.

Fig. 2. Chromatogram showing an extracted human plasma blank obtained immediately before oral administration of 10 mg of prednisolone. Peaks: 0 = injection, 2 = dexamethasone, 3 = cortisol.

Fig. 3. Chromatogram of extracted human plasma obtained from the same volunteer control as in Fig. 2, 1 h after ingesting 10 mg of prednisolone. Peaks: 0 = injection, 1 = prednisone, 2 = dexamethasone, 3 = cortisol, 4 = prednisolone.

In Fig. 2, the chromatogram of an extracted plasma sample, taken before the administration of a 10-mg tablet of prednisolone, is shown. Cortisol and the internal standard peaks are present and it is readily apparent that no interfering compounds were extracted from the plasma.

Fig. 3 illustrates the response of the same patient 1 h after the 10-mg dose of prednisolone. The cortisol peak is considerably suppressed as the prednisolone level approaches its maximum. A peak corresponding to prednisone may also be observed.

The application of the method in clinical practice is shown by the dose—response curve of a human female (74 kg) following the ingestion of 10 mg of prednisolone (Table III).

TABLE III

PLASMA PREDNISOLONE CONCENTRATIONS FOLLOWING A 10-mg DOSE ADMINISTERED ORALLY TO A VOLUNTEER FEMALE CONTROL (74 kg)

Time (h)	Prednisolone (ng/ml)
0.5	115
1.0	232
1.5	279
3.0	225
5.0	114.5
7.0	61
10.0	24
15.0	9

DISCUSSION

The separation and subsequent estimation of steroids in biological fluids by HPLC has been achieved using both silica and reversed-phase columns [3—8]. Separations involving the use of silica packings may be facilitated either by an adsorption mechanism [3, 6, 7] or by liquid—liquid partition, such as the method of Trefz et al. [8] utilizing a solvent system based on that of Hesse and Hövermann [9]. The method described in this paper evolved from attempts to reduce the affinity of the silica packing material for the steroids and so shorten the retention times. This was achieved by employing a water-saturated mobile phase to deactivate the silica [10]. The further addition of 1% glacial acetic acid served to improve resolution and sharpen peak shapes. There are, however, obvious similarities between our method and that of Trefz et al. [8], who proposed that the separation mechanism probably involves liquid/liquid partition.

The determination of prednisolone by this method proves to be efficient, precise, sensitive and selective, offering the advantage of both faster analysis time and greater sensitivity compared to other HPLC techniques [3—8]. Furthermore, the sensitivity limit of the assay (approximately 10 ng/ml) could be enhanced by reducing the amount of internal standard added and injecting larger sample volumes, thus permitting the accurate measurement of concentrations as low as 5 ng/ml.

An additional advantage is the ability to measure simultaneously prednisone, cortisol and prednisolone. Indeed, the appearance of a prednisone peak can be observed in patient samples as the prednisolone level approaches its maximum (see Fig. 3) and disappears as the prednisolone level falls. This confirms the observations of Scott et al. [5] and provides further evidence of the metabolism of prednisolone to prednisone [11]. The decay and eventual disappearance of the cortisol peak may also be observed, indicating that suppression of endogenous corticosteroid production by the adrenal cortex occurs under the influence of increasing plasma prednisolone levels [12].

The chromatograms illustrated in Figs. 2 and 3 are those obtained from the plasma of an adult female volunteer and are representative of the many samples

analysed. Similar peak patterns are present in the extracted plasma from paediatric patients and there appear to be no interfering peaks peculiar to paediatric plasma. In fact, in nearly two years we have not encountered any interferences in either adult or paediatric samples.

The comparison of calibration standards revealed that it is permissible to replace authentic plasma with 3% bovine serum albumin to provide calibration data and this is the practice we have adopted.

The application of this method to the measurement of steroid concentrations in saliva, urine and cerebrospinal fluid is currently under investigation and these findings will be reported later.

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Note

Determination of medroxyprogesterone acetate in plasma by high-performance liquid chromatography

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Medroxyprogesterone acetate (MPA) is a synthetic progestational agent which has proved to be effective for contraceptive purposes [1] and is increasingly used in oncology as an adjuvant treatment for hormone-dependent cancers such as endometrial cancer [2] and especially breast cancer [3, 4]. The plasma measurement of this product in breast cancer, for which repeated high doses of MPA are becoming frequent, appears to be relevant, as was recently demonstrated in a comparative study on kinetics during per os or intramuscular administration [5]. Reports in the literature make mention of gas chromatography [6] or radioimmunology [7] for quantification of MPA concentrations in the blood. We propose a method based on high-performance liquid chromatography in which emphasis has been placed on the ease of plasma extraction and on the speed and quality of the chromatographic resolution.

MATERIALS AND METHODS

Reagents and materials

Bidistilled water was obtained from Laboratoire Aguettant (Lyons, France), R.P. Normapur methanol was obtained from Prolabo (Paris, France), and glacial acetic acid was purchased from Carlo Erba (Milan, Italy). Medroxy-

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progesterone acetate was a generous gift from Carlo Erba (Milan, Italy): batch MP 0004/2149. Cyproterone acetate was a generous gift from Laboratoires Theramex (Principality of Monaco). The internal standard, 19-norprogesterone (19-NPG), and the other steroids tested in the study were obtained from Sigma (St. Louis, MO, U.S.A.). Plasma samples were taken into EDTA tubes.

Extraction procedure

Sep-Pak extraction cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.). Before use they were washed with 2 ml of methanol and 5 ml of distilled water. Use was made of injection syringes with a flow-rate of approximately 2—3 ml/min. A plasma standard enriched with MPA to a concentration of 200 ng/ml was prepared for reference purposes. Plasma samples were tested only once. To 2 ml of plasma (standard or sample) were added 20 μl of 19-NPG $5 \cdot 10^{-5}$ *M* in solution in ethanol. The entire 2 ml were than passed through the cartridge and the eluate was discarded. Five millilitres of water and 2 ml of methanol—water (30:100, v/v) were then passed successively through the cartridge with the eluate being discarded each time. Two millilitres of pure methanol were then passed through the cartridge; the eluate was collected and evaporated at 60°C in a water bath under a flow of nitrogen. The dry residue was recovered in 200 μl of ethanol and an aliquot was injected into the chromatograph. The maximum injected volume necessary to guarantee good separation was 75 μl. We generally used 50 μl.

Apparatus and chromatographic conditions

The liquid chromatograph and column were from Waters Assoc.; the unit included a 6000 A pump and a U6K injector coupled with a radial compression RCM 100 module equipped with a Radial Pak cartridge (8 × 100 mm) containing 5-μm reversed-phase particles of Bondapak C_{18}. An M 440 UV detector (254 nm) connected to a Data Module integrator was used for detection and quantification of the compounds chromatographed. For calculations, peak heights were taken into consideration.

The mobile phase consisted of a methanol—water mixture (75:100, v/v). The pH was lowered to 4.00 with glacial acetic acid. The flow-rate was 2.5 ml/min, and the resulting pressure was 80 bars.

RESULTS

Chromatogram

Fig. 1A shows a chromatogram of blank plasma to which the internal standard 19-NPG (peak 2) had been added; Fig. 1B shows the chromatographic profile of a plasma sample taken 6 h after drug ingestion (1000 mg) and spiked with 19-NPG. MPA was chromatographed and peak 1 corresponds to the retention time of the pure product.

Resolution between MPA and the internal standard 19-NPG appears satisfactory; the blank does not show any interfering peak at the MPA retention time.

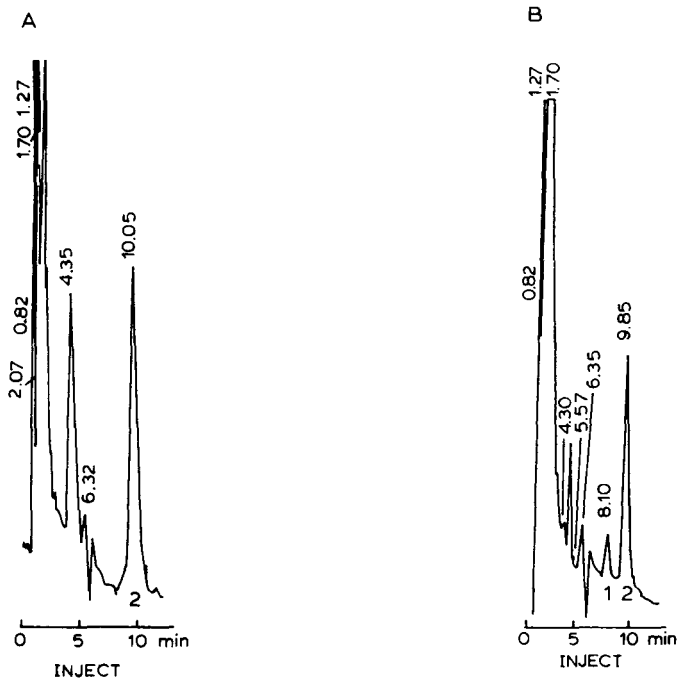

Fig. 1. (A) Chromatogram of a blank plasma sample (2 ml) spiked with 20 μl of $5 \cdot 10^{-5}$ *M* 19-norprogesterone (peak 2); 75 μl injected. (B) Chromatogram of a plasma sample (2 ml) taken 6 h after oral administration of MPA and spiked with 20 μl of $5 \cdot 10^{-5}$ *M* 19-norprogesterone (peak 2); 50 μl injected. Peak 1 corresponds to MPA.

Calibration curve and sensitivity

The calibration curve obtained with MPA concentrations from 20 to 500 ng/ml (six points) was linear, with a coefficient of correlation of 0.987. The limit of sensitivity for plasma samples was reached at a concentration of 4 ng/ml. This represented 2% of full scale detection at 0.005 ABS.

Recovery and reproducibility

Recovery from a reference plasma at a concentration of 200 ng/ml was 76% (the mean of seven samples). Six identical aliquots of plasma (200 ng/ml) were measured in the same series and gave a coefficient of variation [(S.D./mean) × 100] of 3.2%, representing the intra-assay reproducibility.

The same plasma (200 ng/ml) was also evaluated in six different series, and a coefficient of variation of 8.6% was obtained; this represented the inter-assay reproducibility.

Selectivity

The following steroids have been separated using the same chromatographic system: oestradiol, progesterone, promegestone, cortisone, hydroxycortisone, testosterone, dihydrotestosterone and cyproterone acetate. None of them had the same retention times as MPA or 19-NPG.

Application: plasma levels in patients undergoing treatment

MPA was measured for breast cancer patients taking the drug daily, either orally or by intramuscular injection. Table I gives the plasma drug concentrations for the first day and days thereafter. There appear to be wide inter-individual variations in absorption as well as in the steady-state level, as recently mentioned by others [5].

TABLE I

PLASMA LEVELS OF MEDROXYPROGESTERONE ACETATE (MPA) IN BREAST CANCER PATIENTS TREATED ORALLY OR BY INTRAMUSCULAR INJECTION

Means of administration	Patient	MPA concentration in plasma (nmol/l)								
		First day (hours after administration)				Following days (6 h after administration)				
		+2	+4	+6	+12	+1	+2	+3	+4	+5
Intramuscular (500 mg)	1	38	47	22	ND*	5	11	22	28	44
	2	15	30	31	26	30	40	92	27	54
	3	16	54	22	ND	58	50	69	57	53
Oral (1000 mg)	4	118	117	112	12.5	ND	166	170	125	220
	5	11	6	4	<4	14	60	70	75	90
	6	630	530	312	25	120	220	265	147	413

*ND = not determined.

DISCUSSION

The high-performance liquid chromatographic method described allows MPA separation and quantification in plasma. The choice of Sep-Pak cartridges for MPA extraction was based on a previous report [8] concerning estrogen extraction from urine and plasma. The procedure is simple, rapid, quantitative and reproducible, as shown by the relatively low coefficient of variation (below 10%) for intra- and inter-assay reproducibility. It eliminates the need for liquid—liquid extraction, which is often tedious for steroids and can involve classical problems with emulsions and low recovery levels. Radial compression columns represent a new technique in liquid chromatography: the radial compression forms a non-voiding chromatographic bed and applies uniform pressure to the flexible-walled cartridge. The theoretical elimination of channels thus obtained results in objective improvement in separation performance with a greater number of plates when compared to conventionally used stainless steel columns. Furthermore, the back pressure is relatively low: less than 68—100 bar with a flow-rate of 2.5—3.0 ml/min.

We feel that this type of chromatography is particularly suitable for the separation of steroids. All of the major steroids have been tested for possible interference with MPA retention, but none of them showed any superposition of peaks with MPA. It should also be noted that cyproterone acetate, a drug recently shown to have the same retention as MPA [9], did not interfere in this chromatographic system.

While the sensitivity of our method is comparable to that of gas chromato-

graphy [6], our technique has the advantage of being simple and thus easy to perform, especially for sample preparation. Since it allows MPA quantification in patients treated by this drug, it could be used for pharmacokinetics studies. The metabolism of MPA in humans has not yet been studied extensively [10], and our method could be adopted by investigators with a view to identifying and quantifying the products of MPA degradation in man.

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Note

Application of a sodium gradient in dynamic cation-exchange systems for rapid analysis by high-performance liquid chromatography and electrochemical detection of urinary catecholamines after a single purification step with aluminium oxide

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Analysis of urinary catecholamines is of interest in various pathological states [1, 2]. Application of high-performance liquid chromatography (HPLC) has recently renewed interest in the analysis of urinary catecholamines, due to the selectivity and relative simplcity of this technique. Moyer et al. [3], for instance, reported that combined analysis of the catecholamines noradrenaline (NA), adrenaline (A), and dopamine (DA) using HPLC is superior to analysis of metanephrines in detecting pheochromocytoma.

Several methods for the analysis of urinary catecholamines using reversed-phase HPLC have been described in the literature. The abundance of interfering substances in urine necessitates purification of samples before chromatography. Although used by most authors, adsorption onto aluminium oxide has been shown not to be specific enough to be used as a single purification step. Therefore, additional extraction of urine with organic solvents [4] or additional chromatographic procedures like cation-exchange chromatography [5], or boric acid-gel chromatography [3] had to be used.

In this paper we report that rapid analysis of adrenaline, noradrenaline and dopamine in urine after a single purification step with aluminium oxide is pos-

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sible when a dynamic cation-exchange system is used and a sodium gradient at pH 7 is applied.

EXPERIMENTAL

Apparatus

The analyses were performed on a Hewlett-Packard 1084 B chromatograph with an electrochemical detector (Bioanalytical Systems, LC-4 controller and TL-5 cell). The detector was operated at +0.6 V against an Ag/AgCl reference electrode. Prepacked reversed-phase columns were used. An analytical column, 15 cm × 4.5 mm I.D., packed with Hypersil ODS, 5 μm particle size, was obtained from Chrompack (Middelburg, The Netherlands), and in some experiments combined with a 3 cm × 4.6 mm I.D. precolumn, packed with LiChrosorb C_{18}, 10 μm particle size (Brownlee Labs., Santa Clara, CA, U.S.A.). The columns were loaded with sodium dodecyl sulphate (SDS) by pumping through 50 ml of 1% (w/v) SDS in distilled water. After this, columns were equilibrated until the retention of catecholamines was constant, usually with 100 ml of eluent or less.

Materials

Aluminium oxide (Al_2O_3) was obtained from BDH (activity II; BDH, Poole, Great Britain) and prepared according to the method of Weil-Malherbe [6]. All other chemicals were reagent grade. Water was demineralized and distilled in an all-glass apparatus.

Stock solutions (ca. 10—50 ppm) of standards were prepared in 0.05 *M* $HClO_4$ with 0.05% (w/v) tetrasodium EDTA and 0.05% (w/v) $Na_2S_2O_5$ and stored at 4°C for two weeks or less.

Sample preparation

Urine was collected over 0.5 g of disodium EDTA and 0.5 g of $Na_2S_2O_5$ and stored at −70°C. Standard solutions were prepared by diluting 50—200 μl of stock solutions to 25 ml with 0.05 *M* phosphate buffer pH 6.8.

To 0.7 g of aluminium oxide 10 ml of 0.1 *M* Tris buffer pH 8.4 and 0.2 ml of 1 *M* sodium hydroxide were added in a stoppered glass tube. The tube was shaken on a Vortex mixer and the supernatant aspirated off. To 25 ml of urine or of a standard solution were added: ca. 10 mmol of the internal standard dihydroxybenzylamine (DHBA, 100 μl), 2.5 ml of 1 *M* Tris buffer pH 8.4, 1.25 ml of 10% (w/v) disodium EDTA and 0.25 ml of 5% (w/v) $Na_2S_2O_5$. The sample was transferred to the tube containing Al_2O_3. The pH was adjusted to 8.4—8.6 with 5 *M* and 1 *M* sodium hydroxide. The tube was shaken gently for 5 min. After the Al_2O_3 had settled, the supernatant was aspirated off and the Al_2O_3 was washed three times with 10 ml of 0.05% (w/v) disodium EDTA. The catecholamines were eluted with 5 ml of 0.1 *M* $HClO_4$ containing 0.05% (w/v) disodium EDTA and 0.05% (w/v) $Na_2S_2O_5$; 50 or 100 μl of this eluent were injected into the HPLC system.

Chromatographic conditions

The flow-rate was adjusted to 2 ml/min and the temperature of the column

compartment and of the eluents was 30°C. The eluents contained 0.01—0.1 *M* Na_2HPO_4, 0—10% propanol, 0—250 mg/l SDS, and 1.34 mmol of disodium EDTA as specified in the legends to the figures. Addition of EDTA to the eluent eliminated difficulties apparently caused by metal ions from the stainless steel of the apparatus [7]. The pH of the eluent was adjusted with $HClO_4$.

RESULTS AND DISCUSSION

For the analysis of A, NA and DA in Al_2O_3 extracts of urine, use was made of dynamic cation exchange with SDS as anionic surfactant as previously described [7]. It has been shown how the retention of cations in such dynamic cation-exchange systems can be varied by means of the organic modifier concentration, SDS and counterion concentration and pH of the mobile phase [7, 8].

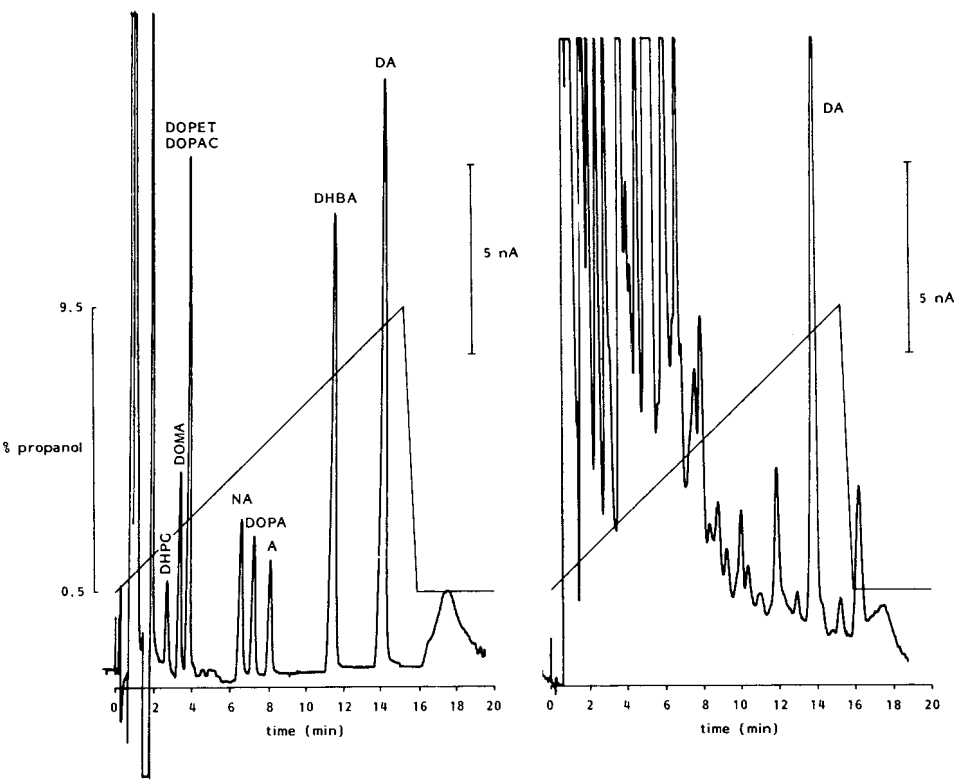

Fig. 1. Application of a propanol gradient for the separation of catecholamines from standard solution or urine, prepurified on Al_2O_3. For preparation of urine samples, see Experimental. The eluent contained 0.05 *M* Na_2HPO_4 and 1.34 mmol/l disodium EDTA; the pH was adjusted to 3 with $HClO_4$. The gradient was 0.5—9.5% propanol and 12.5—237.5 mg/l SDS. Flow-rate was 2 ml/min, the temperature of the eluent and column 30°C. The electrochemical detector was operated at +0.6 V (vs. Ag/AgCl). Abbreviations: DA = dopamine, DHBA = dihydroxybenzylamine, A = adrenaline, NA = noradrenaline, DOPA = dihydroxyphenylalanine, DOPAC = dihydroxyphenylacetic acid, DOMA = dihydroxymandelic acid, DOPET = dihydroxyphenylethanol, DHPG = dihydroxyphenylglycol.

To obtain selective elution of NA, A and DA within a reasonable time, a propanol gradient was applied. Fig. 1, however, shows that many urine constituents are present in the Al_2O_3 extract, preventing the determination of NA and A. It was observed that a considerably cleaner chromatogram was obtained when a high propanol content (4%) was used for the entire chromatography. To obtain a sufficient retention of NA and A with 4% propanol a low sodium concentration can be used. Since under these conditions the k' of DA is very large, the application of a sodium gradient is obvious.

Fig. 2 shows chromatograms of the same standard mixture and urinary Al_2O_3 extract as in Fig. 1, but now obtained by applying a sodium gradient at fixed propanol (4%) content. As can be seen, NA, A, and DA are eluted free of interfering substances, although interfering peaks are close to NA and A and close to the retention time of the internal standard DHBA.

Due to the low pH of the eluent, one can expect that the remaining interferences are acidic compounds. In that case their retention can be drastically reduced by increasing the pH of the eluent. The favourable effect of increasing pH on the background of the urinary Al_2O_3 extract is shown in Fig. 3, which is the same experiment as in Fig. 2 but now at pH 7. The application

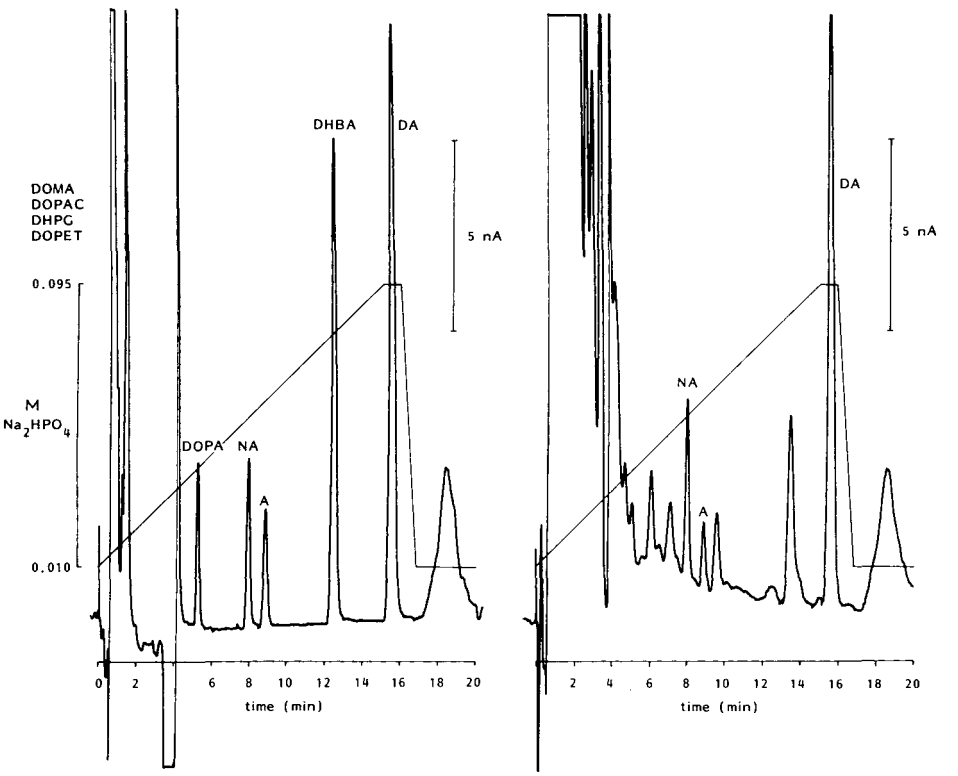

Fig. 2. Application of a sodium gradient for the separation of catecholamines from standard solution or urine, prepurified on Al_2O_3. Same samples as in Fig. 1. The eluent contained 4% propanol, 100 mg/l SDS, 1.34 mmol/l disodium EDTA, and the pH was adjusted to 3 with $HClO_4$. Flow-rate was 2 ml/min, the temperature of the eluent and column 30°C. The electrochemical detector was operated at +0.6 V (vs. Ag/AgCl). For abbreviations see Fig. 1.

of a Na^+ gradient with 4% propanol at pH 7 was found to be the best choice for the analysis of A, NA, and DA directly in Al_2O_3 extracts of urine. The reproducibility of the retention time was determined to be 0.6% (C.V., n=10) for all components, and 3—4 min elution (at 2 ml/min) with the original eluent was sufficient to re-equilibrate the column for the next analysis. The column used to record Figs. 1—3 was one year old and its plate number was ca. 3500 at the time of recording.

The shift of the baseline of the electrochemical detector due to the Na^+ gradient (and also for the propanol gradient) was surprisingly small and of the order of 0.25 nA. This marginally influences the analysis since the expected peak heights of the catecholamines extracted from urine ranges between 5 and 25 nA (see also Fig. 3).

Another favourable effect was noticed when comparing the response of the detector for the catecholamines at pH 3 and 7. It was found that the response of NA, DA, and DHBA (internal standard) was constant in the pH range 3—7 but increased for A by a factor of 1.5 in the pH range 4.5—7.

The electrochemical detector used is suitable for routine use. The cell was cleaned with methanol only after several hundred analyses and polished only every few months.

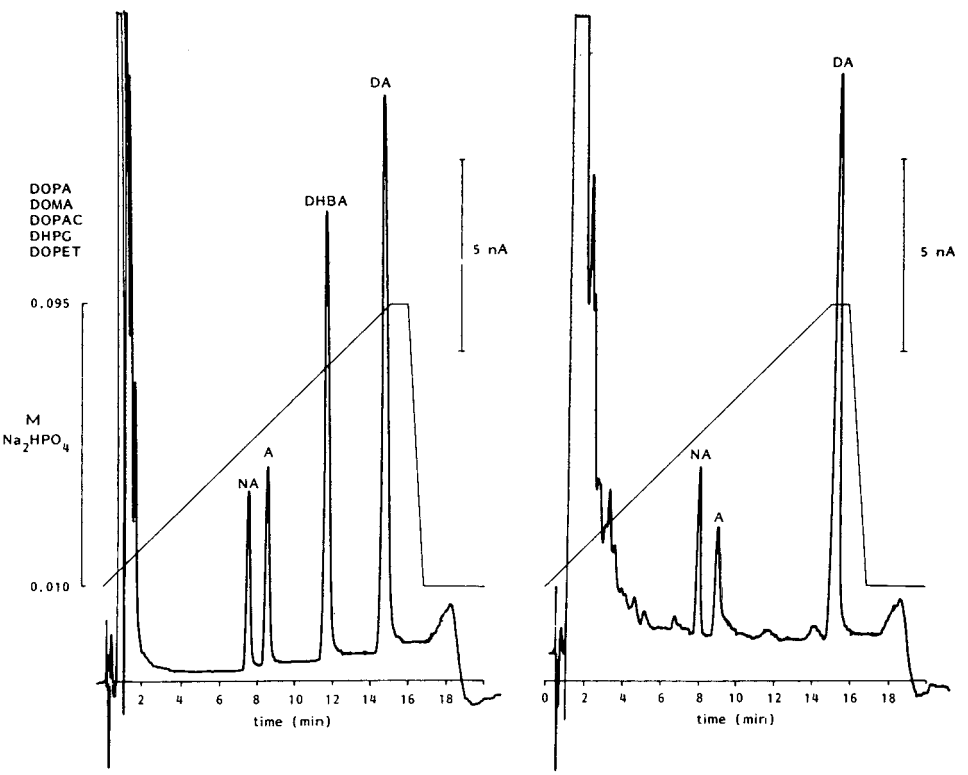

Fig. 3. Application of a sodium gradient for the separation of catecholamines from standard solution or urine, prepurified on Al_2O_3. Same experiment as in Fig. 2, but now at pH 7. For further details see legend to Fig. 2. For abbreviations see Fig. 1.

In conclusion, dynamic cation-exchange chromatography when applying a Na^+ gradient allows a simple and rapid analysis of the three major urinary catecholamines, since sample clean-up can be restricted to a single step using Al_2O_3. The present experiments show the first application of a sodium gradient as an useful chromatographic parameter in combination with electrochemical detection.

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CHROMBIO. 1396

Note

Determination of cytosine arabinoside triphosphate in leukemic cells by isocratic high-performance anion-exchange column chromatography

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Cytosine arabinoside (ara-C, 1-β-D-arabinofuranosylcytosine) is a cytostatic drug, effective in the treatment of acute leukemia [1,2]. In vivo, extracellular ara-C is deaminated rapidly to the inactive metabolite uracil arabinoside (ara-U, 1-β-D-arabinofuranosyluracil) [3]. Ara-C is also taken up by leukemic cells and phosphorylated to cytosine arabinoside triphosphate (ara-CTP) [4, 5], a metabolite interfering with DNA synthesis [5, 6]. The extent of ara-C phosphorylation is one of the main determinants of its cytocidal property [7, 8]. Decreased phosphorylation of ara-C is associated with drug resistance in human and animal tumors [7, 9—14].

Hitherto, phosphorylation of ara-C has been studied by means of radioactive-labeled drug. Consequently, the investigations have been limited to in vitro experiments [11, 12, 14]. Since a method for the determination of small amounts of unlabeled ara-CTP was not available, data concerning intracellular amounts of ara-CTP in vivo are lacking.

Recently, some methods have been developed for ara-CTP determination by means of high-performance liquid chromatography (HPLC) [15, 16]. These methods include gradient elution and laborious sample preparation, making them time-consuming.

In this paper a simple method for cell extraction in combination with a rapid and sensitive HPLC procedure, using isocratic elution, is described. The method is illustrated by data obtained in two patients treated with ara-C.

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MATERIALS AND METHODS

Chemicals

Ara-CTP, cytidine-5′-triphosphate (CTP), uridine-5′-triphosphate (UTP), deoxythymidine-5′-triphosphate (dTTP), inosine-5′-triphosphate (ITP), adenosine-5′-triphosphate (ATP) and guanosine-5′-triphosphate (GTP) were purchased from Brunschwig Chemie, deoxycytidine-5′-triphosphate (dCTP) was from Hoechst and deoxyadenosine-5′-triphosphate (dATP) and deoxyguanosine-5′-triphosphate (dGTP) were from Boehringer. All other chemicals were supplied by E. Merck. All chemicals were of analytical grade.

Preparation of cell extracts

Bone marrow aspirates and peripheral blood samples are collected in ice-cold buffered acid citrate dextrose solution (ACD, formula A, pH 7.4) and immediately placed on ice. Cells are washed once with ACD to prevent fibrin formation, and 10^7 to 10^8 nucleated cells are layered on a Percoll cushion (d = 1.080 g/ml), and centrifugated for 20 min at 800 g and 4°C to remove erythrocytes. The cells are collected from the interface and washed in ice-cold ACD. Cell counting is performed with a Coulter Counter; 2×10^7 to 4×10^7 cells are spun down (10 min, 800 g, 4°C). A 500-μl sample of an ice-cold ethanol—HPLC eluent solution (1:1, v/v) is added to the cell pellet and the intracellular nucleotides are extracted in 15 min at 0°C.

Precipitated protein is removed from the extract by centrifugation (4 min, 8000 g, 0°C). Of these extracts, 100 μl are used for analysis.

HPLC equipment

A Pye-Unicam LC-3-XP pump, equipped with an automatic injection valve (Valco Houston AH 60), a 100-μl loop, and a column (250 × 4.6 mm I.D., Whatman) packed with Partisil-10-SAX anion-exchange resin (particle size 10 μm) is used for separation of the nucleotides. A guard column (30 × 4.6 mm I.D., Partisil-10-SAX) is incorporated in the system to protect the analytical column. Two UV detectors are used: an UV III monitor LDC 1203 (10-μl flowcell, wavelength 280 nm) and a Pye-Unicam LC-UV (8-μl flowcell, wavelength 254 nm).

HPLC procedure

Samples of 100 μl of cell extract are injected into the column. Elution is carried out at a constant temperature (35°C) and at a constant flow-rate of 2.5 ml/min with 0.125 M potassium dihydrogen phosphate solution, containing 0.075 M trisodium citrate as counter-ion, adjusted to pH 4.6 (at 35°C) with phosphoric acid. Helium gas is led through the eluent to prevent development of air bubbles at the low pressure side. Detection of ara-CTP is optimal at 280 nm. Peak heights are used for quantification, whereas ratios of the absorbance at 280 nm and at 254 nm are used for purity control of the peaks.

RESULTS

Chromatographic separation, accuracy, and calibration curves

Separation of ara-CTP from naturally occurring analogues is achieved within 17 min (Fig. 1a). A chromatogram of a blank leukemic cell sample is shown in Fig. 1b. Leukemic cell extracts did not contain compounds which interfere with the ara-CTP peak, as could be checked by the 280/254 nm ratio. The lowest detectable amount in biological samples is about 20 pmol. A chromatogram of a leukemic cell extract containing 40 pmol is shown in Fig. 1c. The calibration curve (not shown) is linear over three decades (from 20 pmol to 20 nmol) and passes through the origin. The coefficient of variation ranges from 3% (1 nmol, n=6) to 11% (50 pmol, n=6).

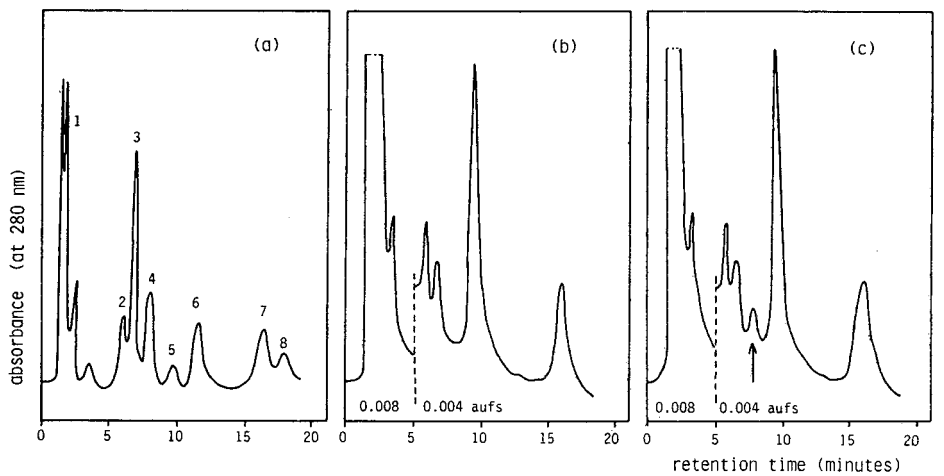

Fig. 1. (a) Chromatogram of a mixture of nucleotides. Detection at 280 nm. Conditions: see under Methods. Peaks: 1 = mono- and diphosphates; 2 = UTP; 3 = dCTP, CTP and dTTP; 4 = ara-CTP; 5 = ATP; 6 = dATP; 7 = GTP; 8 = dGTP. (b) Chromatogram of a blank leukemic cell extract. Detection at 280 nm, 0.004 a.u.f.s. (c) Chromatogram of a leukemic cell extract. The sample was taken 15 min after starting a 1-h intravenous infusion of ara-C (200 mg/m^2). Detection at 280 nm, 0.004 a.u.f.s. The injection sample (100 μl) represents 5.4 × 10^6 cells and contains 40 pmol of ara-CTP. Arrow indicates ara-CTP peak.

Up to 4 × 10^7 cells can be extracted quantitatively with 500 μl of extraction solution (Fig. 2a). To demonstrate linearity of the calibration curve, including the extraction procedure, leukemic cells incubated in vitro with ara-C were mixed with non-incubated cells in different proportions before extraction. This calibration curve appears linear (Fig. 2b).

Application

Ara-CTP has been measured in the leukemic cells of two patients after administration of ara-C. Ara-C (200 mg/m^2 body surface) was administered either as a bolus injection (patient A) or as a constant-rate infusion during 1 h (patient B). At several time intervals blood samples were taken for the determination of the ara-C plasma concentration and for quantification of ara-CTP

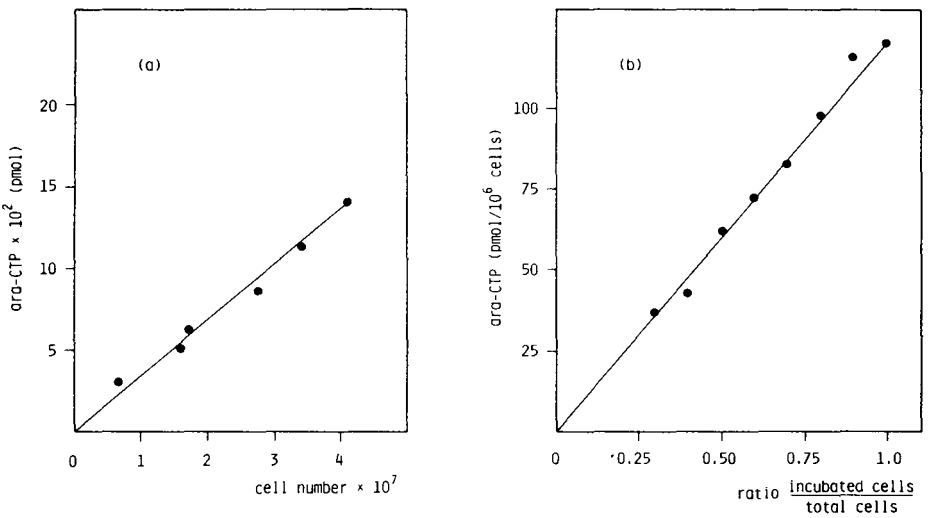

Fig. 2. (a) Calibration curve of the extraction of increasing cell numbers. Cells are incubated with ara-C in vitro and extracted with 500 μl of extraction solution. Chromatographic conditions: see under Methods. (b) Calibration curve of the determination of ara-CTP, including the extraction procedure. Leukemic cells, incubated with ara-C in vitro, are mixed with non-incubated leukemic cells in varying ratios.

accumulated in the leukemic cells. Determination of ara-C was performed as previously described [17].

Five minutes after ara-C administration, ara-CTP was already detected in considerable amounts in the leukemic cells. During the constant-rate infusion

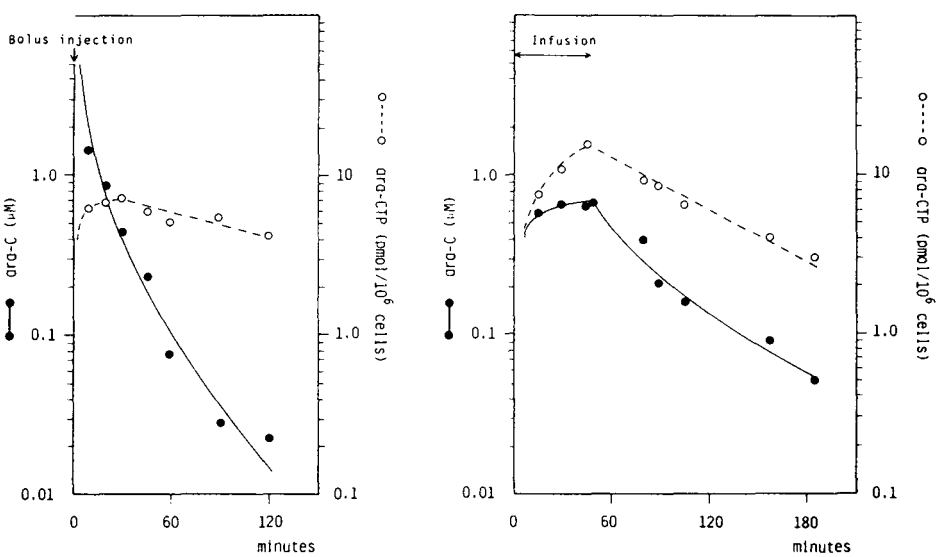

Fig. 3. Time curves of ara-C plasma concentration (●——●) and of amount of ara-CTP in leukemic cells (○ — — — ○) after administration of 200 mg/m² ara-C as a bolus injection and as a constant-rate infusion of 1 h.

ara-CTP showed an almost linear increase. After the bolus injection the intracellular amount of ara-CTP increased during 15 min.

Elimination of ara-CTP from the leukemic cells showed first-order kinetics with half-life times of 60 and 120 min in the two patients studied.

Time curves of ara-C in plasma and of ara-CTP in the leukemic cells are depicted in Fig. 3.

DISCUSSION

Pharmacokinetic studies are performed generally to relate variations in pharmacokinetic parameters to individual therapeutic and toxic effects [18, 19]. In all previous studies of the pharmacokinetics of ara-C, only plasma concentrations have been taken into account. A correlation between the half-life of ara-C and the therapeutic response has been reported [20, 21], but this was not confirmed by others [22]. Since the active compound is not ara-C but the intracellular metabolite ara-CTP, variations in the therapeutic efficacy of ara-C may be related more closely to differences in the pharmacokinetics of intracellular ara-CTP. This hypothesis is supported by the wide variation in the phosphorylating activity of leukemic cells [12, 23].

The intracellular pharmacokinetics of ara-CTP can be monitored by means of the method described in this paper. Extraction of the intracellular nucleotides is performed with an ethanol solution. This procedure is more suitable for application to small samples, since neutralization is not needed. When using perchloric acid careful neutralization is necessary, otherwise ara-CTP will be hydrolyzed. Separation of ara-CTP from the other nucleotides was achieved with the use of an isocratic eluent. This makes the procedure less time-consuming in comparison to methods using gradient elution. In conclusion, this method is sensitive and rapid, and can be readily applied for clinical pharmacological studies. This has been demonstrated in two examples of patients receiving ara-C therapy. The results indicate that after an intravenous bolus injection of ara-C maximal ara-CTP levels are reached after about 15 min, whereas ara-CTP increased linearly during the time period of a constant-rate infusion. Elimination of intracellular ara-CTP in vivo showed first-order kinetics. The observed half-lives of 60 and 120 min are in good agreement with those measured in vitro [14, 23].

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Note

Silicon-selective detection after gas chromatography for the determination of silylated salicylic acid in urine

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(First received January 27th, 1982; revised manuscript received June 11th, 1982)

Silylation techniques followed by gas chromatographic (GC) separation and detection are routine analytical procedures used for the determination of polar and reactive biochemicals containing acid, alcohol or amine functional groups. Derivatives of these compounds are less polar than parent molecules and exhibit reduced retention and adsorption in GC.

A major problem, however, in the determination of these derivatives is the lack of a specific detection system. Currently, the most commonly used detector for silyl derivatives is the flame ionization detector which relies on the response of carbon atoms present in the compounds and provides no response discrimination between silicon-containing compounds of interest and other compounds in the sample matrix. A GC detector which is selective for silicon would extend the scope of silylation methods since silylation would not only serve to deactivate and stabilize compounds of interest, but would also tag these compounds with silicon atoms, providing a more sensitive and selective response.

Such a silicon-selective detector has been recently developed which can be constructed from a standard flame ionization detector by interchanging oxygen and hydrogen inlets so that the flame burns in a hydrogen atmosphere [1—3]. Also, the collecting electrode is removed from the combustion region (its standard position in a flame ionization detector) to a location more than 10 cm above the flame.

Three modes of operation are possible. The first is a non-doped mode in which pure hydrogen gas is introduced into the detector housing. In this mode, silicon-containing compounds produce responses two to three thousand times

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greater than those of hydrocarbons and have a minimum detection limit of about 1 ng/sec. In the second mode of operation, a small quantity (typically 5 ppm or less) of ferrocene vapor is doped into the hydrogen atmosphere, increasing both sensitivity and selectivity. The minimum detectable limit for this mode is 15 pg/sec for silicon-containing compounds and the selectivity against hydrocarbons is on the order of 10,000. A third mode of operation in which silicon-containing compounds produce negative peaks is possible when the concentration of ferrocene vapor in the hydrogen atmosphere is increased to 30 ppm or more. In this mode silicon-containing compounds can easily be identified from hydrocarbons by virtue of their inverted peaks.

This paper demonstrates the use of this silicon-selective detector for the determination of salicylic acid in urine.

EXPERIMENTAL

Materials

The following chemicals were used: potassium hydrogen sulphate (Mallinckrodt, St. Louis, MO, U.S.A.), acetone (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), hexamethyldisilazane (HMDS) (Pierce, Rockford, IL, U.S.A.), salicylic acid and chloroform (J.T. Baker, Phillipsburg, NJ, U.S.A.).

Apparatus

All analyses were conducted on a Hewlett-Packard 5710A gas chromatograph equipped with dual flame ionization detection (FID) with one detector converted to a silicon-selective hydrogen-atmosphere flame ionization detector (HAFID-Si) [3].

Chromatography

Separation was accomplished on a 10-m methylsilicone coated fused silica capillary column (Hewlett-Packard, Avondale, PA, U.S.A.). Operating temperatures were as follows: injection port and splitter, 250°C; oven temperature was programmed from 60 to 200°C at 8°C/min with a final hold period of 15 min; detector temperature, 250°C. The helium carrier gas flow-rate was maintained at 1.2 ml/min. The split ratio was 1:30. HAFID-Si gas flow-rates were as follows: helium make-up, 30 ml/min; air, 240 ml/min; oxygen, 130 ml/min; and hydrogen, 1.6 l/min.

Procedure

Two hours after a volunteer had ingested 900 mg of aspirin, a 150-ml urine sample was collected. Pre-chromatography sample preparation procedures were modified from a method developed for GC by Walter et al. [4]. The sample was treated with 15 ml of a 10% solution of potassium hydrogen sulphate and then extracted with 50 ml of chloroform. After the chloroform had been evaporated to dryness under vacuum, 800 μl of HMDS and 200 μl of acetone were added to the residue. A 5-mg sample of salicylic acid was silylated in a similar manner. Silylated samples were allowed to stand for 1 h at room temperature before injection into the chromatograph.

RESULTS AND DISCUSSION

Fig. 1 represents a typical complicated chromatogram which is often obtained when non-selective detection methods such as flame ionization or thermal conductivity detectors are employed for the analysis of real samples. From retention time comparisons the peak marked S may be the component of interest but ancillary identification methods are required to ensure the accuracy of this assignment.

Fig. 2 is a chromatogram of the same sample with HAFID-Si detection in the positive mode. Since this detector is known to be selective for silicon-containing compounds, peaks which are observed can be attributed to either silylated derivatives or to large quantities of non-silylated components. When operating the detector in the negative mode, however, silicon-containing compounds produce inverted peaks while most compounds not containing silicon still respond in the normal fashion. From Fig. 3, where the sample was detected using the negative mode of the HAFID-Si, responses indicated that most of the peaks detected in the positive mode were silylated derivatives from

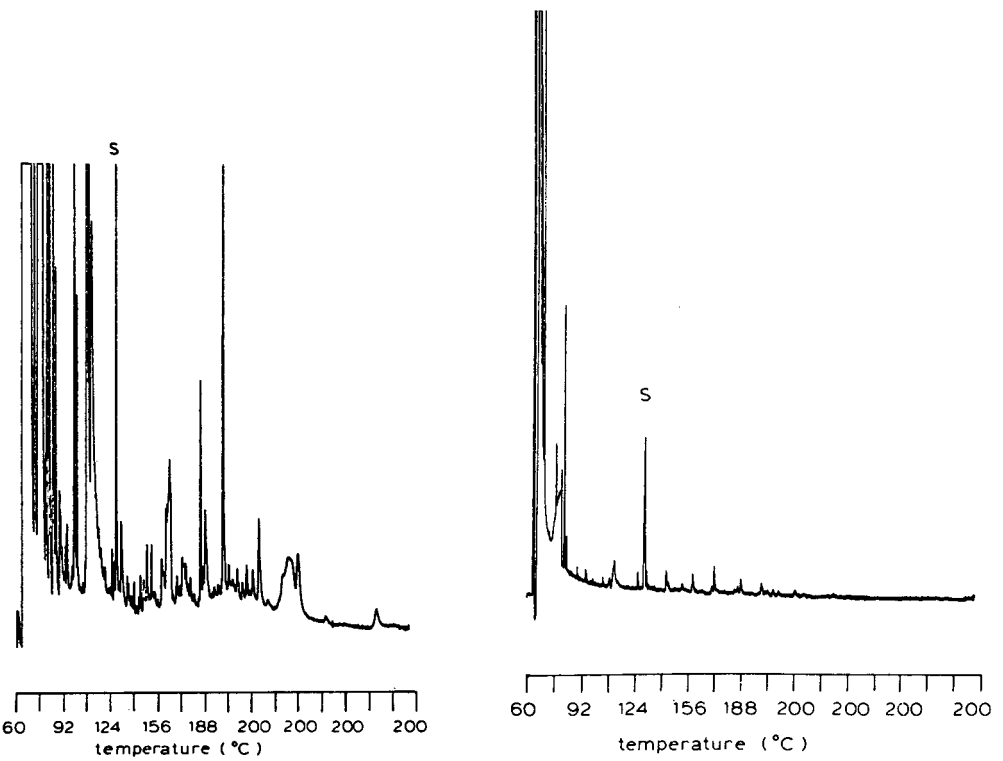

Fig. 1. FID chromatogram of silylated urine sample. S = silylated salicylic acid peak. Amount injected = 3 μl; total sample volume = 1 ml; electrometer attenuation = 8.

Fig. 2. HAFID-Si (positive mode) chromatogram of silylated urine sample. S = silylated salicylic acid peak. Amount injected = 5 μl; total sample volume = 1 ml; electrometer attenuation = 8.

the urine sample. From retention time data, the peak marked S can now be more confidently assigned to the derivatized salicylic acid. Ratios between peak areas in the negative and positive modes for the silylated salicylic acid in the standard (shown in Fig. 4) and in the sample were 3.6 and 3.7, respectively. This negative/positive response ratio provides additional qualitative evidence for the identification of the compound of interest.

Other silyl derivatives from compounds such as aspirin, benzoic acid, hydroxybenzoic acids and phenacetin may also elute from the column under

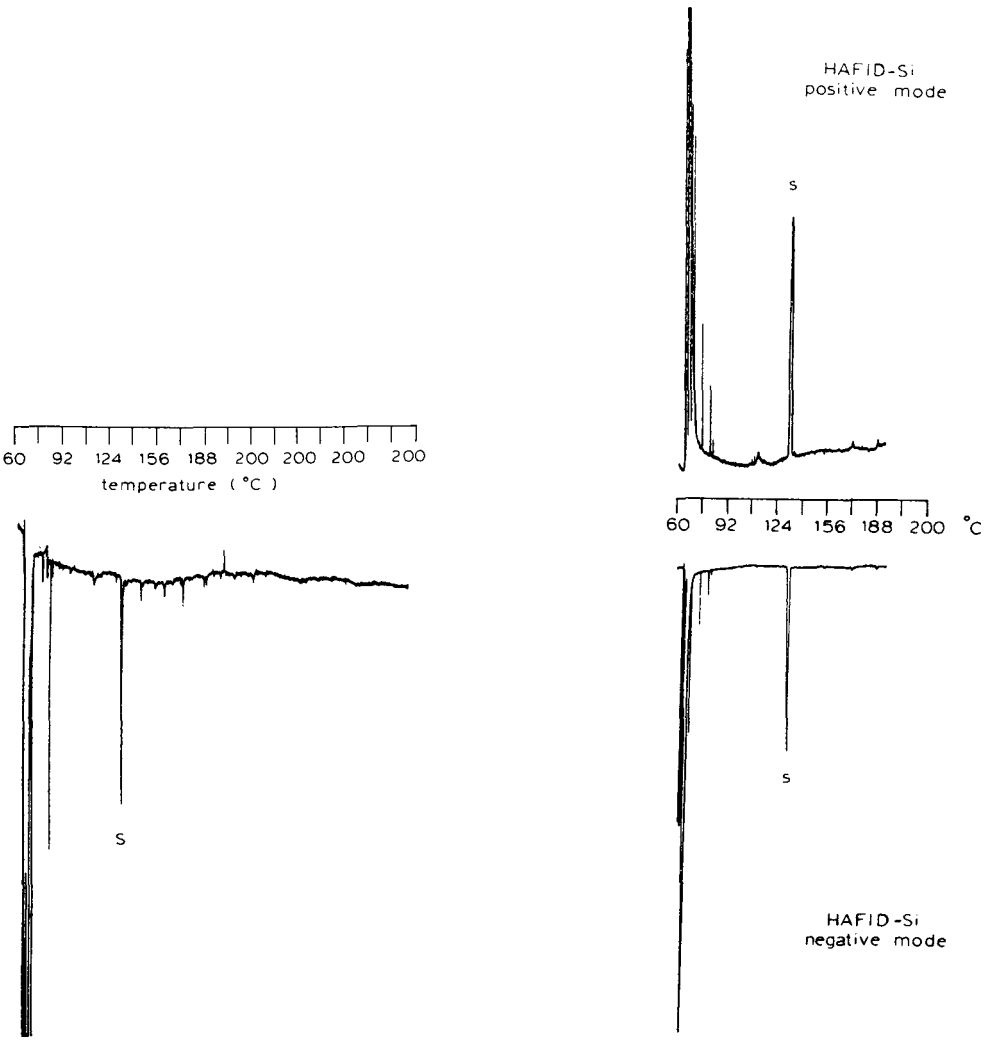

Fig. 3. HAFID—Si (negative mode) chromatogram of silylated urine sample. S = silylated salicylic acid peak. Amount injected = 5 μl; total sample volume = 1 ml; electrometer attenuation = 16.

Fig. 4. HAFID—Si chromatograms of silylated salicylic acid standard. Amount injected = 15 ng per 3 μl. Electrometer attenuation: positive mode = 8, negative mode = 32.

the operating conditions employed. Some of the peaks which were observed in the positive and negative mode HAFID-Si chromatograms may be attributed to these or similar compounds, although none has been confirmed in this study. The large off-scale peaks at the beginning of each chromatogram were due to the silylating reagent and/or silylated contaminants in the reagent (e.g. water) since these peaks were also found in blanks. Acetone, which gives a diminutive positive response in both positive and negative modes, elutes with the derivatizing reagent under these chromatographic conditions and is obscured by the large response of silicon in the reagent.

Two silylated derivatives are possible from the derivatization of salicylic acid, corresponding to 2-trimethylsilyloxybenzoic acid and trimethylsilyl 2-trimethylsilyloxybenzoate. In this study only one predominant peak was observed when the standard was derivatized, indicating that the silylation procedure was quantitative.

The linear response range for silicon compounds has been determined to be about three orders of magnitude for the positive mode [3]. The concentration of salicylic acid in the urine sample was determined to be 9.3 μg/ml from the positive mode and 9.0 μg/ml from the negative mode. These values differ by less than 4% and are in agreement with the level of concentration that is expected for such a sample.

CONCLUSIONS

Silicon-selective detection by both positive and negative modes, when used in conjunction with a standard, provides a more reliable method of identification of silylated derivatives in complex samples than commonly employed non-selective detectors such as flame ionization or thermal conductivity. The ease of conversion from FID to HAFID-Si makes this detector a particularly attractive alternative for qualitative and quantitative analysis when silylation procedures are employed.

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CHROMBIO. 1385

Note

Determination of propafenone in serum or plasma by electron-capture gas chromatography

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Propafenone, 2'-[3-(propylamino)-2-(hydroxy)-propoxy]-3-phenyl-propiophenone, is a new antiarrhythmic agent recently introduced in clinical practice. Its efficacy has been demonstrated in suppressing supraventricular and ventricular arrhythmias [1, 2]. It has been shown that propafenone serum levels are significantly correlated with changes in atrioventricular conduction times [3]. In the literature a high-performance liquid chromatographic (HPLC) method for the determination of propafenone plasma concentrations is described [4] but it requires large blood samples and injection volumes. To overcome these problems a rapid and sensitive gas—liquid chromatographic (GLC) method with electron-capture detection (ECD) which allows the detection of 10 ng/ml of the drug is described in this paper.

MATERIAL AND METHODS

Gas—liquid chromatography

A Perkin-Elmer Model Sigma 4 gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. A glass column (190 cm × 2 mm I.D.) was packed with 3% OV-101 on GCPS 100—120 mesh (Carlo Erba Strumentazione, Milan, Italy). The carrier and purge gas was 10% methane—argon (1:9) at flow-rates of 15 ml/min and 50 ml/min, respectively. The column temperature was 245°C and the temperatures of injection port and detector were 300°C.

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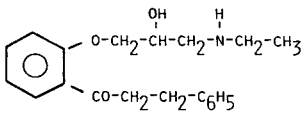
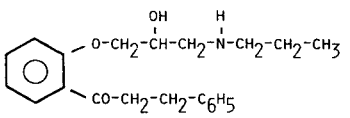
Reagents and materials

Propafenone · HCl and its internal standard Li 1115 (Fig. 1) were supplied by Knoll (Ludwigshafen, Rhein, G.F.R.). Trifluoroacetic anhydride (TFAA) and heptafluorobutyric anhydride (HFBA) were obtained from Pierce Chemicals (Rockford, IL, U.S.A.) and stored at −20°C. All organic solvents were RS grade (Carlo Erba).

Glassware was washed with hot nitric acid, rinsed with distilled water, dried at 105°C and silanized overnight with 5% dimethylchlorosilane in toluene. It was then washed with methanol and dried.



Li 1115



PROPAFENONE

Fig. 1. Structural formulae of propafenone and Li 1115 (internal standard).

Extraction procedure

Plasma samples (100—500 μl) were pipetted into glass stoppered tubes and internal standard (150 ng) was added. The samples were made alkaline (pH 11) with 1 *N* sodium hydroxide and extracted twice by shaking for 10 min with 4 ml of benzene each time. After centrifugation the combined organic extracts were evaporated to dryness under a stream of dry nitrogen at 40°C.

Derivatization

To the dried extract 300 μl of toluene and 50 μl of TFAA were added. The tubes, after being tightly stoppered, were heated for 45 min in a water bath at 40°C. The reaction mixture was then evaporated to dryness, the residue reconstituted with 200 μl of cyclohexane and 0.5—1.0 μl was injected.

The completeness of the reaction was checked by reacting fixed amounts of propafenone and TFAA at the constant temperature of 40°C. The results, shown in Fig. 2, indicate that quantitative derivatization was achieved in the conditions described above. An alternative procedure was also developed using HFBA as derivatizing agent. The HFBA derivatives of propafenone and its internal standard were stable and gave a greater response than TFAA derivatives in the electron-capture detector. However, it was not possible to achieve a satisfactory separation of these compounds on the common stationary phases.

RESULTS AND DISCUSSION

Under the chromatographic conditions used the peaks corresponding to propafenone and internal standard derivatives had retention times of 7.51 and

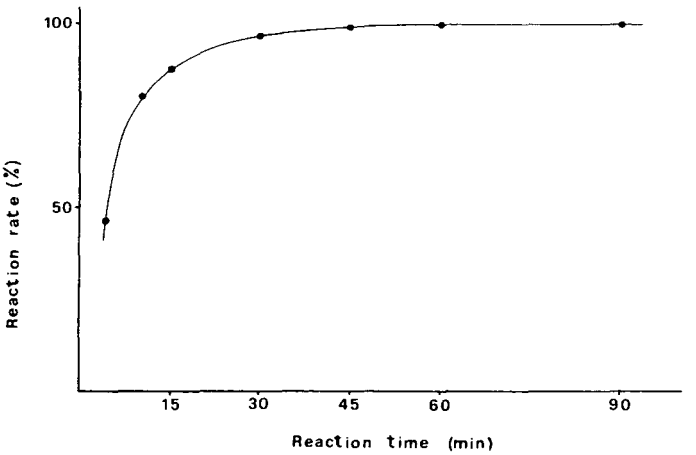


Fig. 2. Time course of TFAA-derivative formation from propafenone and TFAA.

6.54 min, respectively. A typical chromatogram of a plasma sample spiked with 200 ng/ml propafenone is shown in Fig. 3a, while a chromatogram of blank plasma is shown in Fig. 3b. In general no peaks that interfered with propafenone or internal standard peaks were encountered in the analysis of plasma from different patients.

The quantitative determinations of propafenone in plasma were performed by adding known amounts of drug in the range 50—200 ng/ml. The calibration

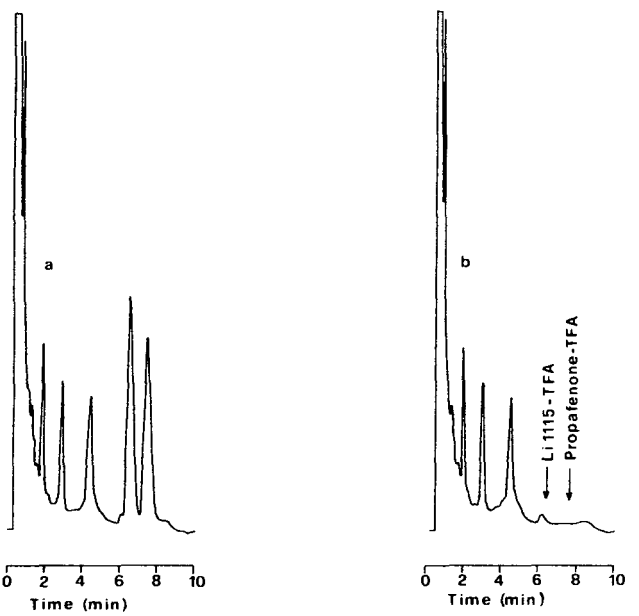


Fig. 3. Chromatograms obtained analyzing (a) an extract from plasma sample spiked with 200 ng/ml propafenone and 150 ng/ml Li 1115, and (b) plasma blank.

curves were linear within the range used and the minimum detectable amount was estimated as 100 pg injected.

To demonstrate the accuracy and precision of this method replicate determinations were carried out; the results are shown in Table I. Restandardization was carried out each day in order to maintain precision, although day-to-day variation in detector response was small.

TABLE I

REPRODUCIBILITY AT A GIVEN PLASMA CONCENTRATION FOR PROPAFENONE

Propafenone added (ng/ml)	*n*	Propafenone found ± S.D. (ng/ml)	C.V. (%)
50	6	52.5 ± 1.0	1.9
100	6	103.3 ± 3.5	3.4
150	6	151.0 ± 3.7	2.4
200	6	204.8 ± 7.6	3.7
Mean			2.8

TABLE II

CONCENTRATION OF PROPAFENONE (ng/ml) IN PATIENT PLASMA SAMPLES AS DETERMINED BY GLC–ECD AND HPLC

Plasma sample	GLC–ECD	HPLC
1	96	65
2	138	122
3	612	571
4	101	110
5	624	569
6	222	200
7	280	265

Moreover, we performed a parallel analysis of patient plasma samples taken after chronic propafenone administration using HPLC [4] and the new GLC–ECD method (Table II). Linear regression analysis of HPLC and GLC values showed good agreement and gave a regression coefficient of 0.998.

The high sensitivity of this method will permit an accurate determination, particularly after the administration of low doses of propafenone, using small blood sample volumes. This feature is most important when the pharmacokinetic study requires many and frequent plasma determinations.

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CHROMBIO. 1371

Note

Measurement of strychnine by high-performance liquid chromatography

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No high-performance liquid chromatographic (HPLC) method has been described for the measurement of strychnine in biological media. Such a method would be of use in toxicology and in monitoring strychnine levels in the blood of non-ketotic hyperglycinemic infants in whom the drug is used clinically [1]. In addition, it would be of use in the elucidation of the metabolism and pharmacokinetic characteristics of strychnine. Achari and co-workers described an HPLC method which measured different basic drugs [2] especially quinidine in human plasma, using strychnine as an internal standard [3]. We have adapted their method to strychnine using quinine as an internal standard.

MATERIALS AND METHODS

Reagents

Strychnine sulfate and quinine were obtained from Sigma (St. Louis, MO, U.S.A.). All solvents were glass-distilled grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). Other reagents were analytical grade.

Extraction procedure

Strychnine is a dibasic alkaloid showing almost identical solubility properties as quinidine and we therefore used the extraction procedure described by Achari et al. [3]: extraction by chloroform at basic pH, evaporation of the chloroformic extract, and reconstitution of the residue in methanol. This procedure resulted in the recovery of 83 ± 5% of added strychnine to plasma. The internal standard, quinine (10 μg/ml), is added with the chloroform.

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High-performance liquid chromatography

The apparatus is composed of a Beckman Model 110A pump, an Altex Model 210 injector and a Beckman Model 153 UV detector set at 254 nm. The chromatographic parameters were controlled by a Beckman Model 421 microprocessor. A Perkin-Elmer silica gel column (10 μm partical size), 25 × 0.26 cm, was used. The eluent, concentrated ammonium hydroxide (28.0—30.0% NH_3) in methanol (0.75:99.25, v/v) was pumped at a flow-rate of 1.1 ml/min.

RESULTS

For each set of measurements, a calibration curve is established. In the case of plasma and urine samples the calibration curve is obtained by taking known amounts of strychnine and extracting them from plasma or urine. In the case of simple solutions, the calibration curve is obtained directly by adding known amounts of strychnine dissolved in methanol. The concentrations of strychnine base used are from 20 to 0.625 μg/ml, the lower limit of sensitivity of the assay. The volume injected is 20 μl. Under those conditions quinine and then strychnine are eluted in less than 6 min, the two peaks being well separated (Fig. 1).

The calibration curve, which is linear (r = 0.999) over the concentration range employed, is established by plotting the peak height ratio of strychnine base to quinine against the concentration of strychnine base. The strychnine concentration in unknown samples is derived directly from the calibration curve. The intra-assay variance is between 3% and 5%; the inter-assay variance is less than 10%.

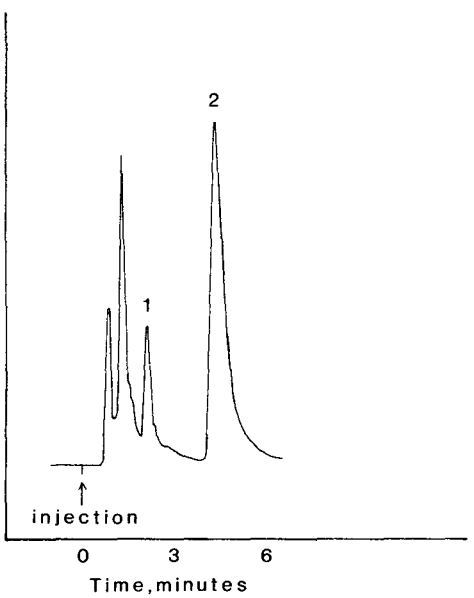

Fig. 1. Chromatogram showing the separation of strychnine base (peak 2) and the internal standard, quinine (peak 1). Chromatographic conditions were as follows: 25 × 0.26 cm silica gel column with a mobile phase of ammonium hydroxide in methanol (0.75%, v/v), flow-rate 1.1 ml/min and the range set at 0.02 a.u.f.s.; detection was at 254 nm.

We have used the above method to analyse the amount of strychnine remaining in Alzet mini-osmotic pumps after 19 days of administration of subconvulsive doses in rats [4]. This method is a definite improvement in sensitivity, accuracy and rapidity over the thin-layer chromatographic method formerly used [5] and is easily capable of monitoring plasma levels of strychnine either as used in pediatric therapy of non-ketotic hyperglycinemia [6] or as encountered in strychnine intoxication [7].

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CHROMBIO. 1389

Note

Sample preparation and a liquid chromatographic assay for misonidazole and desmethylmisonidazole*

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The nitroimidazoles, which include misonidazole (Fig. 1), are potent hypoxic cell radiosensitizing compounds with good correlation between the nitroimidazole serum concentrations and radiation enhancement [1]. The toxicity of these compounds for the central nervous system [2] coupled with their possible carcinogenicity [3] complicates their use. Measurement of serum levels of the compounds to assure adequacy for the desired radiosensitization while minimizing neuropathogenic levels can improve health-care safety standards for the radiation enhancement procedure.

A review of the literature for methods to measure the specific nitroimidazoles indicated that liquid chromatographic methods would be the optimal approach. The high-performance liquid chromatographic (HPLC) method of Marques et al. [4] gave us poor resolution after the ethanol serum sample clean-up procedure. In addition the method did not have an internal standard. The HPLC methods of Workman et al. [5] and Meering and Maes [6] which measure misonidazole and its demethylated metabolite utilize a large volume of methanol per volume of serum for the sample clean-up, which reduces the minimum detectable limits. Neither of the methods [5, 6] could separate misonidazole from metronidazole, and we wanted to use the latter compound as the internal standard. Meering and Maes [6] double the analytical run time to get their internal standard in the assay. None of these authors [4—6] give any summarized patient serum levels of misonidazole or desmethylmisonidazole based on dosage per body surface area.

We describe a study of the effectiveness and volumes of solvent required of

*A preliminary report of this research work was presented at the 34th National Meeting of the American Association for Clinical Chemistry, Anaheim, CA, August 9, 1982.

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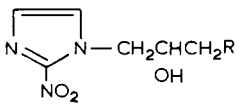
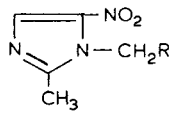


MISONIDAZOLE	R = OCH_3
DESMETHYLMISONIDAZOLE	R = OH
Ro 07-0741 (IS)	R = F
Ro 07-0913	R = OCH_2CH_3
Ro 07-0269	R = Cl



METRONIDAZOLE	R = CH_2OH
Ro 11-6924	R = COOH
Ro 07-0207	R = $CH(OH)CH_2Cl$

Fig. 1. Structural formulae of misonidazole and metronidazole and their related nitroimidazole derivatives.

methanol, ethanol, acetonitrile and methanol—acetonitrile (3:1) for the amount of protein removed from serum samples. The effects of these solvents are also described with regard to sample solvent and volume that is acceptable for column injection on reversed-phase columns. We have been able to reduce the volume of serum required by 80% for the measurement of misonidazole and desmethylmisonidazole in comparison to other authors [5, 6], and to remove 99.6% of the serum proteins prior to the analytical step. We have a four-fold increase in sensitivity with this assay as compared to the method of Meering and Maes [6]. We summarize patient serum levels in relation to the drug dose given based on body surface area.

EXPERIMENTAL

Reagents

We obtain acetonitrile and methanol, both HPLC grade, and potassium phosphate monobasic from J.T. Baker, Phillipsburg, NJ, U.S.A. Absolute ethanol (200 proof) is obtained from U.S. Industrial Chemicals, New York, NY, U.S.A. Water is deionized by repeated passage through a four-cartridge (organic removal, two mixed-bed ion-exchangers, and a 200 μm pore exclusion) filter system (Barnstead Nanopure; Barnstead/Sybron, Boston, MA, U.S.A.). The water supplied to this unit is from a commercial deionizer.

Desmethylmisonidazole, misonidazole and metronidazole are obtained from the National Cancer Institute (Bethesda, MD, U.S.A.) and used as received. The following nitroimidazoles are supplied by Hoffmann LaRoche, Nutley, NJ, U.S.A.: 1-(2-nitroimidazol-1-yl)-3-fluoropropan-2-ol (Ro 07-0741), 1-(2-nitroimidazol-1-yl)-3-ethoxypropan-2-ol (Ro 07-0913), 1-(2-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0269), 1-(2-methyl-5-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0207, and 1-(2-methyl-5-nitroimidazol-1-yl)-acetic acid (Ro 11-6924).

Chromatography

The ambient temperature chromatography is performed with a Model 6000A

solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 7120 injection valve and a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.). The Ultrasphere ODS (octadecylsilane, 5 μm) (150 × 4.6 mm) packed column is obtained from Altex Scientific, Berkeley, CA, U.S.A. The Varian MCH-10 10-μm (300 × 4.0 mm) (MCH is a monomeric octadecylsilane packing), MCH-5 5-μm (150 × 4.0 mm) and the MCH-N-cap-5 (monomeric octadecylsilane that is end-capped with a trimethylsilyl group) 5-μm (150 × 4.0 mm) packed columns are obtained from Varian Instruments, Palo Alto, CA, U.S.A.

Detector 1 is an Hitachi Model 100-30 with an Altex flow cell module Model 155 obtained as a total unit from Altex Scientific. The wavelength setting is 323 nm and the absorbance units full scale (a.u.f.s.) is 0.05. Detector 2 is a Waters Model 440 absorbance detector with a fixed wavelength of 313 nm; the a.u.f.s. is 0.02.

The mobile phase is a mixture of 1 m*M* pH 4.0 potassium phosphate—acetonitrile (93:7, v/v). The flow-rate is 1.5 ml/min. The mobile phase is degassed by bubbling helium through it for 10 min at a moderate rate.

A volume greater than 20 μl is injected to fill the sample loop adequately.

Protein measurements

Protein concentration of the treated sample supernatant is measured using the Lowry phenol method [7]. Reagent blanks were run for each organic solvent used. Absorbance readings were made at 750 nm, with a Model 200 spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.).

Sample preparation

Heparinized plasma or serum is collected in glass containers. All specimens are immediately frozen at −20°C unless the analysis can be done within 6 h. Plastic tubes are not used because of binding reactions between the nitroimidazoles and the plastic surface [8].

A 200-μl volume of standard, control or patient sample is placed into 12 × 75 mm glass test tubes. A 600-μl volume of protein-precipitating solvent consisting of methanol—acetonitrile (3:1) (alternatively 600 μl of ethanol or methanol or acetonitrile are used to establish this method) is added to each sample tube. Tubes are then vortexed for 10 sec, 200 μl of internal standard are added to each tube and then each tube is vortexed for 5 sec at medium speed. The tubes are allowed to stand at room temperature for 15 min to allow precipitation of the proteins to be completed. The tubes are centrifuged for 8 min at 1700 *g*.

RESULTS

Fig. 2 illustrates the effect of methanol, ethanol, acetonitrile and methanol—acetonitrile (3:1), at dilutions of 1/1 (serum to solvent ratio) to 1/10, on the amount of protein remaining in the serum sample. The three solvents and the solvent combination, where 1 volume of serum containing a known concentration of the two drugs was treated with 3 volumes of each particular solvent, were evaluated using the chromatographic system described with an Ultrasphere ODS 5-μm column. The ethanol-treated serum samples gave chromato-

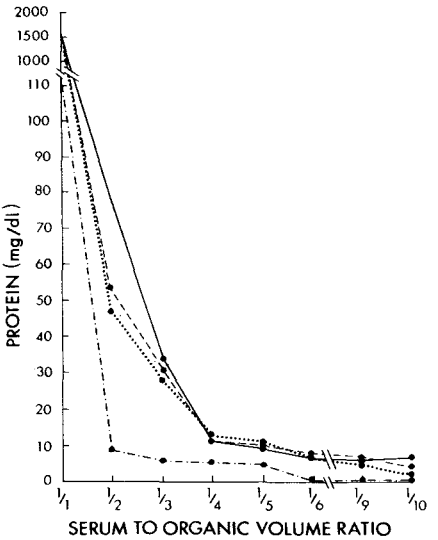


Fig. 2. Graph of the effect of the three organic solvents and a mixture of two of the solvents on the amount of protein remaining in a serum sample. ——, Methanol; – – –, ethanol; · · · ·, methanol—acetonitrile (3:1); —·—·—, acetonitrile.

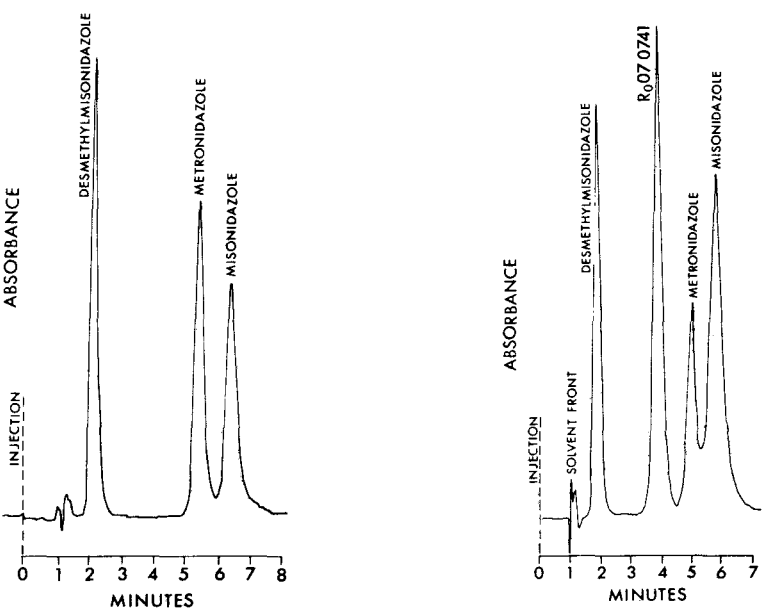


Fig. 3. Chromatogram of the three principle nitroimidazoles separated on an Ultrasphere ODS 5-μm column after serum sample preparation with methanol—acetonitrile (3:1).

Fig. 4. Chromatogram of the principle nitroimidazoles and of Ro 07-0741 on an Ultrasphere ODS 5-μm column.

TABLE I

PRECISION (CV%)

	Conc. (μg/ml)	Within day (n=6)		Day-to-day (n=10)*	
		313 nm	323 nm	313 nm	323 nm
Desmethylmisonidazole	7.5	2.9	1.2	7.3	5.7
	15.0	1.2	1.2	3.8	3.4
	60.0	0.9	2.4	2.2	2.1
Misonidazole	7.5	3.7	1.5	9.2	8.7
	15.0	3.2	2.2	5.7	5.3
	60.0	2.7	1.2	5.5	5.0

*Assayed in triplicate.

TABLE II

RETENTION TIMES

For operating conditions see *Chromatography* section.

Compound	Retention time (min)
Ro 11-6924	1.0
Desmethylmisonidazole	1.8
Ro 07-0741	3.7
Metronidazole	4.7
Misonidazole	5.4
Ro 07-0269	9.5
Ro 07-0913	13.0
Ro 07-0207	25.0

TABLE III

MISONIDAZOLE DOSAGE AND SERUM LEVEL CORRELATIONS

Dose (g/m^2)	n	Serum level (μg/ml)	
		Misonidazole	Desmethylmisonidazole
0.4	5	15.6 ± 2.6	2.0 ± 0.3
1.0	2	27.5 ± 0.5	10.0 ± 0.8
2.5	3	66.7 ± 0.9	13.3 ± 0.7

graphic results with a double peak for desmethylmisonidazole and a front shoulder on the misonidazole peak. Acetonitrile-treated serum samples chromatographed with the major portion of desmethylmisonidazole, co-eluting with the solvent front and a misonidazole peak that had a distorted front side. The methanol-treated serum sample gave chromatographic results with a small diffuse shoulder on the front side of the desmethylmisonidazole peak. When methanol—acetonitrile (3:1) was used as the solvent for serum sample preparation the separations were not interfered with by the injected sample. This is illustrated in Fig. 3, with metronidazole being added to verify the resolution of all three compounds.

The MCH-10 packing gives diffuse broad peaks that are not satisfactory. The MCH-5 packing gives clear resolution of all three compounds in 7 min. However,

the serum sample must be pretreated with methanol alone. The MCH-N-cap-5 packing can accomplish the separation of the three compounds in 4 min. Methanol must be used for the sample clean-up. The mobile phase for all the MCH packing materials is essentially the same as that used for the Ultrasphere ODS 5-μm packing.

Concentration linearity and precision using methanol—acetonitrile (3:1) and the Ultrasphere ODS 5-μm column gives a linear relationship from 0 to 120 μg/ml for desmethylmisonidzole with a CV of 2.4% ($y = 1.001x - 0.1$). Misonidazole is linear from 0 to 240 μg/ml with a CV of 1.2% ($y = 1.003x - 0.2$). Linearity above these values was not verified. The CV for within-day and day-to-day precision for the two wavelengths is shown in Table I. Recovery from serum or plasma is between 98% and 100% for both compounds and the internal standard. The detection limit at two times the signal-to-noise ratio using 200 μl of serum is 0.2 μg/ml for desmethylmisonidazole and 0.4 μg/ml for misonidazole. This represents an on-column injection of picogram amounts.

Fig. 4 shows that Ro 07-0741 elutes at 3.8 min, between desmethylmisonidazole and metronidazole with baseline resolution. Table II lists retention times for eight nitroimidazoles.

The serum levels of desmethylmisonidazole and misonidazole in adults given misonidazole on a g/m^2 basis are shown for three different dose levels in Table III.

DISCUSSION

Distortion of the chromatographic patterns by the small amount of organic solvent in the injection of the sample on to the column is not surprising. However, we have not been faced with this difficulty when we used 10-μm sized packings. Our utilization of 5-μm packings showed us that indeed a great deal of care must be used in the sample solvent to allow for precise chromatographic results. The work of Johnson et al. [9] described some related situations where precautions were indicated concerning the percentage of organic modifiers that could be injected containing the sample over and above which chromatographic distortions were likely to occur with ODS columns. Tseng and Rogers [10] were able to produce two sharp peaks or a sharp peak with a shoulder from a single pure compound that was being chromatographed by reversed-phase methods depending on the solvent combination used for the mobile phase and that which was used to introduce the sample onto the column.

Methanol—acetonitrile (3:1) used as the precipitating and sample solvating agent provides for very satisfactory chromatographic resolution with Ultrasphere ODS 5-μm columns. Methanol as the sample treatment agent provides the best results with the MCH-5 columns.

To avoid the large sample dilution and its accompanying loss of sensitivity of drug measurements, which Workman et al. [5] and Meering and Maes [6] utilize in their 1/9 dilution of serum with methanol, we use a 1/3 dilution of serum with methanol—acetonitrile (3:1). The presence of acetonitrile clears the supernatant whereas methanol alone leaves the supernatant slightly cloudy. We use one-fifth the amount of serum or plasma and we inject one-fifth the amount of the prepared sample onto the column as compared to Meering and Maes [6].

This procedure puts less protein onto the column per injection than these authors by 0.7 mg% (see Fig. 2). We routinely store our chromatography columns in acetonitrile which further removes any protein left on the column from this assay.

With this method we can use either 313 nm at 0.02 a.u.f.s. or 323 nm at 0.05 a.u.f.s. Table I lists the precision at each wavelength.

To our knowledge no one has published serum results for these compounds determined by HPLC from specific doses given to adult patients. The ratio between desmethylmisonidazole and misonidazole varies considerably between patients but is fairly constant for a given patient at a fixed sampling time from day to day.

ACKNOWLEDGEMENTS

The generous supply of 1-(2-methyl-5-nitroimidazol-1-yl)-acetic acid (Ro 11-6924), 1-(2-nitroimidazole-1-yl)-3-fluoropropan-2-ol (Ro 07-0741), 1-(2-nitroimidazol-1-yl)-3-ethoxypropan-2-ol (Ro 07-0913), 1-(2-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0269), and 1-(2-methyl-5-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0207) was given to us by W.E. Scott of Hoffmann-LaRoche.

Technical consultation from Nelson Cook of Altex Scientific and from Ron Majors of Varian Instruments is acknowledged.

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CHROMBIO. 1392

Note

High-performance liquid chromatographic method for the determination of diprophylline in human serum

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(First received November 3rd, 1981; revised manuscript received June 21st, 1982)

Diprophylline [7-(2,3-dihydroxypropyl)theophylline, dyphylline] is a xanthine derivative which has been available for many years as a possible substitute for theophylline in the treatment of bronchospasm, and it has been suggested that it may show a lower incidence of side-effects than does theophylline [1]. The effectiveness of diprophylline therapy remains to be established [2] and blood level measurements are needed in conjunction with clinical investigations in order to establish appropriate dosage regimes which produce the desired therapeutic response.

In early studies of xanthine derivatives, the UV absorption method of Schack and Waxler [3] was used, but because of the poor specificity of this method, interpretation of results can be difficult, especially if dietary xanthine intake is not restricted. Specific gas chromatographic methods have been developed [4] and have been applied to pharmacokinetic studies in human volunteers [5]. These methods have good specificity and sensitivity but sample preparation can be complex or time-consuming. High-performance liquid chromatography (HPLC) has found wide acceptance in the measurement of serum theophylline levels [6—11] and has been applied to the measurement of diprophylline in human serum [12—14] and in serum and urine [15]. The direct injection of a trichloroacetic acid filtrate, as used by Valia et al. [14], results in a short column life and some interference by endogenous metabolites when a C_8 column is used, and the method of Simons and Simons [15] has low sensitivity.

The method described below was devised to allow routine measurement on a C_8 column of diprophylline in small serum samples (250 μl) from patients on a variety of other drugs and from volunteers participating in absorption studies.

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EXPERIMENTAL

Materials and reagents

The following xanthine derivatives were purchased from Sigma, London, Great Britain: 1,3-dimethyluric acid, 8-chlorotheophylline, 3-methylxanthine, theobromine (3,7-dimethylxanthine), diprophylline [7-(2,3-dihydroxypropyl)-theophylline], 2-hydroxyethyltheophylline, theophylline (1,3-dimethylxanthine), proxyphylline [7-(2-hydroxypropyl)theophylline], caffeine (1,3,7-trimethylxanthine). All other reagents used were of analytical grade.

The internal standard used was theophylline at a concentration of 30 mg/l in water. It was prepared fresh daily by dilution from a 3 g/l aqueous stock solution.

The extraction solvent was chloroform—methanol (9:1, v/v).

Calibration standards were prepared by the addition of diprophylline to pooled bovine serum and were stored at −16°C. Peak height ratios of diprophylline and internal standard were plotted to give calibration graphs. Repeatability studies were performed on samples of pooled human serum to which diprophylline had been added.

Diprophylline absorption was studied in four adult male volunteers. After fasting overnight, 400 mg of diprophylline were taken by mouth, and 5-ml blood samples were taken at time zero, and at each half hour for 3 h then hourly until 6 h had elapsed. The samples were allowed to clot and the separated serum was stored at −16°C until analysis.

Samples from patients not taking diprophylline but on a variety of other drugs were residues of samples sent to this laboratory for a variety of tests.

Extraction procedure

Internal standard (50 μl) and 250 μl of sample were placed in a 10-ml conical glass centrifuge tube and vortex mixed. Anhydrous sodium sulphite (1.5 g) was then added and the tube capped and shaken vigorously for 30 sec with 2.5 ml extraction solvent. These steps were carried out with the minimum of delay to avoid the formation of a solid cake of sodium sulphite. After centrifugation at 1000 *g* for 5 min, the aqueous layer was discarded and the solvent filtered into a 10-ml conical centrifuge tube through a Whatman No. 1 filter paper which had been pre-wetted with chloroform. The filter paper was rinsed with 0.5 ml of chloroform. The filtrate was dried under nitrogen at 40°C, redissolved in 100 μl of dichloroethane and back-extracted into 100 μl of 0.1 *M* ammonium carbonate by vortex mixing for 10 sec. Centrifugation at 1000 *g* for 5 min produced a clear aqueous layer which was used for chromatography.

HPLC conditions

Chromatography was carried out on LiChrosorb RP-8, 10 μm, supplied by E. Merck, Darmstadt, G.F.R., packed at 300 bars in a 25 cm × 4 mm stainless steel column as a suspension in methanol. Eluant was delivered by an Altex 110 pump at 2.0 ml/min and absorbance of the eluate was monitored at 275 nm with an Hitachi 100-10 spectrophotometer using an Altex 8-μl flow cell with full-scale deflection set at 0.02. A Rheodyne 7125 syringe loading injection valve fitted with a 20-μl loop was used to introduce 10-μl samples.

The HPLC eluant was prepared by adding 250 ml of methanol to 750 ml of phosphate buffer prepared by adding 1.5 ml of 1 *M* KH_2PO_4 to 750 ml of glass-distilled water and adjusting to pH 3.0 with 0.9 *M* perchloric acid. The eluant was degassed ultrasonically and filtered before use. Chromatography was carried out at ambient temperature (18—22° C).

RESULTS

Representative chromatograms are shown in Fig. 1. Overall recovery was estimated by comparison of the peak heights found when known amounts of aqueous diprophylline standard were injected and the peak heights found when serum standards were carried through the entire procedure. The results shown in Table I are the mean of five measurements at each level. Calibration was achieved by plotting the peak height ratio of diprophylline and internal standard versus added diprophylline concentration and was linear to 20 mg/l with a linear regression equation $Y = 0.484X + 0.27$; the correlation coefficient was 0.9995. Although linearity extended beyond 20 mg/l, the peak height ratio was unacceptably high above this concentration and samples with higher concentrations were diluted with blank serum and re-extracted.

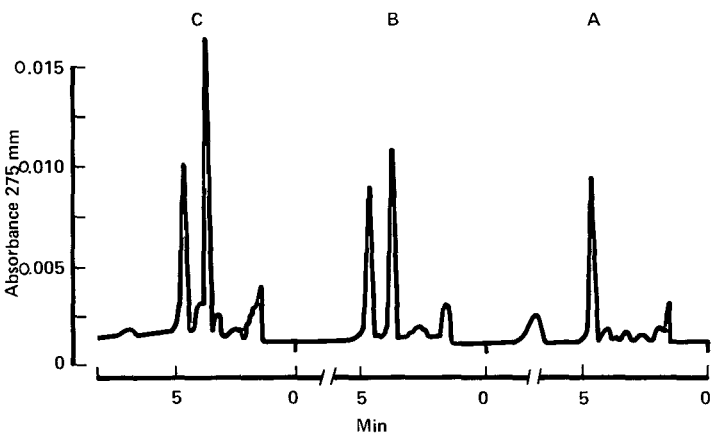

Fig. 1. HPLC of diprophylline standards and a human serum sample. (A) Blank human serum extract with added internal standard. (B) Serum standard containing internal standard and 5 mg/l diprophylline. (C) Patient's sample containing internal standard and 8 mg/l diprophylline. HPLC conditions as stated in the text.

Selectivity was studied in two ways. First, various xanthine derivatives were prepared as aqueous solutions and after injection of 0.1-μg amounts their retention times relative to diprophylline were measured. The results are shown in Table II. Second, in an attempt to exclude possible interference by a wide range of drugs and their metabolites, samples from patients on a variety of drugs but not taking diprophylline were extracted and chromatographed. No interferences were found for the following drugs: acetazolamide, amitriptyline, amylobarbitone, carbamazepine, diazoxide, disopyramide, ethosuximide, isoprenaline, nitrazepam, nortriptyline, oxazepam, paracetamol, pentobarbitone,

TABLE I

PERCENTAGE RECOVERY OF DIPROPHYLLINE ADDED TO HUMAN SERUM

Results shown represent the mean of five measurements at each level.

Concentration added (mg/l)	Calculated peak height* for 100% recovery (mm)	Measured peak height (mm)	Percentage recovery
2.5	18	16.5	92
5	36	35	97
10	72	71	99

*Based on the measured peak height after injection of known amounts of aqueous standard.

TABLE II

RETENTION TIMES OF SOME XANTHINE DERIVATIVES RELATIVE TO DIPROPHYLLINE

1,3-Dimethyluric acid	0.42
8-Chlorotheophylline	0.66
3-Methylxanthine	0.70
Theobromine	0.75
Diprophylline	1.00
β-Hydroxyethyltheophylline	1.22
Theophylline	1.33
β-Hydroxypropyltheophylline	1.90
Caffeine	2.11

TABLE III

WITHIN-BATCH AND BETWEEN-BATCH PRECISION OF DIPROPHYLLINE MEASUREMENTS AT THREE LEVELS OF DIPROPHYLLINE ADDED TO POOLED HUMAN SERUM

Concentration added (mg/l)	Within-batch (n = 10)		Between-batch (n = 15)	
	Mean ± S.D. (mg/l)	C.V. (%)	Mean ± S.D. (mg/l)	C.V. (%)
2.5	2.47 ± 0.13	5.3	2.32 ± 0.18	7.2
5.0	5.03 ± 0.24	4.7	4.95 ± 0.27	5.3
10.0	10.22 ± 0.51	5.0	9.95 ± 0.64	6.3

phenobarbitone, phenytoin, primidone, procainamide, salicylate, salbutamol, sulthiame.

The precision of the method within batch and between batch was assessed at three levels (Table III). The within-batch precision was measured by extracting and chromatographing ten replicate samples within one working day. The between-batch precision was measured over a period of 30—40 days.

Within-batch variation in the peak height of the internal standard was found to be less than ± 10% of the mean peak height.

Fig. 2 shows the mean serum levels found in four normal subjects following an oral dose of 400 mg of diprophylline. The calculated mean half-life was 2.3 h.

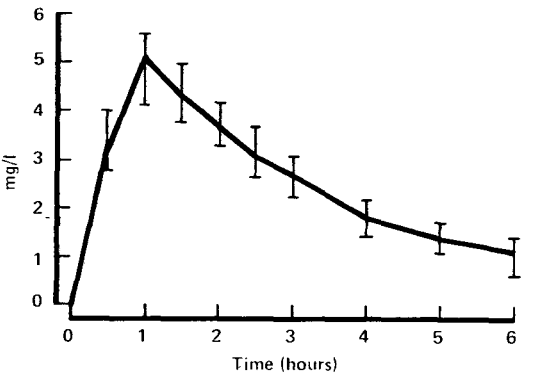

Fig. 2. Mean serum diprophylline concentration found in four subjects after taking an oral dose of 400 mg of diprophylline. The vertical bars indicate the range of concentrations found at each time.

DISCUSSION

Diprophylline has a solubility of 1 part in 10 of water and a correspondingly low solubility in organic solvents [16], so that many commonly used extraction techniques are unsatisfactory and sample preparation methods based on protein precipitation may have advantages. However, it was found in preliminary experiments that direct injection of trichloroacetic acid treated serum on to RP-8 columns resulted in a short column life and interference by endogenous serum constituents. Attempts to purify trichloroacetic acid treated serum by extraction into organic solvents led to low recoveries and loss of sensitivity. The difficulty of low solubility in organic solvents was overcome by the salting-out effect of the addition of sodium sulphite. If salting-out was not used, recoveries of approximately 10—20% were observed rather than the 92—99% recovery shown in Table I. The second extraction step was used to minimise the extraction of other drugs and of endogenous serum constituents. The value of this extraction is illustrated by the wide diversity of types of drug and their metabolites that were shown not to cause interference.

Theophylline was used as the internal standard. It has the required extraction, chromatographic and UV absorption characteristics and it is unlikely that a patient would be treated with these two xanthine derivatives simultaneously. The within-run repeatability of the internal standard peak height was always within ± 10%, so the presence of significant amounts of theophylline in any patient's sample would be indicated by a readily detectable increase in the internal standard peak height. If it is necessary to determine theophylline and diprophylline in the same sample, β-hydroxypropyltheophylline is a suitable alternative internal standard.

Most published work on the HPLC of xanthine derivatives makes use of C_{18} packing materials and acetonitrile-based eluants. In the present method, methanol is used as a much less toxic alternative to acetonitrile and the use of a methanol-based eluant and an octyl bonded silica packing allows good separation of theophylline and diprophylline from endogenous serum constituents. A single RP-8 column has been in routine use for 12 months with only occasional repacking of the top 1 mm being required to maintain resolution.

The sensitivity of the method allows the determination of 1 mg/l concentrations of diprophylline in serum with a coefficient of variation of $< 10\%$. Ten serum samples, three calibration standards and a quality control sample can be extracted and chromatographed in a 2.5-h period.

The results obtained on the four volunteers are in good agreement with previous published results. The short half-life of diprophylline and the low peak serum levels achieved after ingestion of doses comparable to the commonly used theophylline doses suggest that frequent large doses of this drug are likely to be required to achieve the deserved therapeutic effects. Studies on various doses and routes of administration in a variety of clinical situations are proceeding.

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Note

High-performance liquid chromatographic determination of 2-mercapto-propionylglycine (thiopronine) in urine

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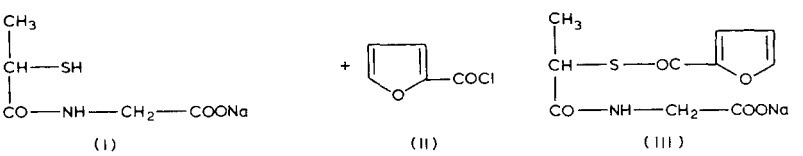
(First received March 25th, 1982; revised manuscript received June 21st, 1982)

Thiopronine (2-mercapto-propionylglycine or 2-thiol-propionamidoacetic acid) is utilized as a hepatotropic [1—3], radioprotective [4] and mucolytic [5—7] drug. The substance has been detected in biological fluids using the amino group—free thiol method [8] or the radioactive ^{35}S-labelled compound [9].

This work describes a more specific and sensitive method suitable for bioavailability studies. Thiopronine (I) sodium salt reacts quantitatively in a pH 7.0 buffer with 2-furoyl chloride (II) to obtain 2-furoyl-thiopronine (III) [10]. Compound III is quantitatively determined by high-performance liquid



chromatography (HPLC). This determination is carried out on urine samples after administration of thiopronine. In this case the interfering substances present in urine are partially eliminated through percolation on a glass column.

EXPERIMENTAL

Materials

The solvents used are all of HPLC grade (LiChrosolv, from Merck, Darmstadt, G.F.R., or Carlo Erba, Milan, Italy). The water was previously bidistilled

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through a glass distiller and filtered on a 0.45-μm membrane (type FH, Millipore). The other reagents are all of analytical grade.

Chromatographic conditions

The high-performance liquid chromatograph is a Varian Model 5000, equipped with a Varian UV 50 detector, a Varian CDS 111L integrator, a Varian 9176 recorder, and a Rheodyne Model 7125 injector. The column used is packed with LiChrosorb RP-18 10 μm supplied by Brownlee Labs. (Santa Clara, CA, U.S.A.); the mobile phase is McIlvaine buffer pH 3.0—acetonitrile (80:20, v/v). The buffer at pH 3.0 is prepared by mixing 15.89 ml of 0.1 *M* citric acid with 4.1 ml of 0.2 *M* disodium phosphate. The flow-rate is 0.8 ml/min, pressure 37 bars, wavelength 290 nm, and the quantity injected is 20 μl.

Standards

Prepare a standard solution of thiopronine in distilled water at a concentration of 1000 μg/ml. From this solution prepare standard solutions in water or urine diluting 1 ml to 10 ml with water or urine. The standard solutions are stable for at least one week if kept at +4°C. To determine the calibration curve and method sensitivity prepare dilutions of thiopronine from 50 to 1 μg/ml in urine.

Procedures

Prepare a chromatographic glass column having an internal diameter of 10 mm, equipped with porous septum, packed with 2 g of LiChrosorb RP-18 with an average diameter of 40—60 μm (Merck). The column so prepared is washed with 5 ml of acetonitrile (LiChrosorb, Merck) and successively with 5 ml of distilled water; the washings are discarded. Apply to the column 1 ml of a standard solution of thiopronine in water or 1 ml of a standard solution in urine, and elute with 5 ml of distilled water. Add to the eluate (collected in a 15-ml capped test tube) 1 ml of buffer pH 7.0 (prepare a 0.5 *M* Na_2HPO_4 solution and adjust the pH to 7.0 with 85% H_3PO_4), stir on a Vortex for 1 min, and then add, under a hood, 1 drop of 2-furoyl chloride (Fluka, Buchs, Switzerland); stir again for 1 min. Add distilled water up to 10 ml, stir and filter on a 0.5-μm Millipore membrane. The solution is ready to be injected.

Quantitative evaluation

In the analysis of thiopronine levels in the urine, peak areas were compared with those of an aqueous solution of thiopronine standard. No internal standard was used owing to the very simple procedure.

Animal study

Male rats (Wistar albino, Morini), weighing 200 g and fasted overnight were used. The animals were treated intravenously or orally with a 100 mg/kg dose of thiopronine dissolved in water. Urine samples were collected 24 h after administration, stored at 4°C and analysed within a week.

RESULTS AND DISCUSSION

Fig. 1 shows a typical high-performance liquid chromatogram of compound III obtained from the reaction of sodium thiopronine and 2-furoyl chloride. Fig. 2 shows a typical chromatogram of thiopronine extracted from urine of treated rats. Fig. 3 shows the profile of urine of rats not treated and not passed through the glass column; and Fig. 4 shows the profile of urine of rats not treated but passed through the glass column. The importance of this passage is obvious to reduce interference between the peaks of substances present in the urine and the peak of thiopronine. The urinary recovery for thiopronine using the described procedure is 95.0 ± 4.3%. The mean inter-assay and intra-assay variability for the compound was 5.38 and 4.76, respectively

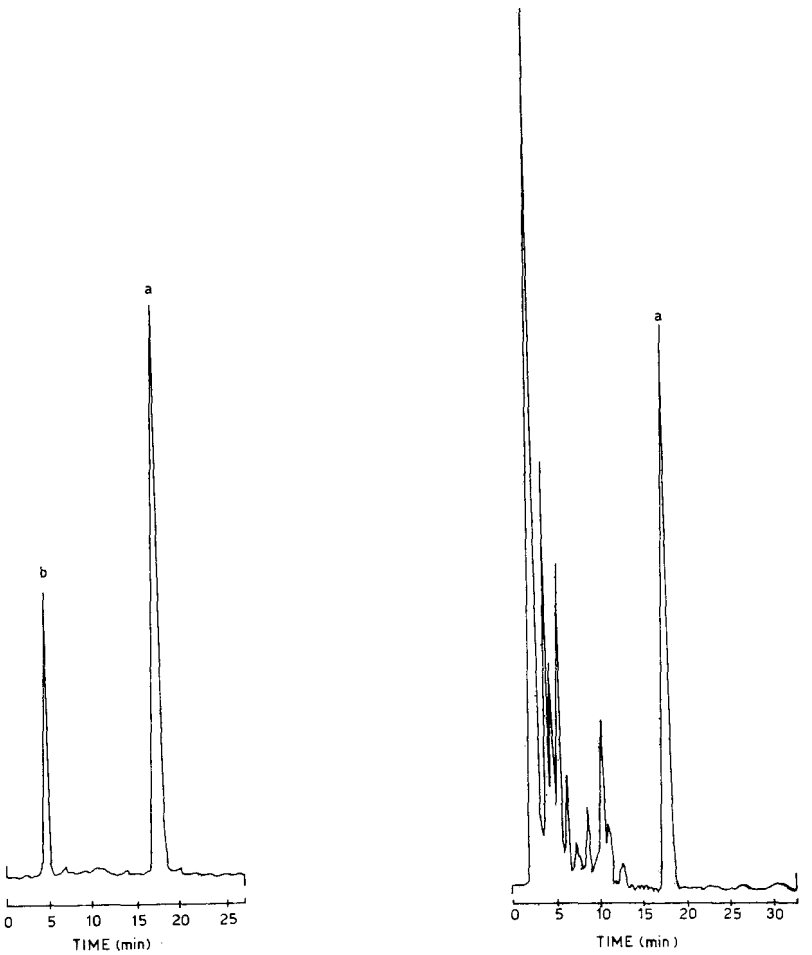
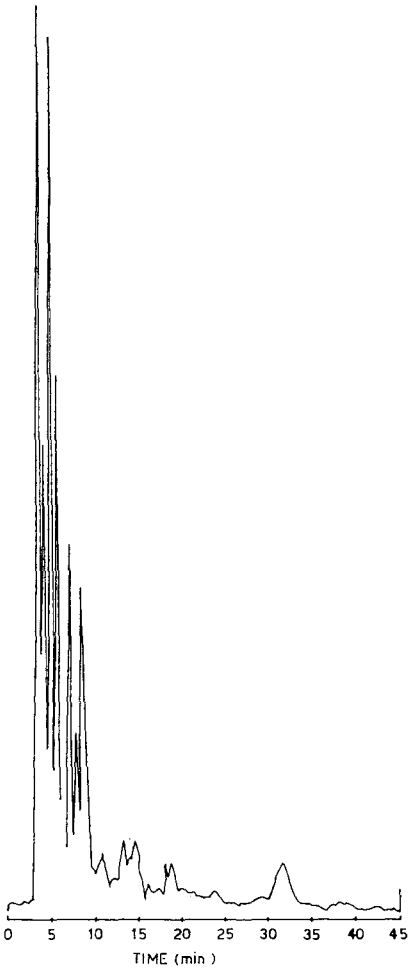


Fig. 1. Chromatogram of compound III prepared from water. a = 2-Furoyl-thiopronine (III), b = 2-furoyl chloride (II) in excess.

Fig. 2. Chromatogram of compound III obtained from urine of treated rat. a = 2-Furoyl-thiopronine (III).



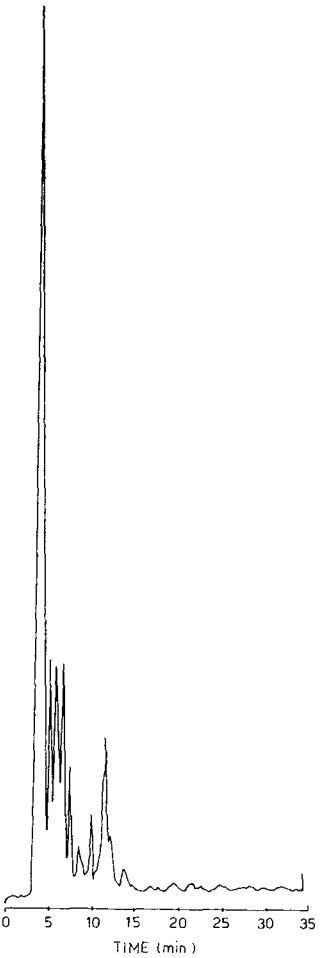


Fig. 3. Chromatogram of blank urines not passed through the glass column packed with 2 g of LiChrosorb 40—60 μm (Merck).

Fig. 4. Chromatogram of blank urines passed through the glass column packed with 2 g of LiChrosorb 40—60 μm (Merck).

(Table I). The minimum detectability of the drug with the described procedure is 1 μg/ml of urine.

The calibration curve for thiopronine shows a good linearity over the concentration range 1—50 μg/ml both in water and in urine. The relationship between thiopronine urine concentration in the range 1—50 μg/ml and the peak area is: peak area (instrument) = 3.0528 × concentration (μg/ml) + 0.03348. The correlation coefficient is 0.9999.

The first results of bioavailability studies on animals are reported in Table II. More extensive results on animals and on humans will be reported soon. In conclusion, the proposed method is suitable for a sensitive and reproducible quantitative evaluation of thiopronine in urine.

TABLE I

RECOVERY AND INTRA- AND INTER-ASSAY VARIABILITY DATA

Aliquots (1 ml) of control urine were spiked with 100 μl of standard solutions and treated as described in the Experimental section. Recoveries were determined from HPLC peak areas from one set of spiked samples. Intra-assay variability was determined from three sets of spiked samples that were extracted and analyzed in one day. Inter-assay variability was determined from three sets of spiked samples that were extracted and analyzed on three different days.

Amount added to 1 ml of urine (μg)	Recovery (%)	Inter-assay variability			Intra-assay variability		
		Amount found (μg/ml)		Coefficient of variation (%)	Amount found (μg/ml)		Coefficient of variation (%)
		Mean	± S.D.		Mean	± S.D.	
1	89	0.90	0.06	6.67	0.93	0.05	5.38
2	93	1.88	0.11	5.85	1.96	0.09	4.59
5	99	5.16	0.27	5.23	5.08	0.24	4.72
10	101	10.19	0.61	5.99	10.09	0.55	5.45
20	97	19.56	0.98	5.01	19.92	1.00	5.02
35	91	34.82	1.42	4.08	35.02	1.32	3.77
50	95	51.07	2.48	4.86	50.85	2.22	4.37
Mean ± S.D.	95.0±4.3			Mean: 5.38			Mean: 4.76

TABLE II

URINARY EXCRETION (0—24 h) OF THIOPRONINE AFTER INTRAVENOUS OR ORAL TREATMENT WITH 100 mg/kg IN THE RAT

Treatment	Rat No.	Weight (g)	Urine volume (ml)	Urinary excretion of thiopronine (0—24 h)		
				Concentration (μg/ml)	Total excretion (mg)	Excretion/administration (%)
Intravenous	1	300	14.5	415.42	6.024	20.08
	2	295	13.5	552.83	7.463	25.30
	3	300	13.8	480.45	6.630	22.10
Oral	4	255	14.0	4.59	0.064	0.25
	5	250	12.5	4.38	0.055	0.22
	6	285	13.5	1.61	0.022	0.08

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Note

Determination of chloramphenicol-glucuronide in urine by high-performance liquid chromatography

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The recently renewed interest in chloramphenicol (CAP) for treating serious infections has created a need for fast, reliable, and accurate measurement of the drug in body fluids [1]. Recently Aravind et al. [2] described a high performance liquid chromatographic (HPLC) method for the simultaneous measurement of CAP and its succinate ester (CAPS) in serum, cerebrospinal fluid, and urine. The purpose of this communication is to extend the original methodology to include analysis of chloramphenicol glucuronide (CAPG) in urine.

MATERIALS AND METHODS

Chromatography

All assays were performed on a Perkin-Elmer Series II high-performance liquid chromatograph and interfaced with a Sigma 10-B data system (Perkin-Elmer, Norwalk, CT, U.S.A.). The chromatographic conditions and data handling were as previously reported [2].

Reagents

Bovine liver β-glucuronidase type B (β-Glu) containing no sulphatase activity was obtained from Sigma (St. Louis MO, U.S.A.). A working β-Glu solution containing 5000 Fishman units of enzyme per ml was prepared in 0.1 *M* sodium acetate buffer (pH 5.0). Saccharo-1,4-lactone monohydrate, a specific inhibitor for β-glucuronidase was obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). A working standard containing 200 μg/ml of CAP was prepared in

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drug-free urine. All other chemicals and reagents were as previously described [2].

Procedure

All urine samples were placed in 10 × 75 mm disposable culture tubes and were analyzed for CAP and CAPG as detailed in Table I.

The tubes were gently vortexed and then covered with Parafilm® and incubated in a water bath at 37°C for 3 h. At the end of the incubation, the tubes were centrifuged for 5 min at 2000 *g*. A 10-μl aliquot of the supernatant was injected onto the column.

TABLE I

INCUBATION CONDITIONS

Tube A	Tube B	Tube C
0.1 ml of standard or patient urine + 0.9 ml sodium acetate buffer (pH 5.0)	0.1 ml of standard or patient urine + 0.9 ml of working β-Glu solution	0.1 ml of standard or patient urine + 0.9 ml of working β-Glu solution + 10 mg saccharo-1,4-lactone

RESULTS AND DISCUSSION

Fig. 1 shows three chromatograms which were obtained after analysis of a patient's urine sample. This patient had been maintained on chloramphenicol palmitate. Fig. 1A shows the chromatogram obtained when the sample was not treated with β-Glu. The retention times for CAPG and CAP were 1.6 and 2.9 min, respectively. Fig. 1B shows the chromatogram obtained after hydrolysis of the same sample with β-Glu. In this chromatogram the CAPG peak is absent and there is a corresponding increase in the CAP peak. Fig. 1C shows the chromatogram after incubation of the sample with both β-Glu and saccharo-1,4-lactone (inhibitor of β-glucuronidase). This chromatogram is virtually identical to that of Fig. 1A, confirming that the peak at 1.6 min was CAPG. Analysis of the area count under the peak provided additional support that the peak at 1.6 min was indeed CAPG. In Fig. 1A, the CAPG and CAP had area counts of 8.6 and 3.5, respectively. In Fig. 1B, the area count for CAP increased to 11.9, an amount (8.4) virtually equal to that of the CAPG peak. In Fig. 1C, the area counts for CAPG and CAP were 8.5 and 3.5, the values obtained in Fig. 1A.

Table II shows data obtained from several patients. Since standard CAPG was not readily available, the CAPG concentrations were reported as CAP equivalents. The data in Table II substantiate our earlier reports [1, 2] of unpredictable and variable excretion of CAPS and CAP in urine. The data indicate that CAP is also glucuronidated to a variable extent as well. The mean percentages determined from the data in Table II for CAPS, CAP and CAPG were 51.4, 22.5 and 26.2, respectively.

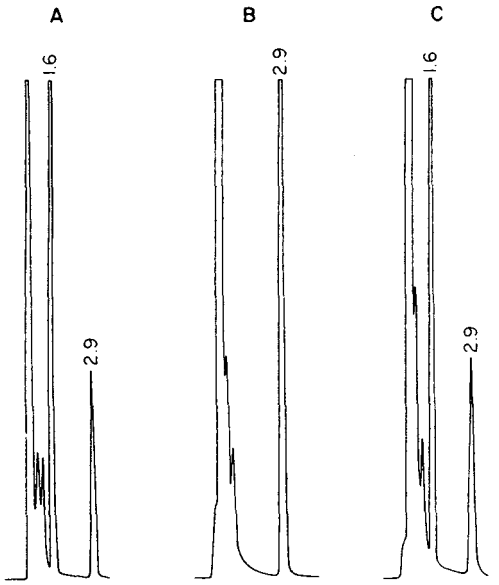

Fig. 1. (A) Chromatogram obtained from urine not treated with β-glu; (B) chromatogram of the same sample after hydrolysis with β-glu; and (C) chromatogram of the same sample treated with saccharo-1,4-lactone with β-glu. The retention times for CAPG and CAP were 1.6 and 2.9 min, respectively.

TABLE II

AMOUNTS OF CAP, CAPS AND CAPG RECOVERED IN PATIENT URINE

The percentage values of CAPS, CAP and CAPG are determined from the total recovered.

Patient no.	Dose of CAP (mg)*	CAPS (mg)	CAP (mg)	CAPG (mg)	Total (mg)	CAPS (%)	CAP (%)	CAPG (%)
1	355	187.5	81.7	77.1	346.3	54	24	22
2	250	108.4	23.1	42.8	174.3	62	13	25
3	200	160.6	23.6	6.2	190.4	84	13	3
4	400	108.5	114.6	241.0	464.1	23	25	52
5	200	56.3	35.4	40.6	132.3	42	27	31
6	200	60.7	24.1	30.6	115.4	53	21	31
7	450	217.0	37.0	108.5	362.5	60	10	30
8	750	258.8	63.6	134.4	556.8	64	11	24
9	50	13.5	19.2	11.2	43.9	31	44	25
10	66	24.6	23.6	15.1	63.3	39	37	24

*Dose administered as CAPS.

Patient 3 (Table II) was a therapeutic failure. Unpon analysis of his urine, it was evident that this failure was not due to excessive glucuronidation of the drug, but rather to almost complete excretion of the unhydrolyzed succinate ester in his urine. Patient 4, on the other hand, initially failed to achieve a therapeutic level of CAP. Analysis of his urine indicated a larger-than-average capacity to glucuronidate the drug. Thus, his dose was subsequently increased

with the knowledge that he was capable of tolerating the increased dose because of his high glucuronidation ability.

The method presented here extends our previously reported method [2] to include the determination of CAPG. It is important to evaluate this metabolite to assess liver function in regards to CAP as well as provide additional information when evaluating toxicity or therapeutic failures.

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2 M.K. Aravind, J.N. Miceli, R.E. Kauffman, L.E. Strebel and A.K. Done, J. Chromatogr., 221 (1980) 176.

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CHROMBIO. 1397

Letter to the Editor

Determination of cinnarizine in plasma by high-performance liquid chromatography

Sir,

Nitsche and Mascher [1] commented recently on our use of 285 nm wavelength for the detection of cinnarizine in our publication [2] on the determination of cinnarizine in plasma by high-performance liquid chromatography. The actual wavelength employed was 258 nm, the last two digits being inadvertently interchanged during the typing of the manuscript. This error was not picked up during proof-readings and we would like to thank the authors for drawing attention to this discrepancy which hopefully has not caused too much inconvenience to other workers.

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H.K.L. HUNDT

1 V. Nitsche and H. Mascher, J. Chromatogr., 227 (1982) 521.
2 H.K.L. Hundt, L.W. Brown and E.C. Clark, J. Chromatogr., 183 (1980) 378.

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Book Review

Handbook of chromatography, Vol. I, Carbohydrates, edited by S.C. Churms (G. Zweig and J. Sherma, Editors-in-Chief), CRC Press, Boca Raton, FL, 1981, 272 pp., ISBN 0-8493-3061-0.

Factography in crystallized form — that would be the best way to describe the present volume. Those who are looking for essays will be disappointed (but I believe these will be very few), but those who are looking for a comprehensive and exhaustive survey of carbohydrate separations (and these are likely to be the majority) will be pleased. This volume is a continuation of the preceding ones. The content is rather simple: gas chromatography (GC), liquid chromatography (LC), paper chromatography (PC), thin-layer chromatography (TLC) and electrophoretic separations. In addition, the reader will find here a whole section on Detection techniques (divided into methods suitable for GC, LC and detection in TLC and PC). The third section is devoted to sample preparation and derivatization, and the fourth section deals with Products and Sources of Chromatographic Materials. Naturally there is a fifth section entitled Literature References. The section on Products and Sources of Chromatographic Materials offers a vast amount of information. But there are other useful tables: a list of monographs or books containing a chapter devoted to carbohydrate chemistry; a list of review articles; a survey of derivatization methods both for GC and high-performance liquid chromatography. From my personal view it is pleasant to find about three pages on the preparation of samples from biological specimens (p. 211).

Certainly one may say that the tabular-form editions of the CRC handbooks have become a part of tradition. But in the present volume this is brought to a stage of perfection by a wise selection of tables presented; adequately classified references cover the rest that would be beyond the scope of this volume. May be that a reference to available bibliographic data on chromatographic (and electrophoretic) separations of carbohydrates would make the present volume even more complete. However, is it possible to manage (as an editor) a volume like this one without *any* comments? I doubt it. Certainly the CRC handbook is a handbook in the very sense of the word and a good buy for many chromatographers — not only those who devote themselves to carbohydrate chemistry.

Prague (Czechoslovakia) Z. DEYL

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CHROMBIO. 1372

Book Review

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$ 72.25, ISBN 0-444-42029-0.

Volume 7, *Recent developments in mass spectrometry in biochemistry, medicine and environmental studies, 7,* is another valuable contribution to the useful *Analytical Chemistry Symposia Series*. Dr. Frigerio and the authors of the thirty-two articles have provided information for both specialists in the various areas of mass spectrometry and also for researchers in a wide variety of other fields to which mass spectrometry has much to offer. Quantitation and identification of drugs, their metabolites, other xenobiotics and endogenous compounds is a major theme in this volume. Harvey's paper on the use of stable isotopes and mass spectrometry in drug metabolism is an excellent short review of the topic. A number of the articles are concerned with environmental studies and advances in methodology [the papers from Cardiff, Great Britain (combined liquid chromatography—mass spectrometry of drugs, etc.) and Bielefeld, G.F.R. (peptide sequencing by mass spectrometry—mass spectrometry) are particularly pertinent]. The wide applicability of mass spectrometry-based methods is exemplified by the articles concerned with measuring anabolic drug residues in meat (Stan et al.), use of cell culture systems for studying drug metabolism (Chessebeuf and co-workers) and indole research (Hooper). Several of the articles are rather short on actual mass spectrometrically-derived information, and a few are too highly detailed for most readers, but this should be expected in a volume based on the papers presented at a "recent developments" meeting.

Rahway, NJ (U.S.A.) W.J.A. VandenHeuvel

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CHROMBIO. 1399

Book Review

Protein—protein interactions, edited by C. Frieden and L.W. Nichol, Wiley-Interscience, New York, Chichester, Brisbane, Toronto, 1981, XI + 403 pp., price £ 39.10, ISBN 0-471-04979-4.

Protein—protein interactions have recently achieved much popularity and represent a fast developing field of biochemistry. This is obviously due to the multiple biological functions of such interactions of which regulation of cell division and cell spreading may be mentioned as randomly chosen examples. Obviously, techniques like affinity chromatography, gel permeation chromatography, affinity and zone electrophoresis would be effective tools for solving problems in this field.

The present volume is a multiauthor book, in which didactically well written chapters are presented by leading scientists. Reading the book requires a fair knowledge of physical chemistry, and certainly the volume is not intended for beginners. Also those expecting practical hints would be disappointed, as the book (amazingly uniform in presentation of the individual chapters written by different authors) is kept very much on the theoretical side and in my eyes is a good source of inspiration. Naturally there are more than separation methods for judging protein—protein interactions. The chromatographic and electrophoretic techniques are summarized in the part called Mass Migration Methods written by D.J. Winzor. This part deals with zonal and frontal analysis, measurement of migration in gel chromatography, and migration of self-associating systems of which representative examples are presented. This involves gel chromatographic studies, the application of porous glass, differential migration techniques and some other procedures. The last part of this section is devoted to the interaction of dissimilar molecules and deals mainly with the electromigration techniques, exclusion chromatography and quantitative affinity chromatography.

The book can be recommended for advanced readers who would like to see the applicability of separation techniques from a slightly different point of view. Certainly the field of protein—protein interactions is a promising one that is likely to be invaded by diverse separation techniques, if not governed by them very soon. The present volume represents a good start in this direction.

Prague (Czechoslovakia) Z. DEYL

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CHROMBIO. 1400

Book Review

Fibrous proteins: Scientific, industrial and medical aspects, Vol. 2, edited by D.A.D. Parry and L.K. Creamer, Academic Press, London, New York, Toronto, Sydney, San Francisco, 1980, XVI + 257 pp., price US$ 35.00, ISBN 0-12-545702-2.

This is a second volume that has emerged from the International Conference on Fibrous Proteins, Scientific Industrial and Medical Aspects, that was held at the Massey University, Palmerston North, New Zealand in 1979. Generally the same that has been said for the first volume is valid for the second one. The organizers succeeded very well in gathering the prominent scientists in the field from all over the world, thus ensuring the level of the meeting. However, the topic of fibrous proteins and the separation methodology applied are two different things. It is amazing how good results in the field of fibrous proteins are achieved with rather primitive and outdated separation means. This is not a fault of the meeting participants, the less of the Editors. This, as I have noted when reviewing the first volume, merely reflects the situation in diverse fields of biochemistry that have not yet been penetrated by modern chromatographic and electromigration methods.

The present volume is divided into several sections devoted to muscle and meat proteins, collagen, elastin, keratin and wool. A considerable amount of information is available about the electrophoretic behaviour of keratin components, repeating units in wool, and last but not least connectin. The volume is, from the point of view of separation science, strictly on the application side, and for those who are involved in the rather specialized field of fibrous proteins would be a valuable source of information.

Prague (Czechoslovakia) Z. DEYL

Related articles published in *Journal of Chromatography*, Vols. 246 and 247

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Erratum

J. Chromatogr., 231 (1982) 255—264
Page 255, the last author's name should read: "JAMES L. McGAUGH".

PUBLICATION SCHEDULE FOR 1982

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