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(Continued overleaf)

Pyrolysis Mass Spectrometry of Recent and Fossil Biomaterials

Compendium and Atlas

by H. L. C. MEUZELAAR, *Salt Lake City, UT, U.S.A.*, J. HAVERKAMP, *Amsterdam, The Netherlands and* F. D. HILEMAN, *Dayton, OH, U.S.A.*

Techniques and Instrumentation in Analytical Chemistry, Vol. 3

Since the 3rd International Symposium on Analytical Pyrolysis in 1976, the importance of pyrolysis mass spectrometry (Py-MS) as an analytical method has increased considerably. Specially designed Py-MS systems using galvanically heated filament or direct probe pyrolysis have been quite successful in structural investigations and kinetic studies involving synthetic polymers and model compounds. The Curie-point Py-MS has, however, made a lasting impression on the field, being unique in those applications that require maximum reproducibility. This book presents an in-depth discussion of basic principles (including sample requirements, short- and long-term reproducibility problems and data processing approaches) of Py-MS techniques.

The Compendium has been put together by authors who have themselves been deeply involved in an extensively used fully-automated Py-MS system. It is thought that this system, particularly useful in characterising bacteria, could revolutionise clinical methods which have not changed since Pasteur.

The advent of commercially available Curie-point Py-MS systems has prompted the authors to include a small Atlas of over 150 reference spectra of carefully selected biomaterials which should help users of these systems to "tune" their instruments to the existing systems and to evaluate unknown spectra.

An asset to any chemistry library and particularly useful to those scientists considering the application of Py-MS techniques to their own specific problems in the analysis and data processing of biomaterials.

Contents: Part I. Compendium of Basic Principles and Applications. 1. Origins and Development of Pyrolysis Mass Spectrometry of Biomaterials. 2. From Fingerprinting to Structural Investigation. 3. Pyrolysis Mechanisms in Biomaterials. 4. The Technique of Curie-Point Py-MS. 5. Reproducibility in Curie-Point Py-MS. 6. Data Analysis Procedures. 7. Selected Applications to Biomaterials. **Part II. Atlas of Selected Pyrolysis Mass Spectra.**

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CHROMBIO. 2181

BRANCHED-CHAIN α-KETO ACID ANALYSIS IN BIOLOGICAL FLUIDS: PREPARATIVE CLEAN-UP BY ANION-EXCHANGE AND ANALYSIS BY CAPILLARY GAS CHROMATOGRAPHY

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(First received December 14th, 1983; revised manuscript received April 13th, 1984)

SUMMARY

A method is given for the quantitative analysis of the α-keto derivatives of the branched-chain amino acids in physiological fluids. A sample containing α-ketovalerate and α-ketocaproate as internal standards is passed through a weak anion-exchange resin at neutral pH. After washing the resin with distilled water, the α-keto acids are eluted with 4 *M* hydrochloric acid—ethanol (50:50). Quinoxalinol derivatives are prepared directly in the eluent, extracted with methylene chloride, and trimethylsilylated. Separation of the derivatives is by capillary gas chromatography on a 30 m fused-silica SE-30 column. Chromatographic separation is superior to that reported for packed column methods, thereby permitting the use of α-ketovalerate and α-ketocaproate as internal standards.

INTRODUCTION

In recent years increased attention has been given to the metabolism of the branched-chain amino acids (BCAA) and to the biological function and clinical uses of their α-keto acid analogues (BCKA) [1]. Gas chromatography (GC) [2, 3] and high-performance liquid chromatography [4] are the current methods of BCKA analysis.

GC methods for determining BCKA concentrations using O-trimethylsilyl-quinoxalinol derivatives are adaptable to most facilities. However, the recovery of BCKA standards added to blood samples is highly variable following procedures which require sample drying after protein precipitation by acetone [2] or require the neutralization of the supernatant following protein precipitation by perchloric acid [3]. In addition, the extra steps of neutralization and evaporation of solvents are tedious when handling a large number of samples.

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In this paper, a simple, rapid procedure for partially purifying α-keto acids from physiological fluids using a weak anion-exchange resin is presented. Conditions required for separating the O-trimethylsilylquinoxalinol derivatives of the BCKA by capillary GC are also presented. The separation of BCKA by the present method provides resolution superior to previously reported packed column methods [2, 3].

EXPERIMENTAL

Apparatus

A Varian Model 3700 gas chromatograph equipped with a flame ionization detector and containing a 30 m × 0.25 mm, fused-silica SE-30 capillary column (film thickness 0.25 μm; J & W Scientific, Rancho Cordova, CA, U.S.A.) was used. Injector and detector temperatures were both 250°C. The oven temperature was programmed to rise from 135°C to 210°C at a rate of 5°C/min. A split injection mode (1:50) maintained helium carrier gas flow at approximately 0.75 ml/min through the column. Make-up gas (helium) flow through the detector was 30 ml/min. Air and hydrogen gas flows to the detector were 300 and 30 ml/min, respectively.

Retention times and peak areas were determined by a Hewlett-Packard Series 3353 Laboratory Automation System (Avondale, PA, U.S.A.). Relative molar responses (RMR) were calculated as the ratio of α-keto acid peak area to internal standard peak area. Results were graphically plotted by a Fisher Recordall Series 5000 recorder (Fisher Scientific, Edmonton, Alberta, Canada).

Chemicals

Sodium salts of pyruvate (PYR), α-ketovalerate (KV), α-ketoisovalerate (KIV), D,L-α-keto-β-methylvalerate (KMV), α-ketoisocaproate (KIC) and α-ketocaproate (KC) and *o*-phenylenediamine (OPD), N,O-bis(trimethylsilyl)acetamide (BSA) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane were purchased from Sigma (St. Louis, MO, U.S.A.). Methylene chloride and 95% ethanol were redistilled before use. Anion-exchange resins (Cl^-) AG1-X8, AG2-X8 and AG3-X4A, 100—200 mesh, were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Standards

Individual 10 m*M* stock solutions of PYR, KV, KIV, KMV, KIC and KC were made and stored at —20°C until working standards were prepared. Working standards consisted of a single solution containing 10 μ*M* PYR, KIV, KMV and KIC and separate working solutions containing 10 μ*M* KV or KC which served as internal standards.

Anion-exchange column

The anion-exchange resins were washed in 4 *M* HCl—ethanol (50:50) prior to use. The exchange column was a 23 mm Pasteur pipette with a glass wool plug in which 0.5 ml of the anion-exchange resin was placed. The column was washed with distilled water until the column effluent was neutral (pH 6—7) to pH paper.

Muscle incubation

A 50-kg wether was anaesthetized with halothane and a small portion of the intercostal muscle removed; 50 mg of intact intercostal muscle fibres were immediately isolated and incubated in 3 ml of Krebs-Ringer bicarbonate—HEPES buffer containing glucose (10 m*M*), acetate (5 m*M*), leucine (0.5 m*M*) and physiological concentrations of the other amino acids found in blood. At the end of a 2 h incubation period at 37°C, the tissue was removed and 0.5 ml of ice-cold 1.5 *M* perchloric acid was added to the incubate; 1 ml of this solution was applied directly to the anion-exchange column without neutralization.

Anion-exchange clean-up of biological fluids

A 1 ml volume of each of KV and KC (10 μM each) were added as internal standards to 1 ml of plasma, whole blood incubate or α-keto acid standard solution. Blood samples were then heated for 5 min in a boiling water bath to denature proteins. Centrifugation (23,000 *g*) was required to remove precipitated blood protein. The samples were then passed through the anion-exchange column, which was subsequently washed twice with 2 ml of distilled water. A 2-ml volume of 4 *M* hydrochloric acid—ethanol (50:50) was then added and the effluent, containing the BCKA, was collected in a 13 × 100 mm screw cap culture tube.

Derivatization

The derivatization procedure was adapted from the procedures of Cree et al. [2] and Schwarz et al. [3]. A 2-ml volume of OPD solution (25 mg/ml) was added to the anion-exchange column effluent in the culture tube, which was tightly capped and heated for 15 min at 110°C. Upon cooling, 4 ml of methylene chloride were added, the tube was capped and shaken vigorously, and the two layers were allowed to separate under centrifugation (1500 *g*). The top aqueous layer was removed by aspiration along with a small portion of the methylene chloride layer. Another 4 ml of distilled water were added and the methylene chloride layer was washed as above. After removal of the water layer, the methylene chloride layer was allowed to evaporate at room temperature overnight or more rapidly with the aid of a stream of nitrogen gas. Then 20 μl of BSTFA were added to the dry residue. The tube was capped and vortexed and then allowed to stand for 10 min; 4 μl were injected onto the capillary column.

RESULTS AND DISCUSSION

Selection of anion-exchange resins

BCKA standards were bound by the strong anion-exchange resins AG1 and AG2, and by the weak anion-exchange resin AG3, at neutral pH. 6 *M* HCl eluted pyruvate from all resins and partially eluted KIV. None of the α-keto acids were eluted from the resins with 95% ethanol; 2 ml of 4 *M* hydrochloric acid—ethanol (50:50) completely eluted all BCKA from the resins. Five to ten times more eluent was required to elute BCKA from columns containing AG1 and AG2 than for columns containing AG3 of similar mesh and bed volume. Since both acid and ethanol were required for BCKA elution, BCKA attraction

to the resin may be hydrophobic as well as ionic. When a less selective form of the resin (OH^-) was used, the chromatogram contained additional peaks which interfered with the quantitative analysis of the BCKA.

Selection of a stationary phase for chromatography

A major difficulty associated with quantitative GC analysis of BCKA is the selection of a stationary phase that adequately separates KMV and KIC. Cree et al. [2] used a multiphase (OV-17, OV-210) packed column but did not achieve complete baseline separation of KMV and KIC. The multiphase (Carbowax 20 M, Silar 5 CP, Lexan) packed column of Schwarz et al. [3] improved the separation of KMV and KIC, but we have found the column to have a short life span and that incomplete removal of OPD from the reaction tube following derivatization results in the coelution of OPD with KIV. Schwarz et al. [3] investigated other phases (OV-1, OV-17, Tabsorb HAC, SP2300) and did not report improved separation of KMV and KIC. We have also investigated OV-1, OV-17 and SE-30 phases in packed columns and have found them to provide inadequate separation of KMV and KIV for quantitative analysis in biological samples. To our knowledge the use of capillary GC has not been reported for

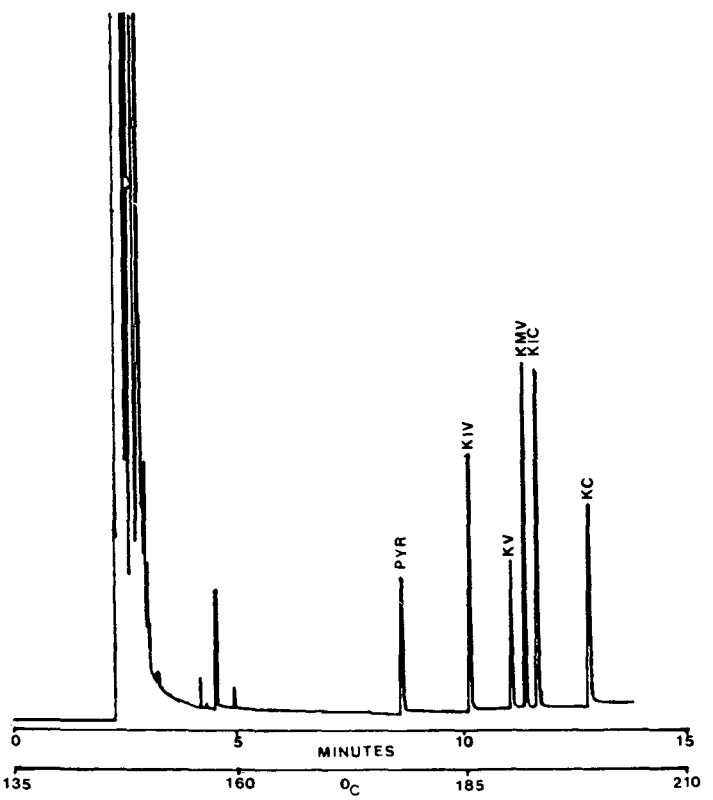

Fig. 1. GC separation of trimethylsilylquinoxalinol derivatives of branched-chain α-keto acid standards on a 30-m fused-silica SE-30 capillary column. Abbreviations are defined in the text. Attenuation: $32 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Standard concentrations: 10 μM each.

the separation of trimethylsilylquinoxalinol derivatives of BCKA. As shown in Fig. 1, there is a stable baseline as well as baseline separation of PYR, KIV, KV, KMV, KIC and KC with an SE-30 capillary column. This separation is superior to that reported by others [2, 3] for packed columns. The method allows the use of KV as an internal standard. In the method reported by Schwarz et al. [3], KV coeluted with KMV. Late eluting substances tend to accumulate on the capillary column, as reported for packed columns [2, 3]. Thus, column baking at 260°C for 5 min following every second sample is required for their removal.

Optimizing derivatization conditions

In order to determine optimal derivatizing conditions we adapted the procedure described by Schwarz et al. [3]. The RMR of the BCKA to either internal standard (KV or KC) was more variable for samples heated with OPD (10 mg in 2 *M* hydrochloric acid) at room temperature overnight, or for 60 min at 110°C, than with standards heated for 15, 30 or 45 min at 110°C (data not shown). Differences in RMR were not observed in the latter three time intervals. Subsequently, derivatization for 15 min at 110°C was adopted. There were no differences in RMR when either 10 or 50 mg of OPD were used, but reduced peak areas were observed for 5 mg of OPD. Mowbray and Ottaway [5] did not recommend excessively high OPD/α-keto acid ratios since the formation of phenazine from OPD might catalyse the decarboxylation of α-keto acid. This did not appear to be a problem in our assay. The effect of HCl molarity is illustrated in Fig. 2. Increasing HCl

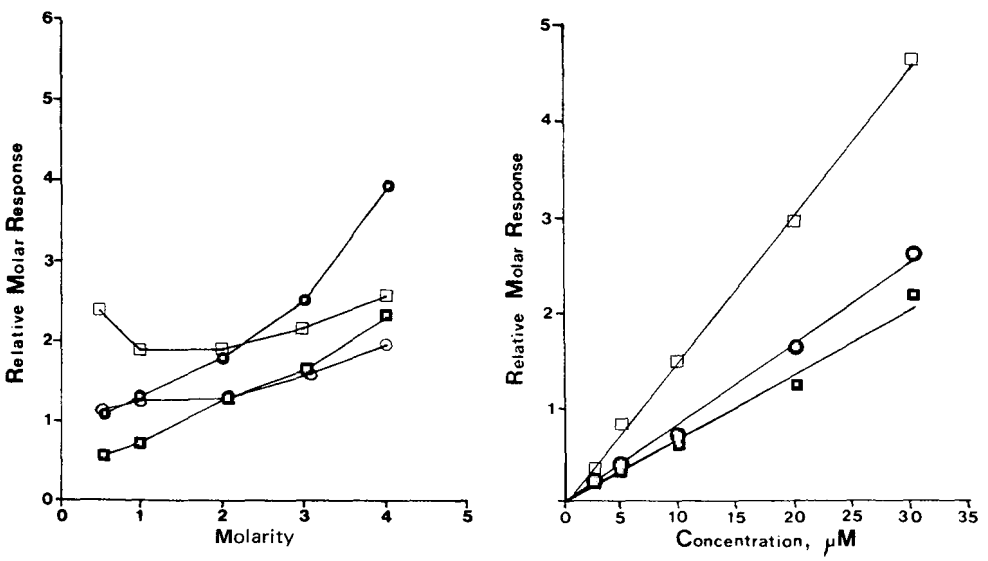

Fig. 2. Effect of varying HCl molarity on the relative molar responses of KIV (▪), KMV (•), KIC (□) and KC (○). Relative molar response is the ratio of α-keto acid area to KV internal standard area. Standard concentrations: 10 *μM* each. Abbreviations are defined in the text.

Fig. 3. Plots of relative molar response values versus concentration of KIV (▪), KMV (•) and KIC (□). Relative molar response is the ratio of α-keto acid area to 10 *μM* KV internal standard area. Abbreviations are defined in the text.

molarity increased the RMR of KIV, KMV and KC and initially decreased and then increased the RMR of KIC relative to KV. The average coefficient of variation for all RMRs presented in Fig. 2 was 4.6% which suggests that, although the RMR changed with HCl concentration, the RMR values were repeatable in HCl of a given molarity. Therefore, 2 *M* HCl was used as recommended by Schwarz et al. [3] and by Mowbray and Ottaway [5] who found this concentration to be optimal for the recovery of PYR and α-ketoglutarate. Ethanol concentrations ranging from 17% to 50% in the reaction medium did not affect the RMR values. Both BSA and BSTFA served as effective donors for trimethylsilylation of the quinoxalinol derivative, but extra peaks were observed with BSA. At least 10 min were allowed for trimethylsilylation. Contrary to other methods [2, 3], pyridine was not required for silylation with this method.

Standard curves of BCKA RMR (10 μ*M* KV as internal standard) versus different BCKA concentrations are illustrated in Fig. 3. A linear response was observed over the concentration range of 2.5—30 μ*M*. The correlation coefficient of each BCKA regression was 0.99.

BCKA analysis in blood and incubation medium

Examples of chromatograms of bovine, rat and human whole blood BCKA

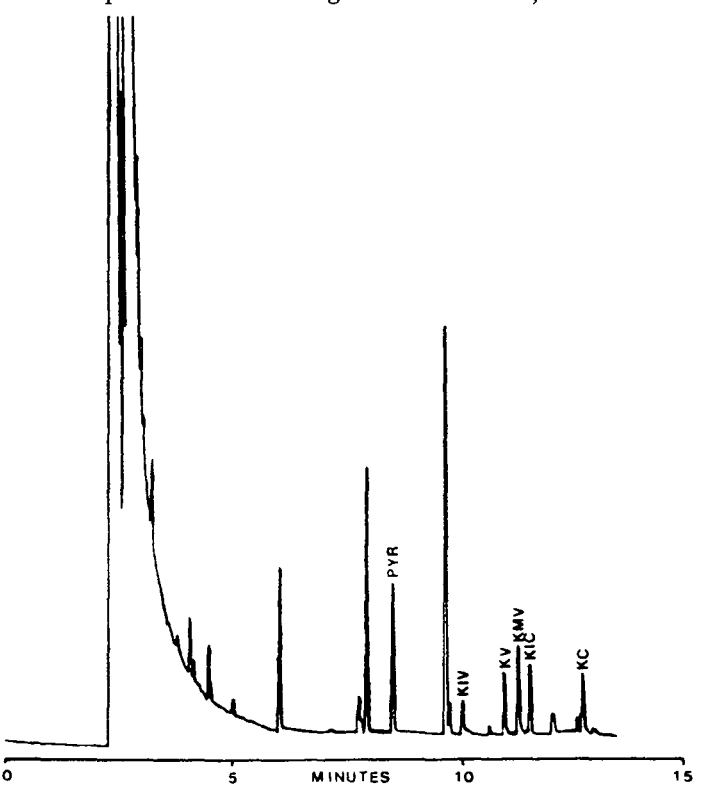

Fig. 4. Chromatogram of branched-chain α-keto acids in bovine whole blood. Abbreviations are defined in the text. Attenuation: $32 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Internal standard concentration (KV and KC): 10 μ*M* each.

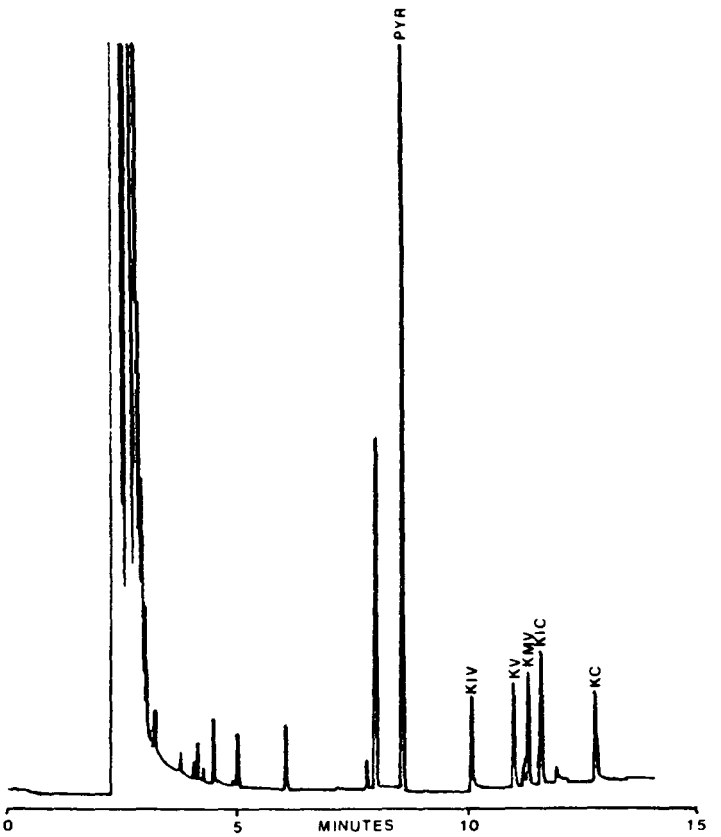

Fig. 5. Chromatogram of branched-chain α-keto acids in rat whole blood. Abbreviations are defined in the text. Attenuation: $32 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Internal standard concentration (KV and KC): 10 μM each.

are illustrated in Figs. 4, 5 and 6, respectively. Unidentified substances in whole blood did not interfere with BCKA separation. Mass spectroscopy of BCKA in rat hindlimb perfusates with a packed column have identified these peaks as O-trimethylsilylquinoxalinol derivatives and have shown them to be free of interfering substances [2]. Both KV and KC were adequate as internal standards in all samples studied. Addition of both internal standards permits calculation of the ratio of KV to KC, which can serve as a means of monitoring the reproducibility of derivatization conditions between samples. That is, the KV/KC ratio should be the same for all samples unless derivatization conditions are different.

Recoveries of BCKA standards added to untreated bovine whole blood (Table I) were less than 80%. However, deproteinization by heating increased the average recovery above 98%. Previous studies [6] have demonstrated the binding of KIC to plasma albumin. Therefore deproteinization may have prevented such binding in these studies and in turn improved the recovery.

The concentrations of BCKA in fed, mature bovine, rat and human whole blood are given in Table II. BCKA concentrations for these species were within the linear RMR range illustrated in Fig. 3.

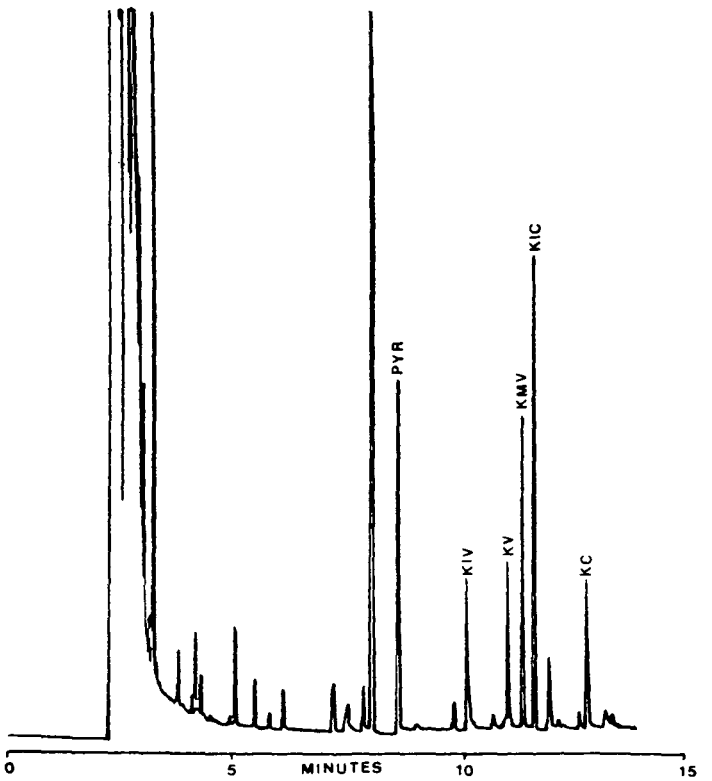

Fig. 6. Chromatogram of branched-chain α-keto acids in human whole blood. Abbreviations are defined in the text. Attenuation: $32 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Internal standard concentration (KV and KC): 10 μM each.

TABLE I

MEAN RECOVERY OF BCKA STANDARDS ADDED TO BOVINE WHOLE BLOOD

	Percentage BCKA recovery (mean ± S.E.M., n = 6)		
	KIV	KMV	KIC
Untreated	76.6 ± 4.7	74.4 ± 2.1	74.3 ± 1.2
Heated*	101.1 ± 1.1	98.0 ± 4.7	102.5 ± 3.4

*Blood heated in a boiling water bath for 1—2 min.

TABLE II

MEAN CONCENTRATION OF BCKA IN BOVINE, RAT AND HUMAN WHOLE BLOOD

	μM BCKA (mean ± S.E.M., n = 6)		
	KIV	KMV	KIC
Bovine	2.53 ± 0.33	8.49 ± 0.96	7.52 ± 0.98
Rat	3.97 ± 0.63	5.11 ± 0.50	5.24 ± 0.58
Human	6.13 ± 0.71	8.09 ± 0.88	10.52 ± 1.21

BCKA in tissue incubation media can also be partially purified and concentrated on the anion-exchange column. An example of a chromatogram of BCKA in 1 ml of medium from an ovine muscle incubation is illustrated in Fig. 7. Schwarz et al. [3] used chromatographic analysis to calculate rates of KIC release from rat epitrochlaris muscle by measuring the concentration of KIC in the incubation media. The rate of KIC release in their preparation was 2.1 nmol/g/min. For the ovine intercostal muscle of Fig. 7, the rate of KIC release, similarly calculated, was 0.77 nmol/g/min.

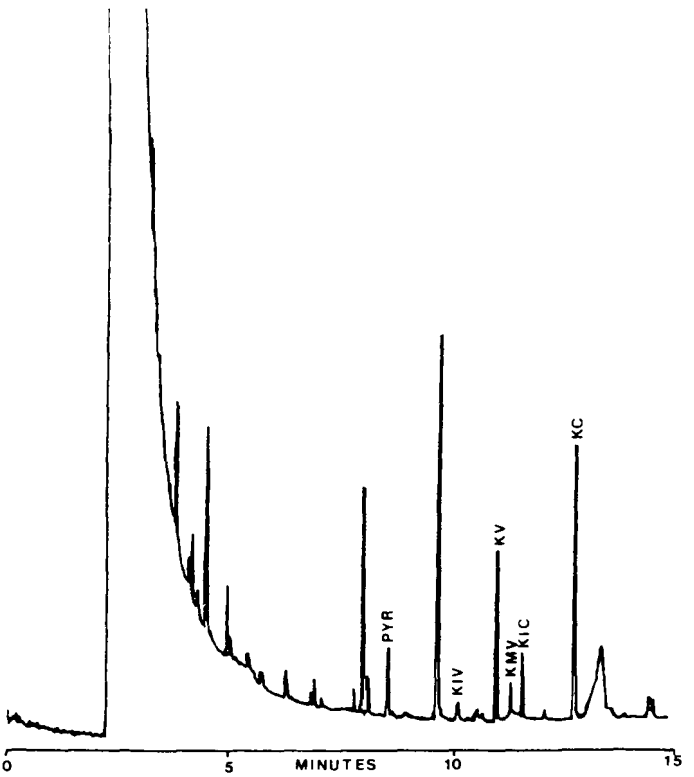

Fig. 7. Chromatogram of branched-chain α-keto acids in incubation medium of ovine intercostal muscle. Abbreviations are defined in the text. Attenuation: $16 \cdot 10^{-12}$ A/mV. Chart speed: 1 cm/min. Internal standard concentration (KV and KC): 10 μM each.

In most incubation systems [2, 3], amino acids are removed from the medium by cation-exchange chromatography. Amino acids did not bind to the anion-exchange column in the present system. Consequently, one need only collect the initial aqueous effluent from the column if an amino acid analysis is required. In this respect, the anion-exchange column can also serve as a means of separating radioactive BCAA and BCKA in incubation media.

In summary, the present method offers several advantages over previous methods. The preparative anion-exchange column permits concentration of BCKA from dilute solutions and provides a means of removing neutral compounds (glucose, triglycerides) and amino acids from the final derivatiza-

tion solution. Evaporation of solvents following acetone or ethanol protein precipitation procedures is unnecessary, avoiding unpredictable losses of BCKA and prolonged preparation time. Heat precipitation of whole blood proteins avoids the problems of additional peaks and variation in the recovery of standards found using perchloric acid, which requires precipitation with potassium hydroxide. However, when perchloric acid is added to protein-free incubation media, the anion-exchange column provides a means of removing the perchloric acid without the addition of potassium hydroxide. The use of capillary chromatography provides superior separation of the BCKA than was achieved by previous packed column methods. It also provides baseline separation of KV and KMV, permitting the use of KV as an internal standard.

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CHROMBIO. 2166

MEASUREMENT OF TYRAMINE IN HUMAN PLASMA, UTILISING ION-PAIR EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION*

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(First received January 12th, 1984; revised manuscript received March 28th, 1984)

SUMMARY

An assay for plasma tyramine has been developed which uses ion-pair extraction, reversed-phase ion-pair high-performance liquid chromatography and amperometric detection. Tritiated tyramine is used as the internal standard. The method can measure down to 0.5 ng/ml of tyramine in 1 ml of human plasma and is thus suitable for monoamine oxidase inhibitor studies involving oral dosing with tyramine.

INTRODUCTION

Tyramine (4-hydroxyphenethylamine) is an indirectly acting sympathomimetic amine found in cheese, fermented foods and red wine [1]. Ingested tyramine is largely inactivated by metabolism to *p*-hydroxyphenylacetic acid catalysed by monoamine oxidase (MAO) enzymes located in the gut, liver and sympathetic nerves [2].

The pressor action of tyramine was found to be dramatically potentiated in some patients, who took cheese whilst receiving MAO inhibitors (MAOI) [3]. This "cheese-reaction" has restricted the use of these drugs in the treatment of depression.

Measurement of plasma tyramine, thus, has an important part to play in the development of safer anti-depressant drugs [4].

Previously, tyramine has been estimated in urine by paper and thin-layer chromatography [5—7] and more recently by high-performance liquid chromatography (HPLC), coupled with fluorimetric [8] or electrochemical detection [9].

In developing a method for the measurement of plasma tyramine, use was

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made of sodium tetraphenylboron [10] to form tetraphenylborate—amine ion-pairs and so enable the extraction of tyramine (at basic pH) into diethyl ether, followed by dissociation of the ion-pair and back extraction into a small volume of acid [11].

Reversed-phase ion-pair HPLC using phosphate-buffered trichloroacetate at pH 5.0 [12] was chosen, so that the retention of tyramine could be controlled by altering the concentration of trichloroacetate (a weak ion-pairing agent) or methanol, depending on the condition of the column. A further degree of selectivity was achieved by use of an amperometric detector to monitor the column effluent as it passed over a glassy carbon thin-layer electrode.

EXPERIMENTAL

Apparatus

An Altex Model 100A solvent delivery pump was used, fitted with an Altex Model 210 valve (Altex Scientific, Berkeley, CA, U.S.A.) and a 100-μl loop. A Shandon Hypersil ODS column (5 μm particle size, 250 × 4.6 mm I.D.) protected by a Co:Pell ODS guard column (25—37 μm particle size, 50 × 2.1 mm I.D.) (HPLC Technology, Cheshire, U.K.) was employed for chromatography. The column effluent was monitored by a BAS LC-4 amperometric detector equipped with a TL-5 glassy carbon thin-layer cell (Bioanalytical Systems, West Lafayette, IN, U.S.A.). Electrochemical detection was performed at 0.90 V vs. Ag/AgCl reference electrode and chromatograms were displayed on Servoscribe Model RE 541.20 chart recorders (Smiths Industries, London, U.K.). Liquid scintillation counting was performed on a Beckman LS 2800 Scintillation Spectrometer.

Reagents

The internal standard (ring ^{3}H) tyramine hydrochloride (specific activity 27 Ci/mmol, 99% pure by paper chromatography, NET-132) was obtained from New England Nuclear (Boston, MA, U.S.A.) and stored at 5°C. The 'cold' tyramine used as primary standard was obtained from Sigma (Poole, U.K.) and sodium tetraphenylboron was purchased from Aldrich (Gillingham, U.K.). Helium and oxygen-free nitrogen were from BOC Special Gases (London, U.K.). All other chemicals were from BDH (Enfield, U.K.).

Method

The method consists of six principal steps: a diethyl ether wash, formation of tetraphenylborate—amine ion-pairs at basic pH, extraction of the ion-pairs into diethyl ether, back extraction into acid, HPLC analysis and fraction collection with scintillation counting.

To 1-ml samples of human plasma add 50 μl of a 1:1000 dilution of the [^{3}H]tyramine internal standard followed by 10 ml diethyl ether. Mix the tubes in a Multivortex shaker (Denley, Bilinghurst, U.K.) for 10 min, aspirate and discard the upper ether layer. Add 0.3 ml of a solution of sodium tetraphenylboron (5 mg/ml in 1 *M* borate, pH 8.0) and 10 ml diethyl ether. Multivortex the tubes for 10 min. After centrifugation (2050 *g*, 5 min) the tubes are frozen in a mixture of solid carbon dioxide and methylated spirits, and the

ether layer tipped into a fresh set of tubes containing 0.2 ml of 0.07 *M* orthophosphoric acid. Multivortex the tubes for 10 min. After centrifugation (2050 *g*, 5 min) the tubes are again frozen but this time the ether layer is discarded and the tubes blown free of diethyl ether under oxygen-free nitrogen. At this stage the tubes can be stored at −20° C overnight if desired, otherwise add 25 μl of 0.56 *M* potassium hydroxide solution and mix briefly, followed by 150 μl of mobile phase and further mixing.

Chromatographic conditions

The mobile phase consists of 0.04 *M* potassium dihydrogen phosphate, 0.03 *M* trichloroacetic acid and 2.5 m*M* disodium EDTA adjusted to pH 5.0. This mixture is filtered through Whatman Gf/f paper and 10% methanol added. The mobile phase is finally helium-degassed prior to pumping at between 1.0 and 1.5 ml/min. At a flow-rate of 1.5 ml/min typical back pressures are 11.03—13.79 MPa. The amperometric detector was set at 0.90 V vs. Ag/AgCl, 1 nA/V, filter C, and three chart recorders connected in series across the signal output to give simultaneous records at three sensitivities: 1 V full scale (1 nA full scale), 2 V full scale (2 nA full scale), and 5 V full scale (5 nA full scale). The column temperature was ambient and in all analytical work constant volumes of 100 μl were injected onto the column by loop over-fill technique. After each injection the column effluent corresponding to the tyramine peak was collected and mixed with scintillation cocktail (Instagel, Packard, U.S.A.) prior to scintillation counting.

Quantitation

A stock standard of 10 μg/ml of tyramine was prepared in 0.1 *M* hydrochloric acid and stored at 5° C. On the day of assay this was diluted with 0.01 *M* hydrochloric acid and then used to spike drug-free plasma and prepare a calibration curve over the range 0—200 ng/ml. An internal standard of [^{3}H]-tyramine was chosen to allow for variable recovery of cold tyramine through the extraction and chromatography. Quantitation was achieved by measurement of the peak height cold tyramine/recovered counts [^{3}H]tyramine ratio, for a range of cold tyramine concentrations.

Plasma samples

Blood samples were obtained from an indwelling intravenous canula in a forearm vein. They were taken into lithium heparin tubes and the plasma spun down in a refrigerated centrifuge, followed by freezing at −20° C until assayed.

Dosing

The bioavailability of oral tyramine was assessed using an 80—800 mg dose, either alone or in combination with an MAO-A inhibitor (Cimoxatone, Delalande, Rueill-Malmaison, France), or an MAO-B inhibitor (MDL 72145, Merrell-Dow, Strasbourg, France). Subjects took no food, drink or drugs that might have contained sympathomimetics during the study periods.

RESULTS AND DISCUSSION

Chromatography

Asmus and Freed [12, 13] showed that simple acids can replace alkyl sulphates or sulphonates as ion-pairing agents for the separation of the catecholamines and their metabolites. Following their example, we found that the retention of tyramine (pKa_1 = 9.5, pKa_2 = 10) on reversed-phase HPLC materials was improved by the use of trichloroacetic acid as the ion-pair. Utilising 0.03 *M* trichloroacetic acid buffered to pH 5.0 with 0.04 *M* potassium dihydrogen phosphate and 10% methanol, tyramine had a retention time of 11 min on a Hypersil ODS (C_{18}) column, and was well separated from the few other peaks that remained after the extraction. The large peak seen after tyramine in chromatograms of extracted plasma samples (retention time 17.2 min) was also present in tyramine-free aqueous extracts and so is presumed to be the tetraphenylborate from the extraction procedure. Typical chromatograms (Fig. 1) illustrate the selectivity of the chromatographic system used. Blank plasma chromatograms showed no interference which corresponded to the retention of tyramine.

Amperometric detection

Mobile phase pH was critical in determining the optimal applied potential. Changing the pH from 5.0 to 4.5 decreased the retention time of tyramine from 11.0 to 9.9 min and decreased the optimum potential from 0.90 V to 0.85 V vs. Ag/AgCl reference electrode. The usual precautions of adding disodium EDTA to the mobile phase, screening the detector by use of a Faraday Cage and helium degassing were observed.

Calibration

The calibration of each assay was achieved by spiking tyramine into plasma obtained from fasting subjects and constructing a plot of peak height/recovered counts ratio as a function of tyramine concentration. Linearity in standard curves of tyramine was established over an extended range of up to 1 μg/ml in plasma.

Recovery

An estimation of the recovery of tyramine was made using a spike of [^{3}H]-tyramine and collecting the column effluent after the tyramine fraction was eluted, followed by counting in scintillation cocktail. This gave an overall recovery of 52% (n = 4). Recovery across the HPLC column was almost quantitative (95%), indicating that most of the loss occurs at the extraction step.

Limit of detection

Measurement of tyramine (following an oral dose of 80—800 mg) in 1-ml plasma samples was possible at a sensitivity of 2 nA full scale at 0.90 V vs. Ag/AgCl reference electrode on the amperometric detector. With such a setting the absolute limit of detection was 0.5 ng/ml at a signal-to-noise ratio of 2.0. It is likely that a more sensitive assay could be developed by increasing the current amplification on the detector, but this would have to be investigated for any possible interferences.

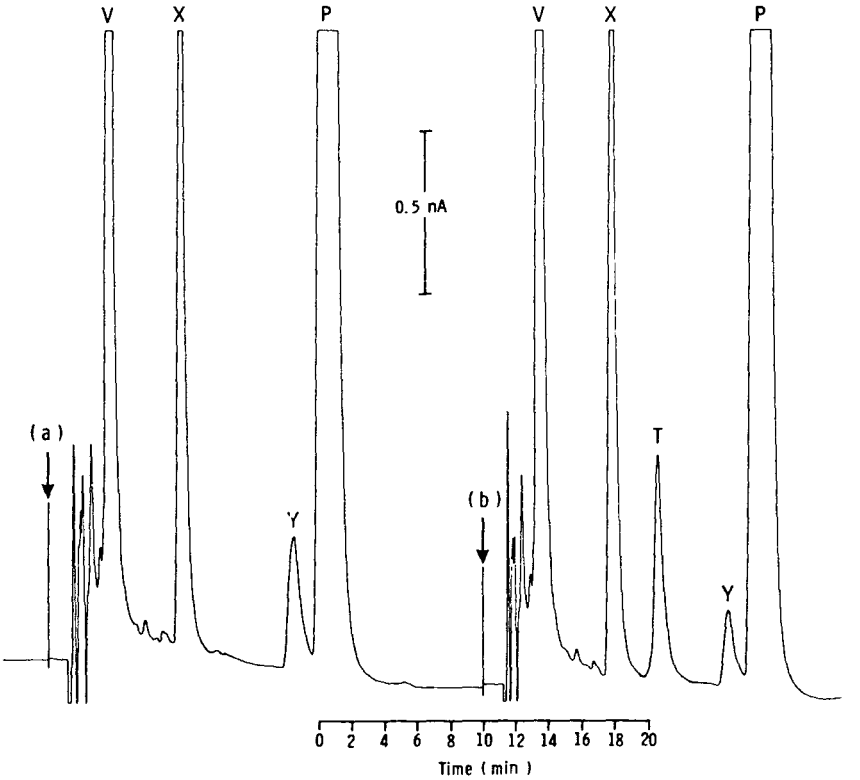

Fig. 1. Chromatograms of (a) blank plasma and (b) plasma taken 30 min after an oral dose of tyramine (400 mg). Concentration of tyramine = 33.5 ng/ml. V = void interference peaks; T = tyramine; P = tetraphenylborate; X and Y = unidentified peaks which do not interfere with the measurement of tyramine. HPLC—amperometric conditions as in text.

Precision

Intra-assay. Replicate analysis of a pooled plasma sample containing tyramine at a concentration of 22 ng/ml gave a coefficient of variation of 5.3% (n = 10, $\overline{X}$ = 21.95 ng/ml, S.D. = 1.1655).

Inter-assay. Assay of the same pooled plasma (stored in portions at —20° C between assays and once thawed not reused) over a two-week period yielded a coefficient of variation of 9.9% (n = 5, $\overline{X}$ = 21.70 ng/ml, S.D. = 2.1389).

Application

The method developed for the amperometric determination of tyramine in plasma, provides a means of investigating the sensitivity of MAOI-treated patients to oral tyramine. Such studies are underway in this department and already there have been some interesting results [4, 14]. The mean plasma tyramine concentrations of two normal volunteers following 400 mg of oral tyramine are given in Table I. In this non-medicated situation, plasma tyramine reached a peak within 30 min of ingestion and rapidly returned to baseline. When the subjects were pretreated with the MAO-B inhibitor MDL 72145 and given either (a) the same 400-mg oral dose or (b) an 800-mg oral dose of tyramine, entirely similar profiles were obtained. (Table II). Thus at a dose of

TABLE I

MEAN PLASMA TYRAMINE AFTER A 400-mg ORAL DOSE

Time after dose (min)	Mean plasma tyramine (ng/ml)
0	3.0
30	33.5
60	19.3
90	5.8
120	3.1

TABLE II

MEAN PLASMA TYRAMINE AFTER 400 OR 800 mg ORAL TYRAMINE AND 20 mg MDL 72145

Time after dose (min)	Mean plasma tyramine (ng/ml)	
	400 mg Tyramine	800 mg Tyramine
0	0	0
30	54.0	41.0
60	33.5	33.0
90	11.5	31.0
120	2.0	24.0
150	0	12.0
180	0	3.0
210	0	0

TABLE III

MEAN PLASMA TYRAMINE AFTER AN 80-mg ORAL DOSE AND FOUR DAYS OF TAKING CIMOXATONE (20 mg)

Time after dose (min)	Mean plasma tyramine (ng/ml)
0	2.0
30	15.7
60	15.4
90	6.1
120	4.9

20 mg, MDL 72145 is unlikely to significantly potentiate the bioavailability of oral tyramine. In another study, the mean plasma tyramine concentrations were recorded after giving two normal volunteers 80 mg of oral tyramine following four days treatment with 20 mg of the MAO-A inhibitor Cimoxatone [15]. These data, shown in Table III, follow a similar pattern, with the MAO inhibitor making no change in the plasma elimination of ingested tyramine. A more detailed report of these studies including clinical details will be published separately.

CONCLUSIONS

We have shown that tyramine can be reliably measured, following its tetraphenylboron ion-pair extraction from plasma and reversed-phase chromatography with trichloroacetate buffer combined with direct amperometric detection. It seems likely that after suitable modifications this HPLC—amperometric assay could be successfully applied to other biological samples such as urine and food [8, 16] which contain higher concentrations of tyramine. The method may also replace the HPLC—radioactivity method for tyramine, previously used in assessing the pre-systemic metabolism of tyramine in isolated intestinal loop preparations [17].

The proposed method for the HPLC—amperometric analysis of tyramine in human plasma would therefore seem to be suitable for studies on the bio-availability of ingested tyramine and should have particular relevance in the development of MAOI drugs.

ACKNOWLEDGEMENTS

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS: PURIFICATION OF α-FETOPROTEIN FROM FETAL CALF SERUM*

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SUMMARY

High-performance liquid chromatography was utilized for the purification of bovine α-fetoprotein (BAFP) from fetal calf serum (FCS). An initial step in the purification involved absorption of charcoal delipidated FCS on Cibacron Blue F3GA gel. The Cibacron Blue pre-purified FCS was then chromatographed on a Polyanion SI weak anion-exchange column. The BAFP isolated had a purity of >93% with an overall yield of 48% from FCS. The procedure was applicable for semi-preparative scale purification of BAFP.

INTRODUCTION

α-Fetoprotein (AFP), a serum glycoprotein produced by the yolk sac, liver and gastrointestinal tract, is the principle plasma protein of fetus during the early gestation period [1, 2]. AFP is maintained at high level throughout the gestation, but drops markly to trace amounts after birth [3, 4]. However, in certain pathological conditions particularly liver cell carcinoma and germ cell tumors [5—8], its concentrations may reappear in the serum to a significant level and is therefore of a great diagnostic significance [5—8].

In recent years, some of the properties of AFP have been unfolded. Its

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proposed functions [4] include bindings to ligands and immunoregulatory effects. However, the biological role of AFP has not yet been fully clarified, largely because homogeneous AFP is still difficult to be purified. For this reason, a simple and rapid purification procedure able to provide homogeneous AFP is desirable.

Various physicochemical methods have been suggested for the purification of AFP [4]. However, because of the structural similarity between AFP and albumin [9, 10] and that the concentration of the latter in serum is usually many folds higher, separation by these methods is rendered particularly difficult involving laborious multi-step procedures which often resulted in low recoveries of the products. In this respect, purification by an immunoadsorbent technique [11—13] is simple and efficient, but unfortunately it is still a long and tedious process and involves the prior availability of purified AFP for the generation of anti-AFP antibodies.

Because of the recently availability of high-performance liquid chromatographic supports specifically designed for the resolution, separation and recovery of biopolymers [14], high-performance liquid chromatography (HPLC) is now being used routinely in protein chemistry laboratories for isolation and purification of native polypeptides and proteins, and for protein structural and sequence analysis. The aim of this paper is to demonstrate the feasibility of using a combination of Cibacron Blue F3GA gel affinity chromatography and anion-exchange HPLC as a fast, simple and efficient means of isolating AFP from fetal calf serum (FCS).

EXPERIMENTAL

Materials

FCS was obtained as a gift from Dr. H.F. Deutsch (University of Wisconsin, Madison, WI, U.S.A.). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, U.S.A.). Bovine α-fetoprotein (BAFP) standard was kindly donated by Dr. E. Ruoslahti (City of Hope National Medical Center, Duarte, CA, U.S.A.). Cibacron Blue F3GA agarose (Affi-Gel Blue) was obtained from Bio-Rad Labs. (Mississauga, Canada). All other reagents used were of analytical or reagent grade and purchased from local suppliers.

Instrumentation

HPLC was performed on a Pharmacia FPLC (fast protein liquid chromatography) system equipped with dual pump capable of generating a gradient or step gradient elution profile. Chromatograms were recorded by monitoring the absorbance at 280 nm using a Pharmacia UV-1 monitor fitted with a 10-nm path length HR-cell. The absorbance unit full scale (a.u.f.s.) was set between 0.1 to 2.0 units, where appropriate. Fractions were collected with a FRAC-100 fraction collector and the operating temperature was ambient.

Delipidation of FCS

FCS was delipidated using a modified method according to Chen [15]. Lyophilized FCS (1 g) was reconstituted with 18 ml of 0.03% citric acid solution and dialyzed against distilled water. Activated charcoal was added

(600 mg), the pH carefully adjusted to 3.0 with 1 *M* hydrochloric acid, and the mixture incubated with shaking at 0°C for 2 h. The solution was then centrifuged at 15,000 *g* for 30 min at 0°C. The supernatant was decanted from the charcoal, adjusted to pH 7 with 0.5 *M* sodium hydroxide, and dialyzed against 0.02 *M* phosphate buffer, pH 7.2.

Cibacron Blue gel chromatography

Affi-Gel Blue was first washed with 8 *M* urea until the eluent became colorless and then washed extensively with 0.02 *M* phosphate buffer, pH 7.2. For quantitative runs, the washed gel was packed into a Pharmacia HR 10/10 column (100 mm × 10 mm I.D.) and chromatographed using the FPLC system. After eluting the unretained protein fraction with 0.02 *M* phosphate buffer, pH 7.2, the retained fraction was next eluted with phosphate buffer containing 1.4 *M* sodium chloride. The unretained and retained protein fractions were dialyzed against distilled water and lyophilized. The column was regenerated with 8 *M* urea and equilibrated with 0.02 *M* phosphate buffer, pH 7.2 before reused. For large scale runs, pre-washed gel was packed into a glass column (Bio-Rad Econo-column, 500 mm × 25 mm I.D.) and equilibrated with 0.02 *M* phosphate buffer, pH 7.2. The unretained and retained protein fractions were eluted with their respective buffers as above. The fractions were concentrated to a volume of 20 ml by means of a Diaflo PM-10 membrane, dialyzed against distilled water and lyophilized.

Polyanion SI chromatography

HPLC was carried out using either a prepacked analytical HR 5/5 Polyanion SI column (50 × 5 mm I.D., 6—7 μm particle size, Pharmacia) or a semi-preparative HR 10/10 column dry-packed with Polyanion SI, 17-μm particle size (Pharmacia). The A buffer for ion exchange was 12.1 g (0.1 *M*) of tris(hydroxymethyl)aminomethane (Tris) per liter, adjusted to pH 8 with 1 *M* hydrochloric acid while buffer B was 12.1 g Tris, pH 8, containing 40.8—68.0 g (0.3—0.5 *M*) of sodium acetate per liter. A preprogrammed linear gradient was used for the chromatography and the appropriate protein peaks were collected, dialyzed against distilled water and lyophilized.

Protein assay

Total protein concentrations were determined by the biuret method [16] using BSA as a standard or by UV absorption at 280 nm. BAFP and BSA concentrations were assayed by the radial immunodiffusion method of Mancini et al. [17]. The purity of the purified protein was determined by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS—PAGE) [18]. The gel was scanned with a DCD-16 digital computing densitometer (Gelman, Ann Arbor, MI, U.S.A.).

RESULTS

HPLC separation of BAFP and BSA

Attempts were made initially to establish an HPLC procedure for the separation of BAFP and BSA, the later being an anticipated major component of the

FCS which might interfere with BAFP in the purification. Fig. 1 depicts the separation of standard BAFP and BSA on a Pharmacia HR 5/5 Polyanion SI (weak anion-exchange) column. By employing a 0.1 *M* Tris buffer, pH 8, as the initial buffer and eluting with a 0.4 *M* sodium acetate gradient, BAFP and BSA were satisfactory resolved. Other eluting salts, such as sodium chloride, sodium sulphate, were tested but they gave a poorer resolution of the proteins. The pH of separation was also studied and found to be critical at around pH 8 with no separation of the two proteins at or below pH 6.

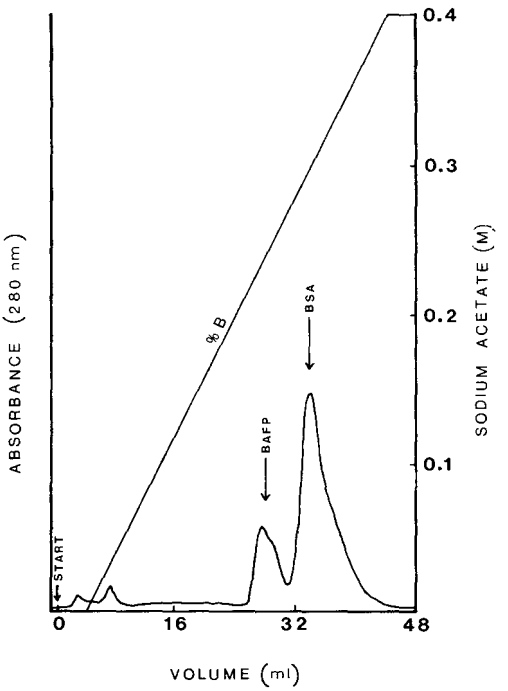

Fig. 1. HPLC separation of standard BAFP (200 μg) and BSA (500 μg) on a pre-packed HR 5/5 Polyanion SI column. Buffer A: 0.1 *M* Tris, pH 8; buffer B: 0.1 *M* Tris, pH 8 containing 0.4 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s.: 0.1.

Cibacron Blue F3GA gel pre-purification of FCS

Chromatography of neat FCS on the above described HPLC procedure (Fig. 2) showed that although BAFP and BSA were resolved from each other, the BAFP peak was massed by a number of other proteins having the same retention volume as BAFP. Thus a pre-purification of the FCS was necessary so as to concentrate BAFP and remove the majority of the interfering proteins.

Fig. 3 shows the chromatography of FCS on a Cibacron Blue F3GA gel affinity column. A single passage through the gel showed that 57% of BAFP and > 97% of BSA were bound (Table I). The retained fraction (fraction B) was subsequently eluted with sodium chloride and analyses revealed that it contained, in addition to BAFP and BSA, only a small amount of other proteins. The remaining BAFP (43%) was associated with the unretained fraction (fraction A) which contained also the majority of the other proteins

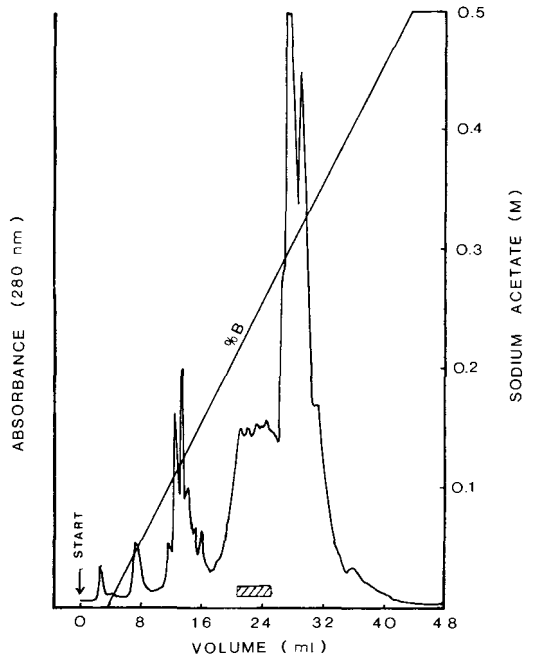

Fig. 2. HPLC separation of FCS (100 μl) on a pre-packed HR 5/5 Polyanion SI column. Buffer A: 0.1 *M* Tris, pH 8; buffer B: 0.1 *M* Tris, pH 8 containing 0.5 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s.: 0.2. The presence of BAFP in fractions is shown in hatched zones.

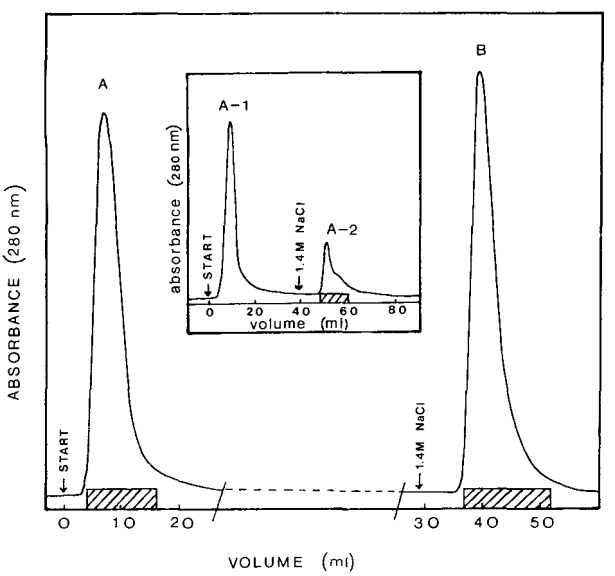

Fig. 3. Chromatography of FCS (1.5 ml) on a HR 10/10 Affi-Gel Blue column using 0.02 *M* phosphate, pH 7.2 as the eluent (1 ml/min). Insert: rechromatography of charcoal delipidated Fraction A on a HR 10/10 Affi-Gel Blue column using 0.02 *M* phosphate, pH 7.2 as the eluent (1 ml/min). The presence of BAFP in fractions is shown in hatched zones.

TABLE I

PURIFICATION OF BAFP FROM FCS BY CIBACRON BLUE F3GA GEL AFFINITY CHROMATOGRAPHY

Fraction	Total protein (mg)	BAFP (mg)	BSA (mg)	Other protein (mg)	Percent BAFP in protein
Whole	48.0	2.0	16.4	30.6	4.2
Unretained (A)	27.0	0.86	<0.05	26.1	3.2
Retained (B)	21.5	1.10	15.75	4.6	5.1
A - 1*	28.4	—	—	28.4	—
A - 2*	10.4	1.2	<0.05	9.2	11.5

*Data obtained from a combination of two unretained (A) fractions, delipidated and rechromatographed on Affi-Gel Blue. A-1 and A-2 were unretained and retained fractions, respectively (see Fig. 3).

in FCS. When fraction A was subjected to delipidation by the charcoal method of Chen [15] and rechromatographed on the Cibacron Blue gel column (Fig. 3, insert), nearly all of the BAFP now was retained by the column but the majority of the other proteins remained unretained (Table I). From these results, it was established that a purified FCS fraction containing predominantly BSA and BAFP could be obtained by subjecting the serum first to charcoal delipidation followed by chromatography on Cibacron Blue gel.

HPLC separation of Cibacron Blue gel purified FCS

When delipidated Cibacron Blue gel purified FCS was subjected to HPLC separation using the procedure described above, the chromatogram (Fig. 4) showed that a total of four peaks were resolved. The retention volumes of peaks 3 and 4 corresponded to that of standard BAFP and BSA, respectively, and their identities were confirmed by radial immunodiffusion plate analyses. A similar chromatogram (Fig. 5) showing the complete resolution of BAFP and BSA was also obtained on chromatography of a non-delipidated Cibacron Blue purified FCS fraction (fraction B, Fig. 3).

The purity of the BAFP thus separated was analyzed by subjecting various fractions of the peak (Fig. 4, fractions a—c) to SDS—PAGE. Fig. 6 shows the densitometer tracing of the gels of the fractions together with that of a standard of BAFP—BSA mixture. Fraction a, which comprised of about 5% of the total peak absorbance, showed the presence of a major BAFP peak (47%) and two other minor peaks. The major b fraction (80%) consisted predominantly of BAFP (> 93%) together with a trace amount of a protein(s) of slower electrophoretic mobility. Fraction c, which comprised of the remaining 15% of the total absorbance, had a BAFP peak with purity of about 70%. The purity of fraction b was further tested by comparing the size of the immunodiffusion ring of a 0.5 mg/ml solution of the protein to that of a standard BAFP sample isolated from an immunoabsorbent method [13]. The result confirmed a protein purity of > 93%. The extinction value $E^{1\%}_{1\,cm}$ (280 nm) of fraction b was 4.2 which was similar to that reported for BAFP isolated from an immunoabsorbent method [$E^{1\%}_{1\,cm}$ (280 nm) = 4.1] [19]. Table II summarizes the

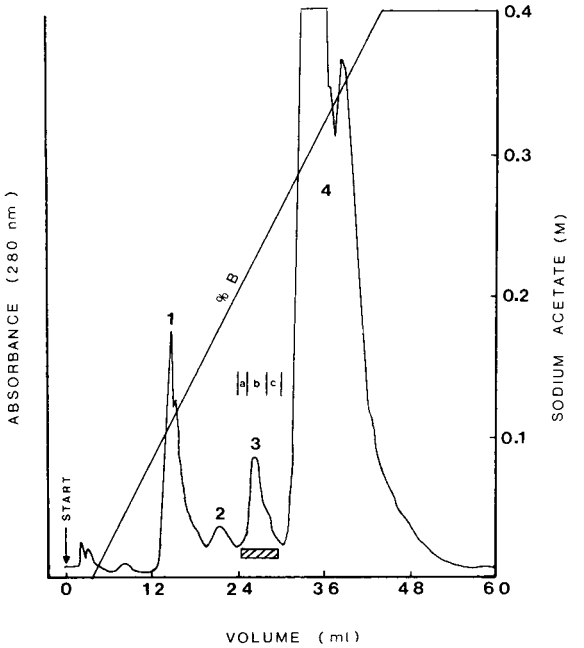

Fig. 4. HPLC separation of delipidated Cibacron Blue gel purified FCS (5 mg) on a pre-packed HR 5/5 Polyanion SI column. Buffer A: 0.1 *M* Tris, pH 8; buffer B: 0.1 *M* Tris, pH 8 containing 0.4 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s.: 0.2. The presence of BAFP in fractions is shown in hatched zones. Peaks: 3 = BAFP; 4 = BSA.

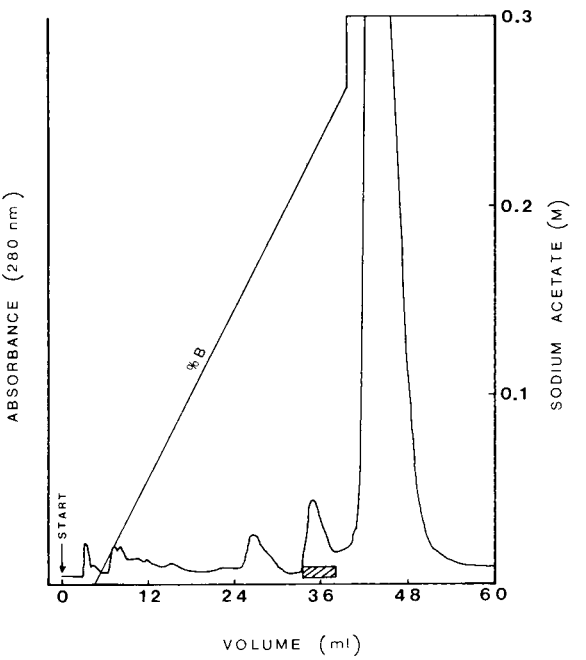

Fig. 5. HPLC separation of non-delipidated Cibacron Blue gel purified FCS (5 mg) on a pre-packed HR 5/5 Polyanion SI column. Buffer A: 0.1 *M* Tris, pH 8; buffer B; 0.1 *M* Tris, pH 8 containing 0.3 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s: 0.2. The presence of BAFP in fractions is shown in hatched zones.

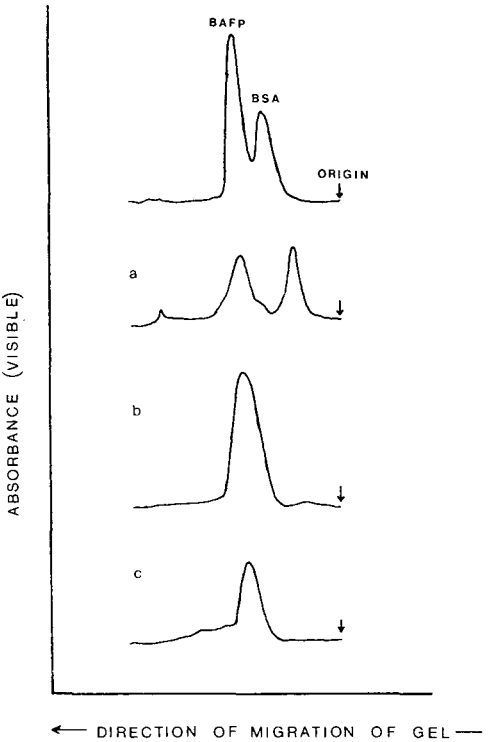

Fig. 6. Densitometer tracing of SDS—PAGE of BAFP peak fractions a, b and c shown in Fig. 4 together with that of a standard BAFP—BSA mixture.

TABLE II

SUMMARY OF PURIFICATION OF α-FETOPROTEIN FROM FETAL CALF SERUM

Method	Total protein (mg)	BAFP (μg)	Percent yield (overall)
Fetal calf serum (neat, 0.33 ml)	9.92	621	100
Delipidation and Cibacron Blue gel chromatography	5.10	460	74
HR 5/5 Polyanion SI chromatography	0.33	302	48.6

recoveries of the two-step BAFP purification procedure. A 48% overall yield of BAFP from FCS was obtained.

Semi-preparative scale purification of BAFP

Fig. 7 depicts the chromatographic separation of a delipidated Cibacron Blue purified FCS on a dry-packed HR 10/10 Polyanion SI (17 μm) column. The column gave a seven-fold increase in loading capacity compared to the analytical HR 5/5 Polyanion SI column with adequate resolution of the BAFP and BSA peaks still being maintained, thereby affording semi-preparative scale

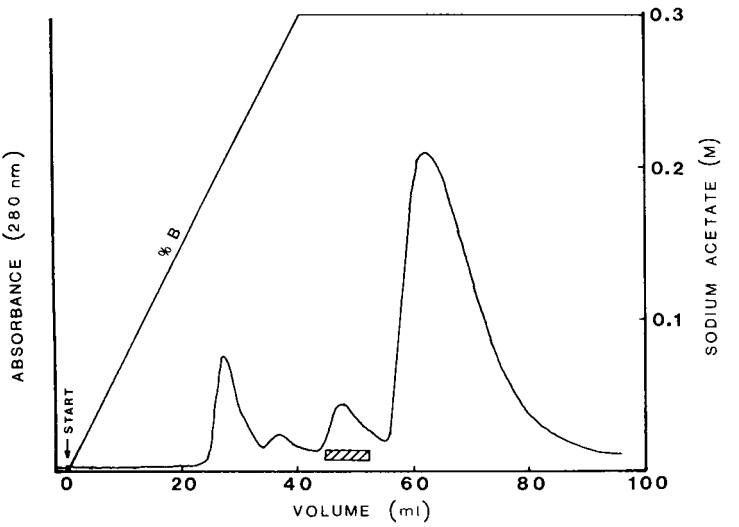

Fig. 7. HPLC separation of delipidated Cibacron Blue gel purified FCS (35 mg) on a dry-packed HR 10/10 Polyanion SI 17-μm column. Buffer A: 0.1 *M* Tris, pH 8; buffer B: 0.1 *M* Tris, pH 8 containing 0.3 *M* sodium acetate; flow-rate: 1 ml/min; a.u.f.s.: 1.0. The presence of BAFP in fractions is shown in hatched zones.

purification of BAFP from FCS. Radial immunodiffusion plate analysis of the BAFP thus isolated showed a purity of 70—80%.

DISCUSSION

BAFP has been isolated from FCS by successive procedures of Con A-Sepharose, DEAE-Sephadex and SP-Sephadex chromatography followed by preparative disc PAGE [20]. More recently, Ruoslahti [13] reported the purification of BAFP from FCS using a combination of immunoadsorbent and gel chromatographic techniques. Although these methods are adequate for the purification of BAFP, they are however laborious and complicated multiple-step procedures.

The use of HPLC for the separation and purification of biopolymers has received considerable attention in recent years. Its advantage over conventional methods of separation is obvious. It is fast, convenient, flexible and also affords semi-preparative scale capability. Herein, we describe a simple two-step procedure involving Cibacron Blue gel pre-purification followed by anion-exchange HPLC separation for the isolation of BAFP from FCS.

Initially, attempts were made to develop an HPLC separation condition for the resolution of BAFP and BSA. Because of the similarity in molecular weight between the two proteins [9, 10], steric exclusion chromatography was not suitable for their separation. Reversed-phase (hydrophobic) chromatography, although being widely used for the separation of peptides and proteins, was also not employed because of the undesirable large amount of organic solvents needed in the separation procedure. The amphoteric character of BAFP and BSA makes ion-exchange chromatography a more suitable tool for their separation. Adequate resolution of the two proteins was attained with the use of an anion-exchange column.

Direct separation of neat FCS using the anion-exchange HPLC procedure showed that although BAFP and BSA could be resolved from each other, the BAFP peak was extensively massed by other protein peaks. Thus a pre-purification of the FCS was necessary so as to remove the majority of the masking proteins before subjecting to HPLC separation. By using a Cibacron Blue gel column which selectively absorbed BAFP and BSA but not the other proteins, a BAFP—BSA enriched fraction of FCS could be obtained. The use of Cibacron Blue gel as an affinity column for the separation of human AFP and serum albumin has recently been reported by Young and Webb [21]. In contrast to our findings in the bovine, these authors found that human AFP from cord serum was unbound while albumin was bound by the blue gel. The exact reason(s) for the discrepancy in binding between bovine and human AFP towards Cibacron Blue F3GA remains unclear and further investigations into these areas are thus warranted.

Although HPLC separation of the Cibacron Blue purified FCS on the HR 5/5 Polyanion SI analytical column could afford BAFP of > 93% in purity, the methodology was adequate only for the isolation of small (low mg) quantities of BAFP by repeated injections. The analytical column had a loading capacity of about 5 mg of the BAFP—BSA enriched protein mixture and therefore approximately 300 μg of BAFP could be purified in a single injection. In order to adapt this methodology for semi-preparative scale purifications of BAFP, a HR 10/10 Polyanion SI (17 μm) column was tested and found to have a seven-fold loading capacity compared to the analytical column. Thus in a single injection using this column, approximately 2 mg of BAFP could be purified. Although the BAFP isolated had a purity of 70—80%, its purity however could be easily upgraded by rechromatography on the analytical column.

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SEPARATION OF HAEM COMPOUNDS BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION IN THE ASSAY OF FERROCHELATASE ACTIVITY

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SUMMARY

The separation of haems and porphyrins was achieved in a reversed-phase ion-pair high-performance liquid chromatography system using tetrabutylammonium hydrogen sulphate as the pairing ion. The concentration of methanol and pH in the mobile phase were determinative parameters for the elution pattern of the compounds. Two isocratic systems — one for the assay of protohaem IX and one for deuterohaem IX — were developed.

The chromatographic systems were applied to the assay of ferrochelatase activity in mitochondria using either protoporphyrin or deuteroporphyrin as the substrate. The ferrochelatase activity was also measured in reticulocytes, which contain high levels of endogenous haem.

INTRODUCTION

The final step in the haem biosynthetic pathway is the insertion of ferrous iron into the tetrapyrrole ring of protoporphyrin IX to form protohaem IX [1]. The reaction is catalysed by the enzyme ferrochelatase (protohaem ferrolyase, EC 4.99.1.1) which in eucaryotic cells is located in the mitochondria [2, 3]. Various procedures based on quantitation of porphyrin substrate consumed or haem product formed have been reported for the assay of this enzyme. The utilization of porphyrin can be measured either spectrophotometrically or fluorometrically [2, 4]. However, absorption and fluorescence spectra of porphyrins change both upon aggregation, which occurs readily in aqueous solutions [5], as well as upon binding to various proteins present in plasma [6], cytosol [7] and mitochondria [8]. Thus, an apparent porphyrin consumption was reported to take place several minutes before any

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formation of haem could be detected when the spectrophotometric approach was used [9].

The haem formed is most frequently measured from the reduced minus oxidized absorption spectra of pyridine haemochromogens [10]. However, these spectra will also have contributions from any endogenous haem in the biological material analysed, and often a small increase in the absorption spectrum has to be detected against a high background. This problem is not encountered in radiochemical methods, which, however, require a rather tedious extraction procedure for the separation of the labelled haem from free radio iron [11].

The purpose of the present study was to develop an assay of ferrochelatase activity in which the haem formed is assayed at high sensitivity and precision based on quantitation of haem by high-performance liquid chromatography (HPLC). Although several HPLC systems for the quantitation of porphyrins can be found in the literature, no system has so far been reported for the assay of haem. Ferrochelatase will accept various porphyrin IX derivatives as the substrate in in vitro assay systems [12]. The activity of the enzyme could therefore be measured in samples containing high concentrations of endogenous protohaem when deuteroporphyrin was used as the substrate since the deuterohaem formed was separated from protohaem on HPLC.

MATERIALS AND METHODS

Chemicals

Protoporphyrin IX and deuteroporphyrin IX were obtained from Porphyrin Products (Logan, UT, U.S.A.). Tetrabutylammonium hydrogen sulphate and protohaem IX were from Sigma (St. Louis, MO, U.S.A.).

High-performance liquid chromatography

Separation of haems and porphyrins were obtained by HPLC using a Constametric III pump from Laboratory Data Control (Riviera Beach, FL, U.S.A.), a valve loop injector from Rheodyne (Berkeley, CA, U.S.A.) and a photodiode array spectrophotometric detector Model HP-1040A connected to an HP-85 microcomputer, a Model 3380A recording integrator and a Model 7470A plotter, all from Hewlett-Packard (Avondale, CA, U.S.A.). The column used was a reversed-phase silica supported prepacked Supelcosil LC-18 column (25 cm × 4.6 mm I.D., particle size 5 μm) equipped with a Pelliguard pre-column (2 cm × 4.6 mm I.D.) prepacked with pellicular LC-18 of 40 μm particle size, both from Supelco (Bellefonte, CA, U.S.A.).

For the quantitation of protohaem the mobile phase contained methanol—water (97:3) with tetrabutylammonium hydrogen sulphate (1 m*M*), acetic acid (10 m*M*) and adjusted to pH′ 6.5 with 5 *M* ammonium hydroxide at ambient temperature. For the assay of deuterohaem the mobile phase was similar, except that the methanol—water ratio was 85:15.

The solvent was delivered at a flow-rate of 1 ml/min and the system operated at ambient temperature. The tetrapyrroles were identified from their retention times (t_R) and their peak absorption spectra.

The spectrophotometer was set at a measuring wavelength of 398 nm with

8 nm spectral bandwidth for monitoring protohaem, whereas the setting was 384 nm with 10 nm bandwidth when deuterohaem was monitored. The reference wavelength was set at 550 nm with 100 nm bandwidth for both of the haems.

Assay of ferrochelatase activity

For the assay of ferrochelatase activity the incubation of mitochondria and reticulocytes was performed as described in ref. 13 in a total volume of 1.2 ml. The reaction was quenched by 5 m*M* *p*-chloromercuribenzoate, and 0.2 ml of this solution was mixed with 0.5 ml of ice-cold methanol containing 1 m*M* of tetrabutylammonium hydrogen sulphate. The tubes were then centrifuged for 15 sec in an Eppendorf centrifuge Model 5414; 50 μl of 1 *M* hydrochloric acid and 0.5 ml of the methanol—tetrabutylammonium hydrogen sulphate solution were added to the pellets which were then sonicated in a well type sonicator and centrifuged for 2 min. The combined supernatants were centrifuged for another 2 min, and 20 μl of the final supernatant were injected into the liquid chromatograph. More than 90% of the haem in the sample was extracted by this procedure (see Results).

pH Measurements

The pH was measured using a PHM52 digital pH-meter equipped with a combined electrode GK 2321C, both from Radiometer (Copenhagen, Denmark). The pH values reported are all apparent pH values (pH′) since no correction was made for the varying amounts of methanol present in the solutions.

Preparation of mitochondria and reticulocytes

Rat liver mitochondria (male Wistar rats weighing 200—300 g) were isolated by differential centrifugation as previously described [14, 15]. Reticulocytosis was induced in rats by injecting phenylhydrazine and the red blood cells were prepared essentially as described in ref. 16 except that 0.15 *M* sodium chloride was used as the isolation medium.

Other analytical and preparative methods

Deuterohaem was synthesized from deuteroporphyrin and ferrous sulphate by refluxing in N,N′-dimethylformamide [17].

In the standard solutions porphyrins were quantitated from their absorption spectra in aqueous hydrochloric acid and haems were measured from the reduced minus oxidized difference pyridine haemochromogen spectra [18].

Protein was measured according to the procedure of Bradford [19] using the dye reagent of Bio-Rad Labs. (Richmond, CA, U.S.A.) and bovine serum albumin as a standard.

RESULTS

Chromatographic systems for separation of haems and porphyrins

The retention (expressed as capacity factor k') of the two haem and porphyrin compounds in the reversed-phase ion-pair HPLC system studied, was

influenced by the amount of organic solvent (Fig. 1) and pH′ (Fig. 2) of the mobile phase. The capacity factor for all four compounds decreased when the methanol concentration was increased (Fig. 1). A decrease in the k' value was also observed for the same compounds when the pH′ of the mobile phase was increased from 4 to 7 (Fig. 2). At low pH′ values a shoulder in the peak representing haem appeared (data not shown), and a pH′ of 6.5 in the mobile phase was therefore selected.

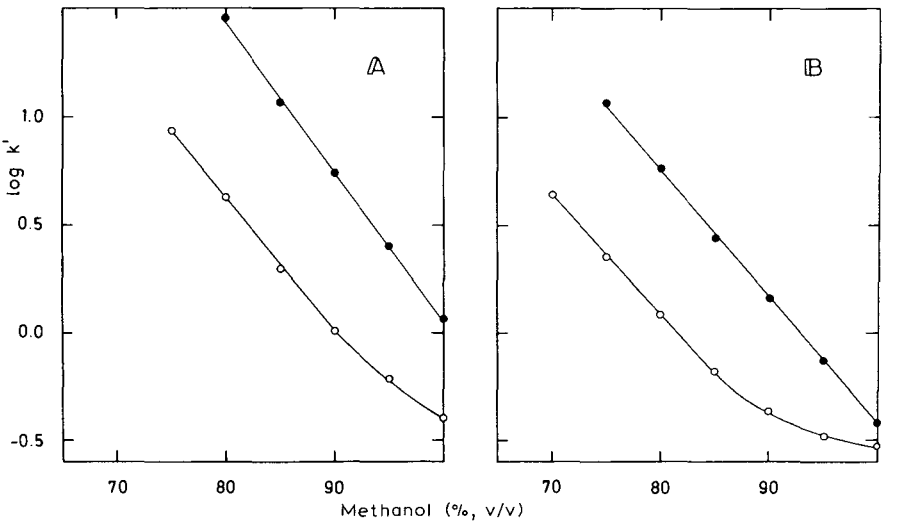

Fig. 1. The effect of the concentration of methanol on the capacity factor (k') in HPLC of porphyrin (•) and haem (○); (A) proto compounds, (B) deutero compounds. The mobile phase contained methanol—water in varying ratios, tetrabutylammonium hydrogen sulphate (1 mM), acetic acid (10 mM), and the pH′ was adjusted to 6.5 with 5 M ammonium hydroxide. The correlation coefficient for the linear regression line was —0.999 for both protoporphyrin (A) and deuteroporphyrin (B).

When the concentration of tetrabutylammonium hydrogen sulphate in the mobile phase was varied between 0 and 5 mM, the symmetry of the peaks improved and showed an optimum at approximately 1 mM, whereas the retention times were only marginally affected (data not shown).

The calibration curves for both protohaem and deuterohaem using the standard HPLC conditions were linear with a correlation coefficient > 0.999, and the detection limit was about 2 pmol at a signal-to-noise ratio of 2.

The recovery of protohaem when extracted from mitochondria containing variable amounts of added haem is shown in Fig. 3. When corrected for the endogenous haem a straight line through the origin with a slope of 0.907 was obtained, indicating that 90% of the added haem was recovered.

The chromatogram of a mixture of protohaem and protoporphyrin, using the conditions for protohaem quantitation (see Materials and Methods), is shown in Fig. 4A, I. When a mixture of deuterohaem, protoporphyrin and deuteroporphyrin was chromatographed using the conditions for deuterohaem quantitation, the chromatogram shown in Fig. 4A, II was obtained.

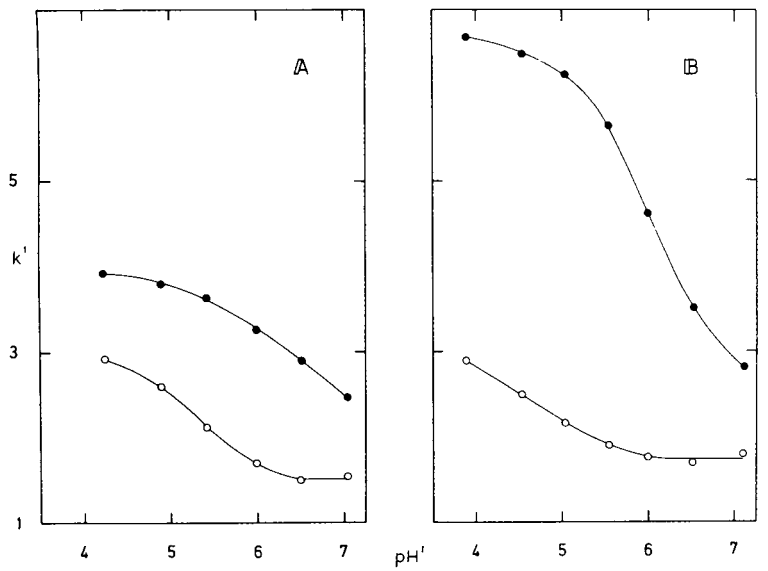

Fig. 2. The effect of pH' on the capacity factor (k') in HPLC of porphyrin (•) and haem (○); (A) proto compounds, (B) deutero compounds. The mobile phase contained tetrabutylammonium hydrogen sulphate (1 mM), acetic acid (10 mM) and methanol—water at a ratio of 97:3 for the proto compounds and 85:15 for the deutero compounds. The pH' of the mobile phase was adjusted with 5 M ammonium hydroxide.

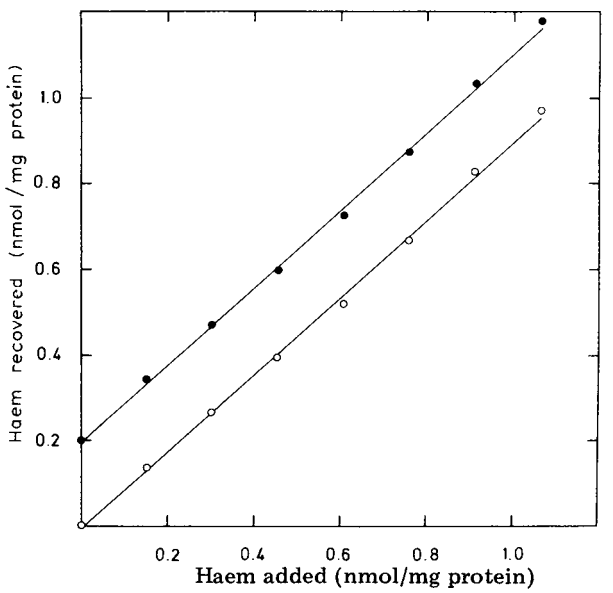

Fig. 3. The recovery of protohaem IX extracted from mitochondria with variable amounts of added protohaem. The total amount of protohaem found (•) and the amount recovered after correcting for the endogenous content of protohaem in mitochondria (○) are shown. Various amounts of protohaem were added to a fixed amount of mitochondria (3 mg of protein) in a total volume of 0.135 ml. This mixture was then extracted and analysed for protohaem as described in Materials and methods. The linear regression line for the data points, representing the recovery of the added protohaem, was $Y = 0.907X - 0.01$ and the correlation coefficient was 0.999.

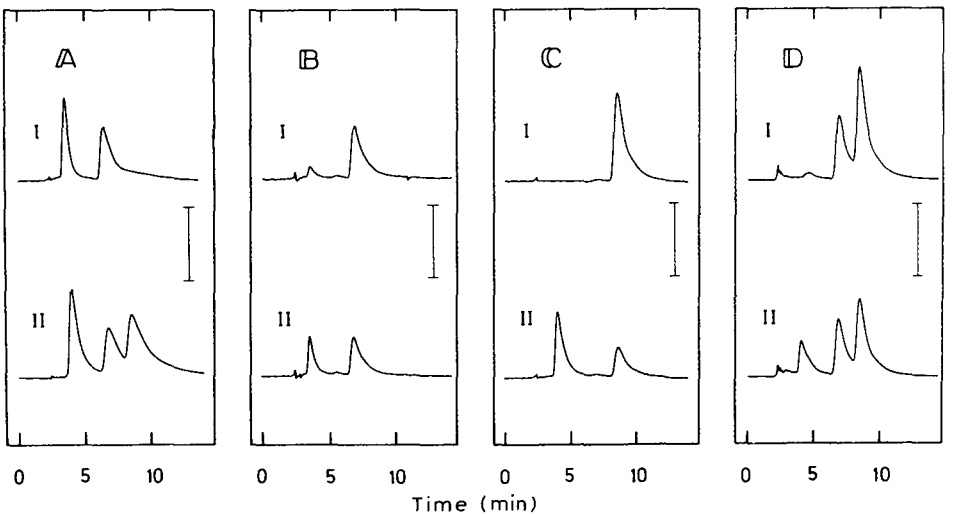

Fig. 4. Chromatograms demonstrating the separation of tetrapyrrole standards (A) and the assay of ferrochelatase activity in isolated liver mitochondria (B and C) and reticulocytes (D). (A) Chromatograms obtained when a mixture of protohaem (80 pmol) and protoporphyrin (200 pmol) was analysed (A, I) and that obtained (A, II) for a mixture of deuterohaem (190 pmol), protohaem (315 pmol) and protoporphyrin (215 pmol). (B) Chromatograms of extracts from mitochondria prior to incubation (I) and following 20 min incubation (II) of the ferrochelatase assay mixture. The porphyrin substrate used was protoporphyrin, and the incubation temperature was 22° C. The amount of mitochondria present in the incubation mixture was 10 mg of protein. For details, see Materials and Methods. (C) Same as B except that deuteroporphyrin was used as substrate and the incubation time was 6 min (II). (D) Chromatograms of extracts from reticulocytes prior to incubation (I) and following 45 min incubation (II) of the ferrochelatase assay mixture. The amount of red blood cells used in the assay mixture contained approximately 60 nmol of protohaem, and the incubation temperature was 37° C. The bars represent an absorption of $A = 0.01$. The chromatographic conditions for the analysis of protohaem were employed in A (I) and B whereas in A (II), C and D the conditions for the analysis of deuterohaem was used (see Materials and methods).

Application of the HPLC procedure in the assay of ferrochelatase

The application of the chromatographic system for the assay of ferrochelatase activity in rat liver mitochondria, using protoporphyrin as the substrate, is shown in Fig. 4B. Three compounds were detected in the chromatogram at zero time (Fig. 4B, I). The peak at t_R = 3.5 min represented endogenous protohaem, whereas the peak at t_R = 6.8 min represented the protoporphyrin substrate. The peak at t_R = 5.6 min is not yet identified, but may represent haem *a* from cytochrome $a + a_3$. The chromatogram of the tetrapyrroles extracted from the assay mixture incubated for 20 min, showed that the peak of protohaem was increased whereas that of protoporphyrin was decreased (Fig. 4B, II). The amount of protohaem synthesized was 0.7 nmol/mg protein.

When deuteroporphyrin was the substrate and mitochondria the enzyme source, one distinct peak (t_R = 8.6 min), representing the porphyrin substrate, appeared in the chromatogram at zero time (Fig. 4C, I). A small peak at t_R = 7.1 min was also seen, representing endogenous protohaem of the

mitochondria. A new peak of t_R = 4 min appeared in the chromatogram following an incubation period of 6 min (Fig. 4C, II) whereas the peak representing the porphyrin substrate decreased. The new peak represented deuterohaem, and the amount formed was 4 nmol/mg protein.

Ferrochelatase activity could also be measured in biological materials containing high amounts of endogenous haem, e.g. reticulocytes, when deuteroporphyrin was used as the substrate (Fig. 4D). In the control chromatogram (Fig. 4D, I) two prominent peaks were observed, one (t_R = 6.7 min) representing the endogenous protohaem from haemoglobin in the red blood cells, and one (t_R = 8.2 min) representing deuteroporphyrin. A small unidentified peak (t_R = 4.5 min) was also seen. Following a reaction period of 45 min, a new peak (t_R = 4 min) appeared in the chromatogram (Fig. 4D, II), representing deuterohaem formed, i.e. 13 nmol/mg haemoglobin (haemoglobin major protein component). The small peak with t_R = 4.5 min in Fig. 4D (I) now appeared as a shoulder in the deuterohaem peak. However, this did not interfere with the estimation of the amount of haem formed when based on peak height, rather than on peak area.

The precision of the ferrochelatase assay is shown in Table I. Of the three steps involved, i.e. anaerobic incubation, haem extraction and assay of haem, the incubation step contributed most to the variation. Thus, the relative standard deviation was 7.8% for the complete assay, 2.0% for the combined extraction and HPLC assay, and 1.0% for this last step alone.

TABLE I

PRECISION OF THE VARIOUS STEPS OF THE ASSAY OF FERROCHELATASE ACTIVITY

Mitochondria were incubated at 25°C for 10 min under the conditions given in Materials and methods using deuteroporphyrin as the substrate. Multiple aliquots of one of the assay mixtures were extracted for haem (parallel extractions) and for one of the extracts multiple HPLC runs were performed (parallel HPLC runs). The mean and S.D. for each series is given.

Deuterohaem found (nmol/mg protein per min)		
Parallel assays	Parallel extractions	Parallel HPLC runs
1.03 ± 0.08 (n = 6)	1.01 ± 0.02 (n = 6)	1.01 ± 0.01 (n = 6)

DISCUSSION

Free porphyrin carboxylic acids have already been separated by HPLC using either ion-exchange, normal-phase, reversed-phase or reversed-phase ion-pair chromatographic systems (see refs. 20—23). In the present study a reversed-phase ion-pair system was selected for the separation of haem and porphyrin compounds since this method is reported to be the most reproducible one for the analysis of tetrapyrroles [24, 25]. The retention time of both haem and porphyrin could be modified either by varying the polarity or the pH of the

mobile phase. A linear relationship between the logarithm of the capacity factor (log k') and the amount of methanol in the mobile phase was found for the porphyrins studied, and this relationship was also obtained for the haem compounds up to a methanol concentration of about 85% (Fig. 1). Such a correlation is frequently observed in reversed-phase ion-pair systems as well as in pure reversed-phase systems [26].

A decrease in k' value was found for both haems and porphyrins when the pH' of the mobile phase was increased (Fig. 2), in agreement with previous studies on porphyrins [25]. From the reported pK values of the tetrapyrroles [27] the effect of pH on the k' values suggests that the retention of the compounds is mainly through hydrophobic forces rather than through an ion-exchange mechanism [26]. A decrease in k' for porphyrins with increasing pH is also found in a reversed-phase system [22]. Thus, for the reversed-phase ion-pair system both the influence of pH' as well as the negligible effect of pairing ion in the mobile phase on the k' value suggest that the separation is largely due to hydrophobic interactions. However, even though an adequate separation of the compounds could also be achieved in a reversed-phase system, an ion-pair system was selected since this improved the peak symmetry.

Under the selected chromatographic conditions, haem was well separated from porphyrin and so was deuterohaem from protohaem. Thus, by using deuteroporphyrin as the substrate for ferrochelatase, the enzyme could also be measured in red blood cells containing high concentrations of protohaem in haemoglobin (Fig. 4D). Ferrochelatase activity in erythroid cells has previously been measured using the pyridine haemochromogen method with meso-porphyrin as the substrate [28]. However, due to a considerable overlap of the spectra for mesohaem and protohaem, this method could only be used when the amount of haem synthesized represented more than 20% of the total amount of haem in the sample [29]. Such a limitation is not present in the method reported here.

The radiochemical method [11] is still the method of choice when very high sensitivity is required. This method can, however, be made more convenient when combined with the present HPLC method. Thus, the tedious extraction procedure used traditionally [11] can be replaced by the much simpler procedure reported here, followed by HPLC. By using protohaem as an internal standard, the recovery of the extraction can be estimated from the same HPLC run.

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SORPTION OF ORGANIC COMPOUNDS FROM URINE IN MUTAGENICITY TESTING: CHOICE OF SORBENT

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SUMMARY

The choice of sorbents for urine mutagenicity testing was investigated. ^{14}C-Labelled compounds were dissolved in urine and extracted from it by sorption with seven different sorbents singly or in combination. The compounds were desorbed with acetone. Sorption was examined under various experimental conditions. Except for two strong ionic compounds satisfactory extraction was achieved with a combination of three sorbents: Amberlite XAD-2, XAD-7, and Sep-Pak Silica. Filtration of the urine or contact of it with the walls of the experimental equipment could lead to substantial losses. Implications of these results for urine mutagenicity testing and possibilities for further optimization of the method are discussed.

INTRODUCTION

Several recent studies have shown that the urine of people exposed to certain work environments, or to cytostatics or tobacco tar (smokers) [1—5] may contain mutagenic substances. In most of this work it was necessary to extract and concentrate the mutagens before they could be studied. For this purpose two techniques were applied: liquid—liquid extraction with dichloromethane or other non-polar solvents; and sorption of the mutagens on Amberlite XAD-2 resin. Although sorption is the more frequently used technique, it has so far not been fully evaluated. One aspect that deserves further study is the type of sorbent used. Yamasaki and Ames [6] were the first to use Amberlite XAD-2 in their experiments with urine of cigarette smokers, and their results led others to follow suit. However, since mutagens are not necessarily chemically similar, the question remains whether other sorbents or combinations of sorbents may not give better results. To answer this question was the first aim of this study, justified by the possible use of urine in routine monitoring of people exposed to mutagenic compounds in their environment.

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We studied the sorption and desorption of ^{14}C-labelled compounds from urine, using seven different sorbents, singly or in combinations, under a variety of experimental conditions. Another aspect studied was the adsorption of the model compounds on the filters and walls of the experimental equipment.

METHODS AND MATERIALS

Sorbents

The sorbents and their salient properties are listed in Table I. A more detailed description is given by Dressler [7] and Saner et al. [8].

The Amberlite XAD sorbents (supplied by Rohm and Haas) were purified by Soxhlet extraction with methanol (24 h) and diethyl ether (24 h) and stored under methanol. They were packed in stainless-steel columns (180 mm, 3 ml; Chrompack, The Netherlands) and later in polyvinylidene fluoride columns, made in cooperation with the TNO Institute for Plastic and Rubber Research (95 mm, 3 ml).

The Sep-Pak sorbents were supplied (Waters Assoc.) already packed in polyethylene cartridges (Sep-Pak Silica 20 mm, 1.5 ml; Sep-Pak C_{18} 10 mm, 0.8 ml).

Just before use all columns were eluted with 50 ml of acetone and 50 ml of distilled water at a flow-rate of 4 ml min^{-1}.

TABLE I

SORBENTS USED

Sorbent	Chemical name*	Surface area per unit weight* ($m^2\ g^{-1}$)	Mean pore diameter* (μm)
Sep-Pak C_{18}	Octadecylsilane	400	—
Sep-Pak Silica	**	**	**
XAD-1	Styrene divinylbenzene copolymer	100	0.01
XAD-2	Styrene divinylbenzene copolymer	300	0.009
XAD-4	Styrene divinylbenzene copolymer	725	0.004
XAD-7	Methacrylate polymer	450	0.009
XAD-8	Methylmethacrylate polymer	160	0.0225

*Information of the manufacturer.
**No information available.

^{14}C-Labelled compounds

The following ^{14}C-labelled compounds were used: benzene, phenol, benzo-(*a*)pyrene, Aroclor 1254, ethylenediamine, dioctylphthalate, chloroacetic acid, *p*-chlorophenol, *p*-nitrophenol, 3,4-dichloroaniline, didecyldimethylammonium bromide.

Except for didecyldimethylammonium bromide, which was synthesized at our institute, all compounds were supplied by the Radiochemical Centre (Amersham, U.K.). Stock solutions of the compounds were made in acetone or distilled water. Of each solution 15 μl were mixed with 50 ml of urine.

Urine samples

Urine was collected in batches from several male employees of our institute who neither smoked nor took medicine. Each batch was at once chilled to 0—4° C upon collection, and its pH was measured: the mean pH was 5.8 ± 0.5. Just before addition of the labelled compounds, the urine was filtered over a regenerated cellulose membrane filter (Sartorius SM 11604, 0.8 μm).

Solvents

All organic solvents (glass-distilled or HPLC grade) were supplied by Rathburn Chemicals (U.K.).

Sorption experiments

Unless noted otherwise in the text, the set-up of the sorption experiments was the same as that used by Yamasaky and Ames [6]. A 50-ml volume of urine was sucked into an evacuated glass vial and was pumped through the column by increasing the air pressure in the vial. The flow-rate was regulated by changing the air pressure (mean value 4 ml min^{-1}). This method of filling the sample vial reduces the amount of gas dissolved in the urine, and promotes better contact between urine and the sorbent.

The bed volume was 3 ml for the XAD sorbents, 0.8 ml for Sep-Pak C_{18} and 1.5 ml for Sep-Pak Silica. Aliquots of urine (2 ml) were taken before and after passage through the column to determine the ^{14}C-labelled compounds by liquid scintillation counting. The sorption percentage is the difference between these amounts as a percentage of the amount before passage.

Desorption experiments

The sorbed compounds were desorbed immediately after passage of the urine, by passing through the column in the opposite direction two 5-ml portions of acetone at a flow-rate of 3 ml min^{-1}. After passage, the two acetone portions were kept separate, and their content of ^{14}C-labelled compound was measured. The desorption conditions were chosen based on the results obtained with ^{14}C-labelled compounds in distilled water which showed that backflush desorption is to be preferred to straight desorption. The desorption percentage is the amount of ^{14}C desorbed as a percentage of the amount sorbed.

RESULTS

Combining sorbents for best results

In a series of experiments we studied the sorption of four compounds (benzo(*a*)pyrene, benzene, phenol and Aroclor 1254) by all sorbents at three urine pH values (pH 2, pH 10 and the physiological pH of urine). The results of these experiments allowed us to select sorbents for further experiments. In these we chose conditions believed to be less favourable to sorption: the concentration of the compound, the volume of the urine, and the flow-rate of the urine were all increased; the volume of the sorbent (bed volume) was decreased. In two experiments the removal of radioactivity from the sorbent was measured when another portion of urine, without labelled compound, was

pumped through the column. A summary of the results is given in Table II.

Phenol is best sorbed by XAD-7 and XAD-4 at physiological pH. At pH 2 the sorption was negligible; at pH 10 it was a little less than at the physiological pH of urine.

The sorption of benzene is not very pH-dependent, and none of the XAD resins emerges as being best for the sorption of this compound. Benzo(*a*)pyrene is best sorbed from urine by Sep-Pak C_{18} and Sep-Pak Silica. The sorption is

TABLE II

PERCENTAGE SORPTION FROM URINE OF FOUR ^{14}C-LABELLED COMPOUNDS WITH VARIOUS SORBENTS UNDER FIVE (I—V) EXPERIMENTAL CONDITIONS

Experiments: (I) basic sorption set-up with lower concentration, (II—V) higher concentration, (II) experiment with distilled water, (III) volume of urine increased to 300 ml, (IV) flow-rate increased to 40 ml min^{-1}, (V) bed volume decreased to one-third with XAD or one-half with Sep-Pak. The XAD resins were packed in stainless-steel columns.

	I	II*	III*	IV	V
Phenol (pH not adjusted, 2 and 200 $\mu g\, l^{-1}$)					
Sep-Pak Silica	5				
Sep-Pak C_{18}	4				
XAD-1	71	61			
XAD-2	69	81			
XAD-4	92	96	66	42	72
XAD-7	94	93	65	81	60
XAD-8	95	86	49		
Benzene (pH 2, 0.6 and 60 $\mu g\, l^{-1}$)					
Sep-Pak Silica	31				
Sep-Pak C_{18}	88	80			
XAD-1	98	99	76		
XAD-2	95	99	77	94	71
XAD-4	93	99	65	97	63
XAD-7	99	99			
XAD-8	98	92			
Benzo(*a*)pyrene (pH 2, 9 and 18 $\mu g\, l^{-1}$)					
Sep-Pak Silica	96	100	66	99	
Sep-Pak C_{18}	93	99	96	96	98
XAD-1	71	46			
XAD-2	61	39			
XAD-4		61	37		
XAD-7		77	56		
XAD-8		88	74		
Aroclor 1245 (pH 2, 2 and 50 $\mu g\, l^{-1}$)					
Sep-Pak Silica	98	99	94	76	
Sep-Pak C_{18}	83	98	87	71	71
XAD-1	71	79			
XAD-2	56	76			
XAD-4	63	74			
XAD-7	83	81			
XAD-8	78	79			

*Experiment in which an extra portion of urine or water was pumped through the sorbent.

TABLE III

PERCENTAGE SORPTION FROM URINE OF ^{14}C-LABELLED COMPOUNDS WITH VARIOUS SORBENTS

The basic sorption set-up was used. The XAD resins were packed in polyvinylidene columns. The pH of the urine was not adjusted.

	Sep-Pak Silica	Sep-Pak C_{18}	XAD-1	XAD-2	XAD-4	XAD-7	XAD-8
Ethylene diamine ($18\ \mu g\ l^{-1}$)	14	5	16	18	21	17	13
Dioctylphthalate ($250\ \mu g\ l^{-1}$)	90	83*	10	11	9	13	15
Chloroacetic acid ($8\ \mu g\ l^{-1}$)	4	1	2	4	5	4	7
p-Chlorophenol ($39\ \mu g\ l^{-1}$)	10	11	84	85	87	89	88
p-Nitrophenol ($12\ \mu g\ l^{-1}$)	6	3	77	89	98	100	100
3,4-Dichloro-aniline ($20\ \mu g\ l^{-1}$)	16	90**	74	74	70	67	70
Didecyldimethyl-ammonium bromide ($20\ \mu g\ l^{-1}$)	93	77	85	85	85	86	87

*Duplicate value was 26 (see also Table V).
**Duplicate value was 46.

somewhat better at pH 2 or pH 10 than at the natural pH. Likewise, Sep-Pak cartridges are the best sorbents for Aroclor 1254. Also with this compound the sorption is not very pH-dependent.

The extra portion of urine without ^{14}C-labelled compound resulted in a considerable loss (> 10%) of phenol when XAD-1, -2 or -8 were used and of benzene when Sep-Pak C_{18} was used.

The sorption of the other labelled compounds was investigated less thoroughly. Using the basic sorption set-up, we extracted them with each of the sorbents only at the physiological pH of urine. The ionic compounds were also extracted at pH 2 or 10. Results are given in Table III. The sorption of ethylene diamine did not improve at a higher pH; that of chloroacetic acid increased to 14—28% with the XAD resins when the pH decreased to 2.

In the next series of experiments all the labelled compounds were sorbed on combinations of two, three or four sorbents; the pH of the urine was not adjusted. The choice of those sorbents was based on the earlier experiments: Sep-Pak Silica, Sep-Pak C_{18}, XAD-2 and XAD-7. In this way it could be established whether two suboptimal sorbents could replace the optimal one, and whether the pH-dependence of the extraction was reduced by combination. Results with combinations of suboptimal sorbents are shown in Table IV.

It is clear from this table that a combination of XAD-2 and XAD-7 is sufficient for almost all compounds. To reach a high sorption percentage with benzo(*a*)pyrene the Sep-Pak Silica can be added to the two XAD sorbents. The

two strong ionogenic and polar compounds are poorly sorbed even with a combination of all four sorbents (10—20%). With this combination the sorption of the other compounds lies in the 90% range. This was also the case when the combination XAD-2—XAD-7—Sep-Pak Silica was used.

TABLE IV

PERCENTAGE SORPTION FROM URINE OF ^{14}C-LABELLED COMPOUNDS WITH COMBINATIONS OF SORBENTS

Apart from the use of more sorbents, the basic sorption set-up was used. The pH of the urine was not adjusted. The XAD resins were packed in polyvinylidenefluoride columns.

Compound	Concentration ($\mu g\ l^{-1}$)	Combination of sorbents*	Percentage sorption
Phenol	200	C_{18}—XAD-2	38
		C_{18}—XAD-2—Sil	45
Benzene	60	XAD-2—XAD-7	34
Benzo(*a*)pyrene	18	C_{18}—XAD-2—XAD-7—Sil	79
Aroclor 1254	50	XAD-2—XAD-7	90
Ethylenediamine	18	C_{18}—XAD-2—XAD-7—Sil	28
Dioctylphthalate	250	XAD-2—XAD-7	96
Chloroacetic acid	8	C_{18}—XAD-2—XAD-7—Sil	10
p-Chlorophenol	39	C_{18}—Sil	14
p-Nitrophenol	12	C_{18}—Sil	6
3,4-Dichloroaniline	20	C_{18}—Sil	51
		C_{18}—XAD-7—Sil	85
Didecyldimethylammonium bromide	20	All combinations	>90

*C_{18} = Sep-Pak C_{18}; Sil = Sep-Pak Silica.

TABLE V

PERCENTAGE DESORPTION OF ^{14}C-LABELLED COMPOUNDS FROM VARIOUS SORBENTS

The concentrations of the compounds were equal to those listed in Table IV. The XAD resins were packed in stainless-steel columns.

Compound	Sorbent	pH*	Adsorption	Desorption	Total extracted**
Benzene	XAD-2	2	95	87	83
Phenol	XAD-4	n.a.	82	77	63
Benzo(*a*)pyrene	Sep-Pak C_{18}	2	96	63	60
Aroclor 1254	Sep-Pak Silica	2	91	104	91
Dioctylphthalate	Sep-Pak C_{18}	n.a.	21	96	20
Chloroacetic acid	XAD-7	2	29	68	20
p-Chlorophenol	XAD-4	n.a.	90	77	69
p-Nitrophenol	XAD-4	n.a.	84	79	66
3,4-Dichloroaniline	XAD-2	n.a.	94	75	71
Didecyldimethylammonium bromide	XAD-4	n.a.	74	70	52

*n.a. = natural pH of urine, not adjusted.
**Percentage of the amount of compound present in the urine that is extracted.

Desorption of the sorbed compounds

Table V shows the results of a number of experiments in which we measured desorption of the labelled compounds. The results indicate that, for optimum desorption, 10 ml of acetone are sufficient. Nearly all the radioactivity was desorbed by the first 5 ml of acetone. It is not clear, however, whether the proportion not desorbed remains behind in the sorbent or on the walls of the sampling vial.

Adsorption on glass, cartridge material and membrane filter material

In the adsorption experiments the urine inevitably comes into contact with materials that are not sorbents, but which may nevertheless act as adsorbents. We briefly investigated the resulting spurious sorption in experiments in which such materials were "exposed" to the urine in the same way as in the basic set-up. Table VI lists the results, which show that a considerable proportion of a compound can be lost through spurious adsorption. Adsorption experiments with stainless-steel columns showed that adsorption of benzo(*a*)pyrene or Aroclor 1254 from distilled water was negligible. The adsorption of benzo(*a*)pyrene with the cartridge material is very high, which can account for the high percentages found with the complete Sep-Pak Silica.

TABLE VI

ADSORPTION OF ^{14}C-LABELLED COMPOUNDS ON GLASS, CARTRIDGE MATERIAL AND MEMBRANE FILTERS

The concentrations of the compounds were equal to those listed in Table IV.

Compound	pH*	"Adsorbent"**	Percentage adsorption	Percentage desorption
Benzene	2	Glass	24	
Phenol	n.a.	Glass	1	
Benzo(*a*)pyrene	2	Glass	14	
		Cartridge	93	
		Filter	50	85
Aroclor 1254	2	Glass	22	
		Cartridge	18	
		Filter	33	111
Dioctylphthalate	n.a.	Filter	44	100

*n.a. = pH of urine not adjusted.

**Glass = vial used in the sorption experiment; cartridge = empty Sep-Pak Silica cartridge; filter = regenerated cellulose membrane filter.

DISCUSSION

An important aspect of mutagenicity studies with urine from humans, thought to have been exposed is the uncertainty about the identity of the mutagen that has to be extracted and concentrated. Mutagens occur in widely different classes of chemicals which implies that the techniques applied should extract a broad spectrum of chemicals. Available techniques are liquid–liquid partition and sorption. With the former the polarity of the extracted

compounds depends on the solvent and is thus rather low because partition must be possible; the latter technique has the advantage that sorption can be varied by changing the type of sorbent used. Combination of appropriate sorbents will enable the extraction of organics of widely varying properties.

The results presented in this paper illustrate the suitability of sorption for several classes of compounds. Except for compounds that are both ionic and polar (ethylenediamine and chloroacetic acid), compounds could be extracted satisfactorily by a combination of sorbents followed by desorption with acetone. It follows that, if one wishes to extract a wide range of mutagens from urine, a combination of sorbents is superior to a single sorbent (e.g. XAD-2).

Most compounds were not fully desorbed by acetone; this phenomenon could be explained by spurious adsorption on materials other than the sorbents themselves. Special care must be taken in filtering the urine, which may result in the loss of a large proportion of the material one wishes to isolate.

The results show that satisfactory extraction of mutagens from urine can be achieved with the combination XAD-2—XAD-7—Sep-Pak Silica.

Urine can be regarded as a rather concentrated solution of polar organic compounds. Their presence may limit the extraction of mutagens to those having a lower polarity, because they may cause toxicity problems in mutagenicity tests, they necessitate large bed volumes to avoid overloading of the sorbent, and they may limit the possible concentration factor of the mutagens.

Attempts aimed at the sorption of more polar mutagens could result in too big a share of the "natural" polar urine constituents in the extract, which in turn could reduce the advantage of being able to sorb the more polar mutagens.

Should urine be found to contain mutagens in such low concentrations — compared to those in our experiments — that it is necessary to aim at higher concentration factors, one might increase the urine volume. This may well cause part of a mutagen already sorbed to be supplanted by the larger amount of "natural" urine constituents that now passes the sorbent. Increasing both bed volume and urine volume, however, leads to sorption of larger amounts of "natural" urine constituents, thus limiting the concentration factor.

These considerations make clear that the polarity of the mutagens in urine that can be extracted and the concentration factor that can be reached are both limited by the "natural" polar urine constituents. Further investigations are therefore necessary to establish optimum experimental conditions for extraction in urine mutagenicity studies. The results of this study indicate which combination of sorbents should be used.

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CHROMBIO. 2170

GAS—LIQUID CHROMATOGRAPHIC EVALUATION OF LOFEMIZOLE IN BIOLOGICAL SAMPLES FOR PHARMACOKINETIC INVESTIGATIONS

COMPARISON OF TWO ANALYTICAL METHODS

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SUMMARY

The present paper reports the analytical conditions allowing lofemizole, a new non-steroidal anti-inflammatory drug, to be evaluated in biological fluids for pharmacokinetic and bioavailability investigations. The first approach led to an N-methyl derivative of lofemizole which could be successfully analysed by gas—chromatography employing a flame-ionization detector, reaching a sensitivity of 2 μg/ml. The second approach led to the N-(2-chlorobenzoyl) derivative of lofemizole which was suitable for pharmacokinetic investigation using gas—liquid chromatography with electron-capture detection, and reaching a much higher sensitivity of 10 ng/ml of plasma. Recovery of the extraction, reproducibility and specificity were all satisfactory with both methods. Since the first method employing flame-ionization detection was suitable for pharmacokinetic investigations in animal species, this paper describes both methods on a comparative basis.

INTRODUCTION

Lofemizole [1H,4-(4-chlorophenyl)-5-methylimidazole, $C_{10}H_9N_2Cl$] has the molecular structure shown in Fig. 1, and its chemical identity was confirmed by mass spectrometry. Lofemizole has proved to possess an interesting anti-phlogistic activity in both animals and humans, associated with a favourable

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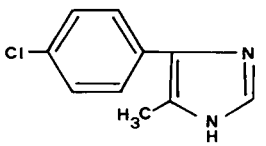

Fig. 1. Molecular structure of lofemizole.

gastric and systemic tolerability [1]. From the point of view of the structure—activity relationship, lofemizole is comparable with other compounds having an imidazole [2, 3], thiazole [4] or oxadiazole [5, 6] structure and possessing anti-inflammatory activity.

The aim of this paper was to investigate the analytical conditions that would allow a pharmacokinetic or bioavailability study to be carried out on lofemizole in both animals and humans.

EXPERIMENTAL

Drugs, chemicals and instruments

Solvents and chemicals, all of analytical grade purity, were supplied by E. Merck (Darmstadt, F.R.G.). 2-Chlorobenzoyl chloride and methyl iodide of an excellent grade purity were prepared at B.T.B. Industria Chimica, Chemical Department; zomepirac methyl ester and miconazole were also supplied by B.T.B. The supports and stationary phases for the gas—liquid chromatography (GLC) columns were supplied by Supelchem (Milan, Italy). A Varian 3700 gas chromatograph and a VG Micromass MS 30/70 mass spectrometer were employed for the analysis. Rats were supplied by Charles River Italia (Calco, Italy). The statistical evaluation was performed on a Hewlett-Packard HP 86 personal computer.

Evaluation by GLC with flame-ionization detection (GLC—FID)

Extraction. A 1-ml volume of sodium hydroxide solution (1 mol/l) and 5 ml of diethyl ether were added to 1 ml of plasma (or urine) in a glass-stoppered test tube. The mixture was vigorously stirred for 5 min and then centrifuged at 2400 *g* for 10 min. Methyl iodide (1 ml of a 10% solution in diethyl ether), 0.2 ml of tetrabutylammonium hydroxide (0.1 mol/l in benzene—methanol, 1:1) and 1 ml of sodium hydroxide solution (1 mol/l) were added to 4 ml of the organic layer in another test tube. The test tube was stoppered and kept at 90°C with stirring for 30 min, after which it was cooled to room temperature and centrifuged. An aliquot of the organic layer containing the N-methyl derivative of lofemizole was dried with anhydrous sodium sulphate, evaporated to dryness and finally dissolved in 100 μl of acetone. Zomepirac methyl ester was added as the internal standard. The sample was then injected into the gas chromatograph.

Analytical conditions. A glass column (1.5 m × 6 mm O.D., 2 mm I.D.) filled with 3% SP-2250 DB on 100—120 mesh Supelcoport was used. The temperatures of the injection port, oven and flame-ionization detector were maintained at 280°C, 245°C and 350°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 35 ml/min.

Evaluation by GLC with electron-capture detection (GLC—ECD)

Extraction. A 1-ml aliquot of plasma (or urine) was placed in a glass-stoppered test tube with 1 ml of sodium hydroxide solution (1 mol/l) and 5 ml of diethyl ether. The test tube was vigorously stirred for 5 min and then centrifuged at 2400 *g* for 10 min. An aliquot (4.5 ml) of the organic layer was added to 0.5 ml of 2-chlorobenzoyl chloride (400 μg/ml in diethyl ether) and to 1 ml of sodium hydroxide solution (1 mol/l) in another test tube. The mixture was vigorously stirred for 5 min and then centrifuged at 2400 *g* for 10 min. An aliquot of the organic layer was dried over anhydrous sodium sulphate and evaporated to dryness under a gentle nitrogen stream at 60° C. The residue was redissolved in 0.5 ml of benzene. Miconazole was added as the internal standard. The solution was then ready for GLC analysis.

Analytical conditions. A glass column (1 m × 6 mm O.D., 2 mm I.D.) was filled with 10% OV-11 on 100—120 mesh Supelcoport. The temperatures of the injection port, oven and electron-capture detector were maintained at 380° C, 300° C and 380° C, respectively. Nitrogen was employed as the carrier gas at a flow-rate of 35 ml/min.

RESULTS

GLC—FID method

The retention times observed were 58 sec for the N-methyl derivative of lofemizole, the chemical identity of which was confirmed by mass spectrometry, and 2 min 4 sec for the internal standard (zomepirac methyl ester, I.S.). Linearity was investigated in the range of 2.5—200 ng for each injection (Table I) by means of the detector response factor (d.r.f.) evaluated as follows:

$$\text{d.r.f.} = \frac{\text{lofemizole weight}}{\text{I.S. weight}} \times \frac{\text{I.S. peak area}}{\text{lofemizole peak area}}$$

This factor allows the different response of the detector to the analytical substance and the internal standard to be counterbalanced. Table II shows the d.r.f. with a lofemizole derivative/internal standard ratio ranging from 1:4 to 4:1.

TABLE I

LINEARITY OF THE DETECTOR RESPONSE TO N-METHYLLOFEMIZOLE EVALUATED BY GLC—FID

Linearity was confirmed by a constant detector response factor (d.r.f.). The lofemizole derivative and the internal standard were injected at a 1:1 ratio.

Lofemizole injected (ng)	Mean d.r.f. ± S.D. (n = 4)
2.5	1.10 ± 0.07
5	1.08 ± 0.01
10	1.08 ± 0.03
20	1.07 ± 0.02
50	1.09 ± 0.03
100	1.08 ± 0.03
200	1.08 ± 0.02

TABLE II

DETECTOR RESPONSE FACTOR AT DIFFERENT LOFEMIZOLE DERIVATIVE/I.S. RATIOS (GLC—FID METHOD)

Lofemizole/I.S. weight ratio	Mean d.r.f. ± S.D. (n = 4)
1:4	1.15 ± 0.11
1:2	1.12 ± 0.07
1:1	1.06 ± 0.02
2:1	1.04 ± 0.03
4:1	1.04 ± 0.05

TABLE III

RECOVERY AFTER THE EXTRACTION PROCEDURES FOR ANALYTICAL EVALUATION OF LOFEMIZOLE (GLC—FID METHOD)

Lofemizole added to 1 ml of plasma (μg)	Lofemizole found (μg/ml) (mean ± S.D., n = 4)	Recovery (%)
1.0	0.92 ± 0.22	92.0
2.5	2.44 ± 0.32	97.6
5.0	4.93 ± 0.27	98.6
10.0	9.82 ± 0.40	98.2
25.0	24.24 ± 0.78	97.0
50.0	45.80 ± 2.15	91.7
100.0	93.70 ± 4.39	93.7
	Mean ± S.D.:	95.5 ± 3.0

The recovery of lofemizole was investigated in the range 1—100 μg/ml of plasma, the analysis being performed in quadruplicate at each concentration (Table III). The mean recovery in the whole range investigated was 95.5%. The amount of lofemizole added correlated well with that found, as shown by the following relationship obtained by linear regression: $\text{lofemizole}_{\text{found}}$ (μg/ml) = 0.221 + [0.932 × $\text{lofemizole}_{\text{added}}$ (μg/ml)], r = 0.9999, $p < 0.0001$. The slope of the resulting linear function can be considered as a significant expression of the extraction recovery; in this respect the slope (0.932) is very near to the mean recovery value.

Reproducibility was evaluated by the standard deviation (S.D. %). It was 2.5% when 1 ml of solution containing 50 ng of N-methyllofemizole and 50 ng of internal standard was injected seven times. Reproducibility for the entire analytical procedure, including the extraction, was about 4—5% in the range 5—100 μg/ml of plasma and rose to 13% at a concentration of 2.5 μg/ml and to 24% at a concentration of 1 μg/ml. On this basis the sensitivity of the method appears to be about 2 μg/ml.

GLC—ECD method

Retention times were 3 min 42 sec for the 1-N-(2-chlorobenzoyl) derivative of lofemizole, the chemical identity of which was ascertained by mass spectrometry (Fig. 2), and 6 min 15 sec for the internal standard (miconazole

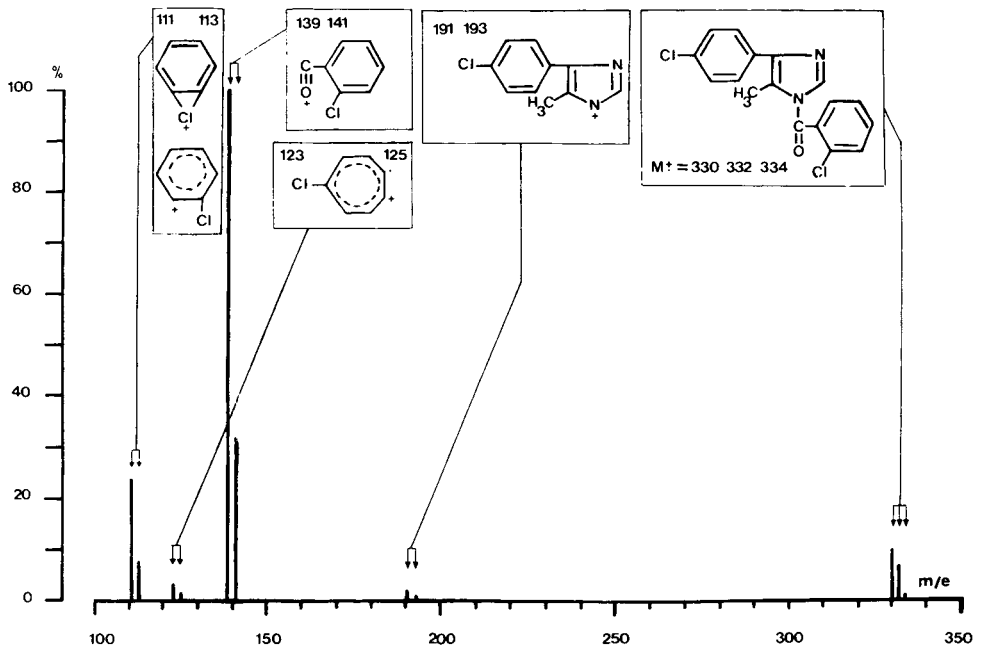

Fig. 2. Mass spectrum of 1-N-(2-chlorobenzoyl)lofemizole obtained in the electron-impact ionization mode (70 eV), the product being introduced into the source through the gas chromatograph.

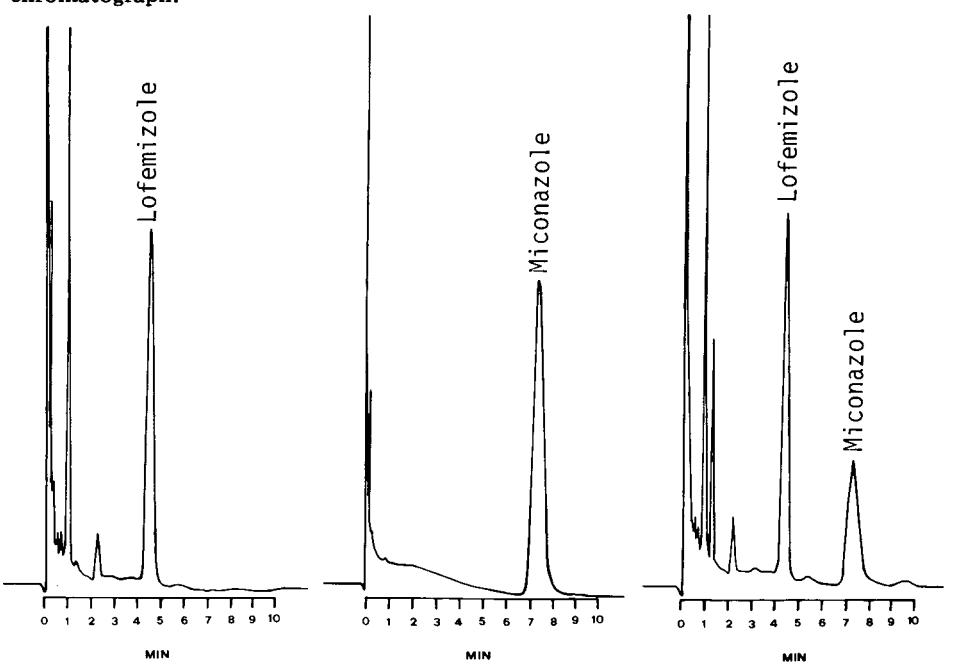

Fig. 3. Gas chromatograms of lofemizole, miconazole (I.S.) and, in the right panel, of a mixture of lofemizole and miconazole. The peaks appearing in the first 2.5 min are due to the reactants employed for derivatization and are missing in the case of miconazole, which had not undergone derivatization.

as such). The related gas chromatograms are shown in Figs. 3 and 4. Linearity was investigated in the range 20—4000 pg injected. Table IV shows the good linearity verified in the range 20—1000 pg. The d.r.f. obtained by varying the lofemizole/I.S. ratio is shown in Table V. Recovery of lofemizole was investigated in the range 10—5000 ng/ml of plasma and proved to be 94.5% on average (Table VI). The linear relationship between lofemizole added and that

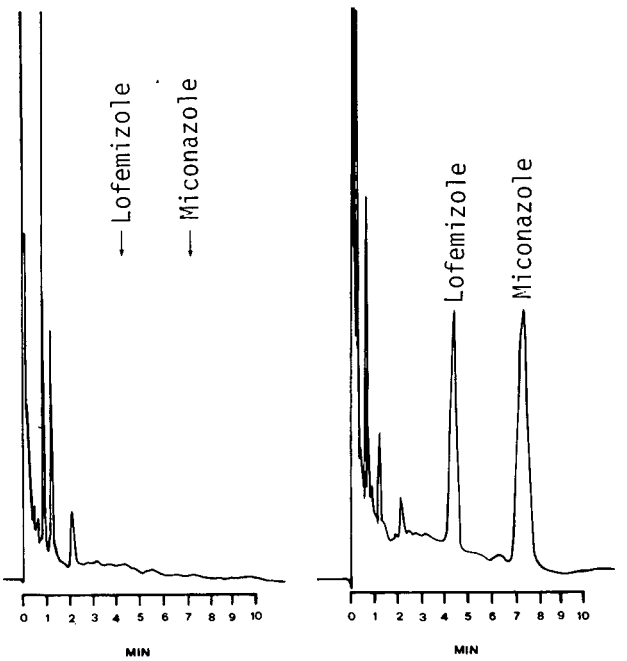

Fig. 4. Left: gas chromatogram of a blank plasma with neither lofemizole, nor miconazole, after being taken through the whole analytical procedure. Right: gas chromatogram of an analysis performed on a plasma sample from a volunteer treated with the drug, carried through the entire analytical procedure. No interference by endogenous peaks could be detected in the blank sample.

TABLE IV

LINEARITY OF DETECTOR RESPONSE TO 1-N-(2-CHLOROBENZOYL)LOFEMIZOLE EVALUATED WITH THE GLC—ECD METHOD

Linearity was confirmed by a constant detector response factor (d.r.f.). The lofemizole derivative and the internal standard were injected at a 1:1 ratio.

Lofemizole injected (pg)	Mean d.r.f. ± S.D. (n = 4)
20	0.97 ± 0.08
50	0.98 ± 0.04
100	0.98 ± 0.02
200	0.98 ± 0.03
500	0.94 ± 0.03
1000	0.97 ± 0.02
2000	1.22 ± 0.08
4000	1.75 ± 0.07

TABLE V

DETECTOR RESPONSE FACTOR AT DIFFERENT LOFEMIZOLE DERIVATIVE/I.S. RATIOS (GLC—ECD METHOD)

Lofemizole/I.S. weight ratio	Mean d.r.f. ± S.D. (n = 4)
1:4	0.92 ± 0.08
1:2	0.94 ± 0.03
1:1	0.94 ± 0.02
2:1	0.98 ± 0.04
4:1	0.87 ± 0.08

TABLE VI

RECOVERY OF THE EXTRACTION PROCEDURES FOR THE ANALYTICAL EVALUATION OF LOFEMIZOLE (GLC—ECD METHOD)

Lofemizole added to 1 ml of plasma (ng)	Lofemizole found (ng/ml) (mean ± S.D., n = 4)	Recovery (%)
10	9.4 ± 1.5	94.0
20	18.9 ± 1.8	94.5
50	47.0 ± 2.0	94.0
100	95.0 ± 6.3	95.0
200	187.0 ± 8.4	93.5
500	480.0 ± 16.4	96.0
1000	940.0 ± 31.0	94.0
5000	4753.0 ± 177.0	95.0
	Mean ± S.D.: 94.5 ± 0.8	

found, using linear regression, was $\text{lofemizole}_{\text{found}}$ (μg/ml) = —1.209 + [0.951 × $\text{lofemizole}_{\text{added}}$ (μg/ml)], r = 1.000, $p < 0.0001$. In this case too the slope of the resulting linear function agrees with the mean recovery. Reproducibility, evaluated as S.D. % on repeated assays, ranged between 2% and 3% when standards of lofemizole derivative and I.S. were injected into the gas chromatograph in the range 100—1000 pg without extraction, the test being repeated four times. Over the whole analysis, including the extraction procedures, reproducibility ranged between 3.3% and 6.6% in the concentration range 50—5000 ng/ml, and increased to 9.5% at a concentration of 20 ng/ml and to 16% at 10 ng/ml. The sensitivity of this method therefore seems to be around 10 ng/ml.

DISCUSSION

In the very first approach lofemizole was analysed without derivatization on non-polar stationary phases such as SP-2100 DB. The tailing, low sensitivity and poor reproducibility prompted us to develop an appropriate N-derivatization of the molecule, as others have done with other 1H-imidazoles [7]. This in fact proved to be a very difficult process, because the more aggressive reactants such as anhydrides or acyl chlorides cause imidazole ring cleavage in lofemizole.

An extractive N-methylation, like that introduced by Ervik and Gustavii [8] for sulphonamide diuretics, led to a stable derivative with a quantitative yield. Despite the aromatic chlorine in the lofemizole molecule it could not be revealed by using the electron-capture detector, and the thermoionic-specific detector did not attain a sensitivity high enough to be suitable for our purposes. Thus the first analytical method employing a flame-ionization detector was performed with the aim of starting the pharmacokinetic studies on the rat. The distribution volume data obtained in the rat clearly indicate that the sensitivity achieved with the FID method was not high enough for pharmacokinetic studies to be carried out on human beings. A new N-derivatization method with a chlorinated reactant therefore needed to be developed.

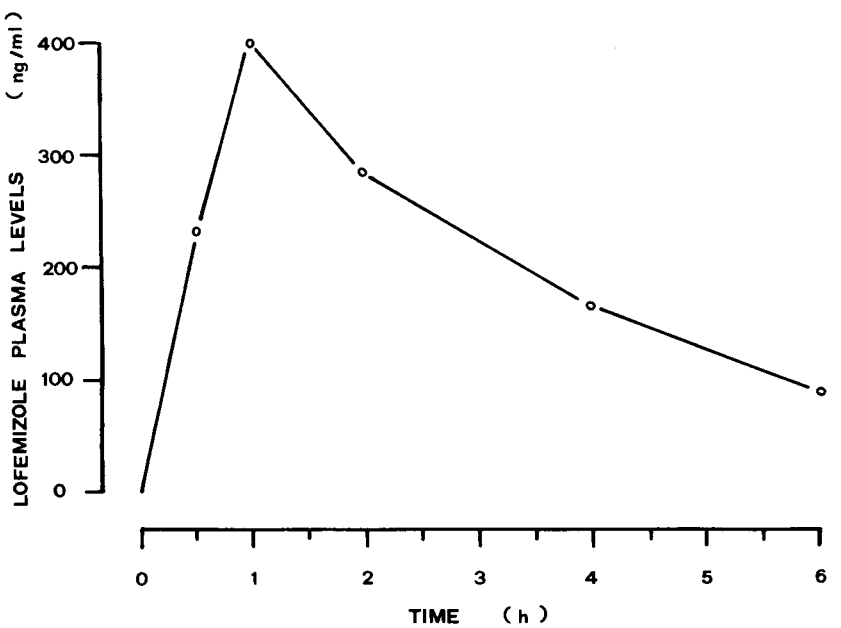

Fig. 5. Lofemizole plasma levels evaluated in a human volunteer orally treated with 100 mg of the drug.

The acyl chloride of 2-chlorobenzoic acid showed rather slow reaction kinetics, thus any undesired cleavage of the imidazole ring was avoided. The derivative was obtained in an extractive acylation process with a quantitative yield and could be revealed by ECD with a very high degree of sensitivity. This method allowed quantitative evaluation to be performed on humans who had been orally or rectally treated with 50 or 100 mg of lofemizole for bioavailability investigations. Fig. 5 shows the plasma levels of lofemizole in one subject treated with a 100-mg dose of lofemizole per os. An extensive investigation is now in progress using this method on both animal species and human beings in order to clarify the pharmacokinetic behaviour of lofemizole.

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GAS CHROMATOGRAPHIC DETERMINATION OF PENTOXIFYLLINE AND ITS MAJOR METABOLITES IN HUMAN BREAST MILK

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SUMMARY

A method has been developed for determination of the xanthine drug, pentoxifylline, and three of its metabolites (a secondary alcohol and two carboxylic acids) in human milk. The method requires pre-extraction with hexane to remove lipids followed by extraction with dichloromethane or dichloromethane—isopropanol (4:1). Absolute extraction recoveries were between 76—90%. Pentoxifylline and its alcohol metabolite (as the trifluoroacetate) and the carboxylic acid metabolites (as ethyl esters) were measured in separate gas chromatographic steps using a nitrogen detector. Determinations of pentoxifylline and its three metabolites were 96—99% accurate and standard deviations of 5—10% were observed for samples at or above the lower practical sensitivity limit (10 ng/ml) for the assay. Pentoxifylline and its metabolites were stable in breast milk for three weeks when stored at —15°C.

INTRODUCTION

Pentoxifylline [P; 1-(5′-oxohexyl)-3,7-dimethylxanthine] (Fig. 1) is used extensively in the treatment of cerebrovascular and peripheral vascular diseases [1—3]. The principle metabolites found in plasma after oral administration of P are a secondary alcohol metabolite (metabolite I) and two carboxylic acid metabolites (metabolites IV and V) (see Fig. 1). Since some mothers who

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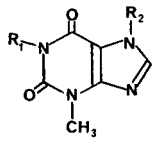

P, R_1 = $CH_2CH_2CH_2CH_2COCH_3$; R_2 = CH_3

I, R_1 = $CH_2CH_2CH_2CH_2CHOHCH_3$; R_2 = CH_3

II, R_1 = $CH_2CH_2CH_2CH_2CH_2CH_2OH$; R_2 = $CH_2CH_2CH_3$

III, R_1 = $CH_2CH_2CH_2CH_2CH_2CO_2H$; R_2 = CH_3

IV, R_1 = $CH_2CH_2CH_2CH_2CO_2H$; R_2 = CH_3

V, R_1 = $CH_2CH_2CH_2CO_2H$; R_2 = CH_3

Fig. 1. Chemical structures of pentoxifylline (P), its principal metabolites (I, IV and V) and internal standards (II and III).

require therapy with P may wish to breast-feed their infants, the amounts of P and its metabolites that are excreted into breast milk must be assessed to evaluate potential drug-related risks to newborns.

Wilson [4] and Findlay [5] have recently reviewed the literature on drug excretion in breast milk. Few reports have appeared on the secretion of methylxanthine-related substances in milk. Different investigators, however, note that the milk levels of theobromine [6], theophylline [7] and caffeine [8] are similar to those found in plasma. Pentoxifylline is closely related structurally to these compounds, thus, its excretion into mother's milk may be significant.

Procedures for determining P and its metabolites have been developed for plasma [9, 10] and urine [11]; however, a suitable method has not been published for the corresponding determinations in human breast milk. We now report a gas chromatographic (GC) method which has been validated through analyses of spiked human milk samples.

MATERIALS AND METHODS

Reagents

Pentoxifylline, the internal standards II and III, and metabolites I, IV and V (Fig. 1), were supplied by Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). These compounds were used as received and were homogeneous when submitted to thin-layer chromatography [12], high-performance liquid chromatography [12] and GC as described herein. Dichloromethane (glass-distilled), toluene (glass-distilled), and trifluoroacetic anhydride were used as obtained from MCB Manufacturing Chemists (glass-distilled i.e. Omni Solv; Cincinnati, OH, U.S.A.). Hexane (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). All other solvents and reagents were reagent grade or better. Control human breast milk was obtained from local hospitals and was generally colostrum.

Pentoxifylline and metabolite I

The method developed for the assay of P and metabolite I in milk is a modification of that described for plasma by Bryce and Burrows [9]. The procedure involves GC with alkali flame ionization (nitrogen-selective) detection. A P-analogue, compound II, was used as the internal standard. Metabolite I, and

compound II were converted to their trifluoroacetate derivatives to affect complete separation from P in the GC step.

Standard solutions of P and metabolite I (5 and 50 μg/ml) were prepared in toluene (glass-distilled). These solutions were stored at −15° C and were stable for several months. In screw-capped 12-ml centrifuge tubes (silylated with 2% trimethylchlorosilane (TMCS) in toluene on alternate use), 10-μl portions of a stock solution of II and sufficient volumes of stock solutions of P and metabolite I were added to yield the final concentrations shown in Table I. Following evaporation of the organic solvent at 40° C under a gentle stream of nitrogen, the residues were mixed with 1-ml portions of milk. The resulting spiked milk standards and samples were washed with single 5-ml portions of hexane to remove lipids and the aqueous phases transferred to clean tubes, taking scrupulous care to avoid transfer of interfacial material. To the washed aqueous phases were added 0.5-ml portions of 1 *M* sodium hydroxide and 5-ml portions of dichloromethane (glass-distilled) and the samples were extracted for 10 min on an inversion mixer (18 cycles per min). Following separation of the phases by centrifugation at 1875 *g* for 5 min, the milk layers were discarded and the organic phases transferred to 5-ml PTFE-capped reaction vials. Organic solvent was removed as noted above and the residues taken up in 1-ml portions of freshly prepared 5% trifluoroacetic anhydride in hexane. After mixing on a vortex shaker, the samples were heated at 60° C for 5 min to form the trifluoroacetyl derivatives of metabolite I and compound II. Following evaporation of excess derivatizing agent and solvent, the residues were taken up in 50-μl portions of glass-distilled toluene and 5-μl portions were chromatographed. GC was performed on a Varian 3700 gas chromatograph equipped with a nitrogen–phosphorus detector. The glass column (2 m × 2 mm I.D.) was packed with 3% OV-17 on Chromosorb W-HP (100–120 mesh). Flow-rates of helium carrier gas, air and hydrogen were 25 ml/min, 100 ml/min and 3.3 ml/min, respectively. Detector bias voltage was set at 4 and bead

TABLE I

STANDARD CURVE CHARACTERISTICS FOR MILK ANALYSIS OF TRENTAL AND METABOLITE I

All samples contained internal standard (II; approximately 500 ng/ml).

Concentration (ng/ml)		Normalized, integrated peak area ratios*	
Pentoxifylline	Metabolite I	Pentoxifylline	Metabolite I
10.0	10.0	0.019 (0.007)	0.022 (0.009)
20.0	20.0	0.038 (0.009)	0.044 (0.006)
51.0	51.0	0.102 (0.022)	0.113 (0.011)
102	102	0.210 (0.035)	0.228 (0.025)
255	256	0.564 (0.060)	0.587 (0.028)
511	511	1.19 (0.131)	1.19 (0.056)
1020	1020	2.41 (0.198)	2.34 (0.250)
2040	2050	4.80 (0.380)	4.88 (0.156)

*Peak area drug or metabolite divided by peak area internal standard. Mean of four determinations. Standard deviation given in parentheses.

current at 300—600, depending on bead age. Oven, injector and detector temperatures were 245°C, 300°C and 300°C, respectively. Under these conditions, chromatograms were obtained as shown in Fig. 2. Chromatograms of blank milk samples were devoid of interferences at retention times corresponding to P, metabolite I and compound II.

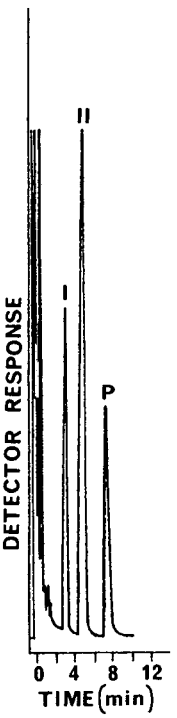

Fig. 2. Gas chromatogram of pentoxifylline (P; equivalent to 26 ng), metabolite I (I; equivalent to 26 ng as trifluoroacetate) and internal standard (II; equivalent to 52 ng as trifluoroacetate) extracted from human breast milk. Chromatographic conditions are as described in Materials and methods.

Metabolites IV and V

The assay method for P-carboxylic acid metabolites, IV and V, was a modification of the procedure described by Bryce [10] for the determination of these compounds in blood plasma. Standard stock solutions (100 μg/ml) of metabolites IV and V and internal standard III were prepared in the minimum amount of 0.1 *M* sodium hydroxide required for dissolution, the remainder of the required volume being water. These stock solutions were diluted with water to yield working stock solutions of 5 μg/ml (metabolites IV and V) and 2.5 μg/ml (III). Standard curve samples were prepared using serial dilutions of the working stock solutions. The following were added to 12-ml screw-capped centrifuge tubes (silylated with 2% TMCS on alternate use): 1-ml portions of milk, 0.1-ml portions of internal standard stock solution, and in the case of standard curve samples, sufficient amounts of working stock solutions of metabolites IV and V to give the final concentrations listed in Table II. Samples were washed with 5-ml portions of hexane to remove lipids and the aqueous

TABLE II

STANDARD CURVE CHARACTERISTICS FOR MILK ANALYSIS OF METABOLITES IV AND V

All samples contained internal standard (III; approximately 250 ng/ml).

Concentration (ng/ml)		Normalized, integrated peak area ratios*	
Metabolite IV	Metabolite V	Metabolite IV	Metabolite I
10.0	10.0	0.046 (0.017)	0.039 (0.008)
25.1	25.0	0 093 (0.012)	0.099 (0.017)
50.3	50.1	0.209 (0.013)	0.232 (0.062)
100	100	0.542 (0.040)	0.532 (0.042)
251	250	1.06 (0.087)	1.07 (0.092)
503	501	2.18 (0.137)	2.22 (0.146)
1000	1000	4.50 (0.434)	4.45 (0.427)
2010	2000	9.56 (1.52)	9.28 (0.996)

*Peak area metabolite divided by peak area internal standard. Mean of five determinations. Standard deviation given in parentheses.

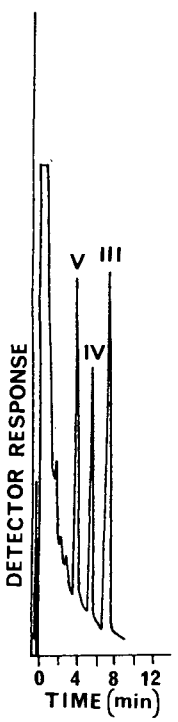

Fig. 3. Gas chromatogram of metabolite IV (IV; equivalent to 10 ng as ethyl ester), metabolite V (V; equivalent to 10 ng as ethyl ester) and internal standard (III; equivalent to 25 ng as ethyl ester) extracted from human breast milk. Chromatographic conditions are as described in Materials and methods.

phases transferred to clean tubes, taking scrupulous care to avoid transfer of interfacial material. In order to remove interfering P and metabolite I, hexane-pre-extracted milk samples were treated with 0.5-ml portions of 1 *M* sodium

hydroxide and extracted by shaking for 10 min with single 5-ml portions of dichloromethane on an inversion mixer (18 cycles per min). Following centrifugation at 1875 *g* for 5 min to separate phases, the aqueous phases were transferred to clean tubes and acidified with 1-ml portions of 1 *M* hydrochloric acid. The carboxylic acid metabolites were then extracted into 5 ml dichloromethane—isopropanol (4:1) using the technique outlined above. The organic phases were transferred to 5-ml PTFE-capped reaction vials, evaporated as above, and the metabolites were esterified with 0.1-ml portions of acidified ethanol (1 *M* hydrochloric acid in ethanol prepared by bubbling dry hydrogen chloride through ethanol and adjusting the hydrochloric acid molarity by addition of ethanol) at 60°C for 30 min. At the end of the reaction period, excess derivatizing reagent was removed by evaporation as above and the residues taken up in 50-μl portions of butyl acetate, of which 5-μl portions were injected into the chromatograph.

GC was performed as described for P and metabolite I (above). Under these conditions, chromatograms were obtained as shown in Fig. 3. Chromatograms of blank milk samples were devoid of interfering peaks at retention times corresponding to metabolites IV and V and the internal standard III.

RESULTS AND DISCUSSION

The determination of drugs and their metabolites in human breast milk presents unique challenges. Human breast milk contains large amounts of endogenous lipids [4] that can compromise both sample extraction and chromatographic separation steps. To circumvent losses in extraction efficiency of pentoxifylline and its metabolites, we pre-extracted all milk samples with hexane and scrupulously avoided contamination of organic extracts with interfacial matter from separated phases. The detector used in the GC separation step was a nitrogen-selective detector. This also favored selectivity since more than 95% of milk lipids are di- and triglycerides, sterol and sterol esters, and free fatty acids [13], all compounds that are transparent in the detection system.

TABLE III

ABSOLUTE RECOVERIES OF PENTOXIFYLLINE AND ITS METABOLITES FROM HUMAN BREAST MILK

Compound	Absolute recovery* *(%)*	*n*
Pentoxifylline	89.7 (4.75)	11
Metabolite I	86.5 (4.30)	10
Internal standard II	81.8 (2.87)	11
Metabolite IV	92.1 (16.3)	11
Metabolite V	76.4 (5.57)	12
Internal standard III	85.8 (7.34)	11

*Mean (standard deviation in parentheses) of *n* determinations, expressed as percent. Concentration of internal standard II was 517 ng/ml; concentration of internal standard III was 254 ng/ml. Concentrations of pentoxifylline and its metabolites were sampled over the concentration range 10—2000 ng/ml.

TABLE IV

EFFECT OF SODIUM HYDROXIDE ON AQUEOUS PENTOXIFYLLINE SOLUTIONS AS A FUNCTION OF TIME

From a 10-ml aqueous solution of pentoxifylline (approximately 1000 ng/ml), replicate 1-ml aliquots were removed, extracted and chromatographed. To the remaining 8 ml of solution, a 4-ml portion of 1 *M* sodium hydroxide was added and the solution thoroughly mixed. At the time intervals indicated in the table, replicate 1-ml samples were withdrawn, extracted and chromatographed according to the procedure outlined in Materials and methods. Values for non-zero time samples have been corrected for dilution by base.

Time (min)	Pentoxifylline remaining at 25°C (%)	*n*	Pentoxifylline remaining at 4°C (%)	*n*
0	(100)	3	(100)	6
5	95.2	3	94.8	4
10	92.8	3	95.1	5
20	101	3	97.5	5
30	94.6	3	90.9	4

TABLE V

SUMMARY OF REGRESSION DATA

Values of correlation coefficient (*r*), slope and ordinate for lower and upper concentration ranges. Standard deviation given in parentheses. Values for concentration ranges and *n* as in Tables I and II. See text for details.

	r	Slope × 10^3	*Y*-Intercept × 10^3
Pentoxifylline			
Lower	0.9989 (0.0009)	2.08 (0.323)	−3.16
Upper	0.9997 (0.0002)	2.36 (0.173)	−26.4
Metabolite I			
Lower	0.9994 (0.0004)	2.25 (0.196)	−1.28
Upper	0.9990 (0.0016)	2.40 (0.085)	−12.0
Metabolite IV			
Lower	0.9916 (0.0025)	5.63 (0.572)	−38.7
Upper	0.9984 (0.0015)	4.76 (0.793)	−113
Metabolite V			
Lower	0.9923 (0.0044)	5.55 (0.542)	−31.5
Upper	0.9989 (0.0012)	4.63 (0.520)	−59.6

After pre-extraction with hexane, separate milk samples are extracted with dichloromethane for P and metabolite I, or dichloromethane–isopropanol (4:1) for metabolites IV and V. The assay of metabolites IV and V requires a dichloromethane pre-extraction of P and I which interfere in the GC step. No interferences from metabolites IV and V are observed in the determination of P and I because P and I are extracted at an alkaline pH which prevents contamination by the carboxylic acid metabolites. Table III contains absolute recovery values of P, its metabolites and internal standards. Extraction efficiencies for all of these compounds are acceptable [14] when the prescribed extraction schemes are used.

The possibility existed that pentoxifylline undergoes an aldol condensation

when exposed to the strong alkali of the extraction step. This could result in losses of detectable parent drug. We, therefore, tested the stability of P in sodium hydroxide at both 4°C and room temperature. The results of these experiments are shown in Table IV. As indicated, P appears to be stable for at least 20 min at both temperatures. Thus, the extraction methods reported herein can be performed without significant loss of drug.

Standard curve samples for P and metabolites I, IV and V were prepared as described in Materials and methods. Tables I and II contain standard curve behavior and Table V summarizes regression analysis data for these compounds determined in milk. Excellent correlations were obtained for the regressions of peak area ratios versus concentrations. Day-to-day variations in detector response due to detector bead aging, however, necessitated daily preparation of standard curves.

Our experience with analyses of P and its metabolites in plasma indicated a statistically significant difference (95% confidence level) between slopes of the lower range of standard curve concentrations (typically 10—100 ng/ml) and those for the upper range of standard curve concentrations (typically 100—2000 ng/ml). Thus, concentrations of P and its metabolites have been calculated from two separate regression line analyses, the first bracketing the lower concentration range, and the second bracketing the upper concentration range. The lower sensitivity limits for P and its metabolites were approximately 2 to 6 ng/ml at a signal-to-noise ratio of 5; however, the practical lower limit of detection was routinely 10 ng/ml for these compounds.

TABLE VI

ACCURACY AND PRECISION OF GC ASSAYS FOR PENTOXIFYLLINE AND ITS METABOLITES IN HUMAN BREAST MILK

Assay	Concentration range (ng/ml)	Accuracy*	*n*
Pentoxifylline	26.0—1760	96.1 (8.73)	19
Metabolite I	26.0—1750	98.9 (4.69)	19
Metabolite IV	15.1—1610	96.9 (10.2)	10
Metabolite V	15.0—1600	97.4 (8.07)	10

*Amount found/amount added × 100%; standard deviation given in parentheses.

Table VI contains percent accuracy and standard deviations for analyses of spiked milk samples containing P and its metabolites, I, IV and V. It can be concluded from these data (reflecting within-day variations) that the accuracy and precision of the GC assays are good.

In summary, the sensitivity, selectivity, accuracy and precision of the methods described herein are good, and compare favorably with the same characteristics of determinations of pentoxifylline and metabolite I in plasma described by Bryce and Burrows [9] and Chivers et al. [15]. An extended study of the stability of P and its metabolites in milk samples stored at −15°C was initiated. Preliminary data indicate that little or no loss of these compounds occurred over a period of three weeks.

ACKNOWLEDGEMENT

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DETERMINATION OF IMIPENEM (N-FORMIMIDOYL THIENAMYCIN) IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, COMPARISON WITH MICROBIOLOGICAL METHODOLOGY AND STABILITY

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SUMMARY

High-performance liquid chromatographic (HPLC) methods using ultraviolet (UV) detection have been developed for the assay of the antibiotic imipenem (N-formimidoyl thienamycin) in human plasma and urine. A reversed-phase analytical column is employed in the plasma assay method and a cation-exchange column is used in the urine assay method. Both methods use borate buffer in the mobile phase. The method of preparation of human fluid samples for HPLC injection has been optimized with respect to the stability of imipenem in aqueous buffers, in morpholine buffer—ethylene glycol stabilizer, and in urine and plasma. Preparation of the samples before injection into the HPLC systems involves deproteination/filtration of the plasma/urine samples. The open lactam metabolite and the coadministered dehydropeptidase inhibitor, cilastatin sodium, do not interfere with the 313-nm detection of imipenem in either the plasma or the urine assay. Thienamycin, the precursor of imipenem and an impurity in imipenem formulations, is separated from the drug using both of these methods. Concentrations generated from the HPLC analysis of plasma and urine samples from two healthy volunteers compare favorably with results using a microbiological assay method. Correlation of the two methods gives $r \geqslant 0.990$ for both fluids.

INTRODUCTION

Imipenem* (N-formimidoyl thienamycin) (I) is a stable derivative of thienamycin (II), a naturally produced carbapenem antibiotic [1]. Chemical structures are shown in Fig. 1. It has an unusually high degree of activity against a broad spectrum of bacteria [2, 3].

*The Merck Sharp & Dohme Research Laboratories corporate identification code for imipenem is MK0787.

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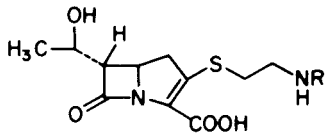
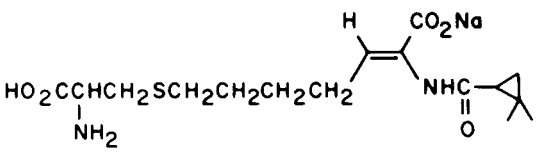


I. Imipenem R = CH=NH

II. Thienamycin R = H



III Cilastatin Sodium

Fig. 1. Chemical structures of imipenem, thienamycin and cilastatin sodium.

The urinary recovery of imipenem following intravenous administration in humans is low, typically 6—38% of the dose [4]. This is because imipenem is highly metabolized in the kidney by the renal dipeptidase, dehydropeptidase-I (DHP-I) [4, 5]. The renal metabolism occurs by cleavage of the beta-lactam. Coadministration of imipenem with the dehydropeptidase inhibitor, cilastatin sodium [(*Z*)-S-(6-carboxy-6-[([2,2-dimethyl-(*S*)-cyclopropyl]carbonyl)amino]-5-hexenyl)-L-cysteine monosodium salt*, (III)], increases the urinary recovery of the antibiotic and makes inter-subject urinary excretion more uniform at about 72% [6].

The determination of imipenem in human fluids in the presence and absence of cilastatin sodium has been achieved by a large plate, disk-diffusion, microbiological method [4, 6]. Although sensitive, the microbiological assay is not specific and does not differentiate imipenem from other antibiotics which could be coadministered. High-performance liquid chromatographic (HPLC) assay provides an alternative method for the determination of imipenem in urine and plasma. HPLC analysis is sensitive, rapid and specific, permitting the separation of imipenem from thienamycin, cilastatin sodium and the open lactam form of the drug. The HPLC methods used involve reversed-phase chromatography for plasma and cation-exchange chromatography for urine, both using UV detection at 313 nm. The UV $(H_2O)\lambda_{max}$ of imipenem is 298 nm [1]. Good agreement between the microbiological and the HPLC assays would indicate the absence of significant quantities of active metabolites or precursor.

*The Merck Sharp & Dohme Research Laboratories corporate identification code for cilastatin sodium MK0791.

EXPERIMENTAL

Materials and reagents

HPLC-grade methanol used in the mobile phase was purchased from Bodman Chemical (Burdick & Jackson Labs.). Boric acid, ethylene glycol, citric acid, sodium phosphate dibasic, sodium phosphate monobasic and anhydrous sodium carbonate were obtained from Fisher Scientific. Sodium bicarbonate was obtained from J.T. Baker. 4-Morpholinepropanesulfonic acid (MOPS), 4-morpholineethanesulfonic acid (MES), serotonin creatinine sulfate monohydrate and 5-methoxyindole-3-acetic acid (MIAA) were procured from Aldrich. Sodium hydroxide (pellets) was purchased from Mallinkrodt, and triethylamine from Pierce. Amicon's Centriflo ultrafiltration membrane cones (CF50A) were used for deproteination of urine and plasma samples, and Milli-Q water was supplied from the Millipore Reagent Water System.

Imipenem, thienamycin and cilastatin sodium were supplied by Merck Sharp and Dohme Research Labs. (Rahway, NJ, U.S.A.).

Apparatus

The HPLC system consisted of a fixed-wavelength absorbance detector (Waters Assoc., Model 440) using a 313-nm filter for the detection of imipenem. A variable-wavelength detector (Schoeffel Instruments Spectroflow monitor SF770 and monochromator GM770) was used to determine the UV absorption of the open ring product of imipenem at 214 nm. The solvent delivery system used was a double-reciprocating pump (Waters Model 6000A). Injections were made with an automated injector (Waters Intelligent Sample Processor, WISP Model 710A). A microprocessor (Waters System Controller 720) provided automated control of the pump and the injector. Integration of peak area or height detected at 313 nm was performed by a computing integrator (Spectra Physics Model SP4100). A chart recorder (Houston Instruments Omniscribe) was used to record the chromatographic profile detected at 214 nm.

Centrifugation was carried out in a refrigerated table top centrifuge (International Equipment Company Centra 7R).

Chromatographic conditions

Plasma assay was performed on a reversed-phase, 5-μm ODS Hypersil, 100 mm × 5 mm I.D., analytical column (Shandon Southern Instruments). A 30 mm × 4.6 mm I.D. Brownlee 10-μm LiChrosorb C_{18} guard column cartridge (Rainin Instrument Co.) fitted into an MPLC guard holder (Rainin) was placed in-line before the analytical column. Pump flow-rate was set at 4 ml/min, injection volume was 10 μl, and run time was 6.0 min. Integration parameters were set at 0.5 cm/min for chart speed and 8 mV full scale for attenuation.

Chromatographic conditions for the urine assay consisted of a 250 mm × 4.6 mm I.D. analytical column packed with Altex 10-μm Ultrasil CX cation-exchange packing (Rainin). A 30 mm × 4.6 mm I.D. Brownlee 10-μm Partisil SCX guard column cartridge (Rainin) was fitted into an MPLC guard holder and placed in-line prior to the analytical column. A 250 mm × 4.6 mm I.D.

stainless-steel preconditioning column dry packed with 37—53 μm silica particles (Whatman Chemical Separation) was used to saturate the mobile phase with silica and was fitted between the pump and the injector. (The pH of the buffer used in the urine assay (pH 7.5) borders on the maximum suggested pH for buffers used with silica-based column packings. A silica preconditioning column is used to saturate the mobile phase and prevent breakdown of the silica backbone of the cation-exchange packing in the analytical column.) To protect the automated injector from particulates from the preconditoning silica, a 2-μm Hastelloy C filter (Scientific Systems) was inserted between the silica column and the injector. Mobile phase flow-rate was 2 ml/min, injection volume was 30 μl and run time was 10 min. Chart speed of the integrator was 0.5 cm/min and attenuation was 16 mV full scale. The recorder chart speed was set at 0.5 cm/min.

Prepared solutions

Stabilizing solutions. (Imipenem is most stable at neutral pH. MES (pK_a = 6.15) and MOPS (pK_a = 7.2) buffers are used to bring the pH of plasma and urine, respectively, to approximately 6.8. Ethylene glycol is essential to the stability of imipenem for long-term storage because it disrupts the organized water structure that leads to breakdown of beta-lactams in the frozen state.) Plasma stabilizer was prepared by dissolving 194.2 g of MES in 700 ml Milli-Q water, adjusting the pH to 6.0 with 1.0 *M* sodium hydroxide and diluting to 1 l with Milli-Q water. The MES buffer was then diluted 1:1 with ethylene glycol. Similarly, 209.3 g of MOPS were dissolved in 700 ml Milli-Q water, adjusted to pH 6.8, diluted to 1 l, and diluted again 1:1 in ethylene glycol. This solution was used as the urine stabilizer.

Mobile phase. Mobile phase for the plasma assay was 0.2 *M* boric acid, adjusted to pH 7.2 with 1.0 *M* sodium hydroxide. The solution was degassed by vacuum filtering through a 0.45-μm membrane filter (Waters Assoc.).

The urine assay mobile phase was prepared by mixing 52 ml methanol with 2 l of 0.5 *M* boric acid containing 130 μl of triethylamine and adjusting to pH 7.5 with 1.0 *M* sodium hydroxide. Degassing was carried out as described above for the plasma assay mobile phase.

Standard solutions

Urine and plasma imipenem calibration standards in the range of 0.30—72 μg/ml for plasma and 1—100 μg/ml for urine were prepared daily from stock solutions of the antibiotic in stabilizer. Standards were prepared by spiking the appropriate volume of stock solution into 500 μl of biological fluid mixed with 500 μl of appropriate stabilizer.

Analytical procedure

Samples were thawed and prepared for HPLC analysis as follows. To 1.0 ml of stabilized plasma sample (500 μl plasma in 500 μl stabilizer) was added the internal standard MIAA to a final concentration of 168 μg/ml. Serotonin (136 μg/ml final concentration) was used as the internal standard in 1 ml stabilized urine. The same concentrations of each internal standard were also added to urine and plasma standards. Samples and standards were mixed on a

Vortex mixer and deproteinated through Amicon CF50A ultrafilters by centrifugation at 1500 *g* (2700 rpm) for 10 min at 5°C. The filtrates were analyzed by direct injection into the HPLC system. Drug concentrations were calculated from the linear regression of the standards using peak height response ratio (imipenem to internal standards) vs. concentration of imipenem. Urine samples with imipenem concentrations of more than 100 μg/ml were diluted appropriately with a 1:1 mixture of urine and its stabilizer. All plasma sample concentrations fell below the 75 μg/ml upper limit of the standard curve.

Study protocol

Urine and plasma samples from an imipenem clinical study which were analyzed by a microbiological assay [4] were re-analyzed using the HPLC method. All samples were assayed by the microbiological method in February and March 1981. Plasma samples were analyzed by HPLC in April 1981. The HPLC analysis of urine samples was completed in May 1983. All samples had been stored at −70°C to −80°C in the interim period.

The samples were from an open study conducted to determine the tolerance, safety and pharmacokinetic properties of single doses of imipenem per se and imipenem coadministered with a dehydropeptidase inhibitor (cilastatin sodium). Subjects were given an intravenous infusion of 500 mg of imipenem in treatment 1, and 500 mg of imipenem plus 250 mg of cilastatin sodium were given in the same manner in treatment 2. There was seven-day washout period between the two treatments. Blood samples were collected at 0 (preinfusion), 20 and 40 min, and 1, 2, 4, 6 and 8 h after infusion. The blood samples were chilled immediately and centrifuged at 2–5°C; the plasma was collected. Urine collections were made at the following time intervals: 0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–8 and 8–10 h after infusion. The volumes of the urine samples were recorded. A 2-ml aliquot of both urine and plasma from each collection period was stabilized by a 1:1 dilution with the appropriate stabilizer to decrease the possibility of degradation. All samples were stored at −70° to −80°C until just prior to analysis.

Stability

The short-term stability of imipenem in plasma and urine at 24°C was investigated using the following four preparation methods:

(a) Unstabilized, unfiltered sample. A stock solution of 2 mg/ml imipenem was spiked into a blank pool of plasma to a concentration of 100 μg/ml. A 500-μl aliquot of plasma was removed from the pool, and internal standard and stabilizer were added to each sample. The sample was mixed on a Vortex mixer, filtered (ultrafiltration), and peak height ratio was determined by HPLC analysis. This procedure was repeated every hour up to 5 h.

(b) Stabilized, unfiltered sample. A second pool was prepared by adding stabilizer (1:1) to a portion of original pool. The final concentration was 50 μg/ml. Every hour for 5 h 1 ml of stabilized plasma was removed from the pool and prepared and analyzed as indicated in *a*.

(c) Unstabilized, filtered (deproteinated) sample. Imipenem was spiked into 1 ml of plasma to a concentration of 50 μg/ml. After the addition of internal

standard, the sample was filtered at zero hour of the study and analyzed. The same sample was then analyzed hourly for 5 h.

(d) Stabilized, filtered (deproteinated) sample. Imipenem was spiked into 1 ml of stabilized plasma to a concentration of 50 μg/ml. The sample was then prepared and analyzed as indicated in *c*.

All samples in this study were prepared in duplicate. The stability of imipenem in urine was determined similarly to that described for plasma substituting the urine stabilizer for the plasma stabilizer where necessary.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms of blank and spiked plasma and urine matrices are shown in Figs. 2 and 3, respectively. The detection of imipenem in urine and plasma subject samples has been determined to be specific. No background peaks which would interfere with imipenem or internal standards are present. The stabilizers also do not interfere. The renal dehydropeptidase inhibitor, cilastatin sodium, does not absorb at 313 nm and does not appear in the chromatograms under the assay conditions used for plasma and urine.

An investigation to identify the metabolites and degradation products of imipenem is currently being conducted. The major metabolite, formed by hydrolysis or enzymatic reaction is known to be the open lactam [5]. It has been established that the hydrolysis product can be formed in dilute acid [5].

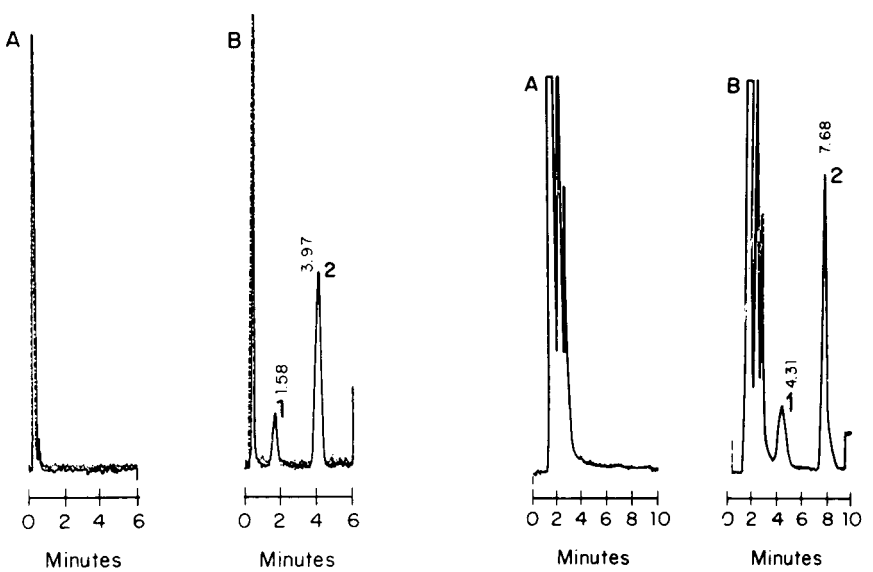


Fig. 2. Chromatograms of (A) a blank plasma—stabilizer matrix and (B) of imipenem, 7.2 μg/ml (1) and internal standard, 168 μg/ml (2) in stabilized plasma. The injection volume was 10 μl. Chromatographic conditions are given in the text.

Fig. 3. Chromatograms of (A) a blank urine—stabilizer matrix and (B) of imipenem, 10 μg/ml (1) and internal standard, 136 μg/ml (2) in stabilized urine. The injection volume was 30 μl. Chromatographic conditions are given in the text.

Therefore, to determine specificity, imipenem (1.5 m*M*) was reacted in citrate—phosphate buffer at pH 3.1. The same concentration was also reacted in 0.05 *M* carbonate buffer, pH 10.8 and 0.1 *M* phosphate buffer, pH 7.25. The degradation of imipenem and the formation of the reaction products were followed simultaneously by HPLC analysis with detection at 313 nm and 214 nm, respectively, as described for urine analysis using a flow-rate of 2.5 ml/min (Fig. 4). A schematic diagram showing the possible reaction sequence is given in Fig. 5. None of the reaction products, A—E, interfere with the detection of imipenem. Product II, which is detected at both 313 nm and 214 nm (as is imipenem) and is present at time zero of the study, has been identified as thienamycin by HPLC analysis of pure thienamycin. Thienamycin is an impurity found in

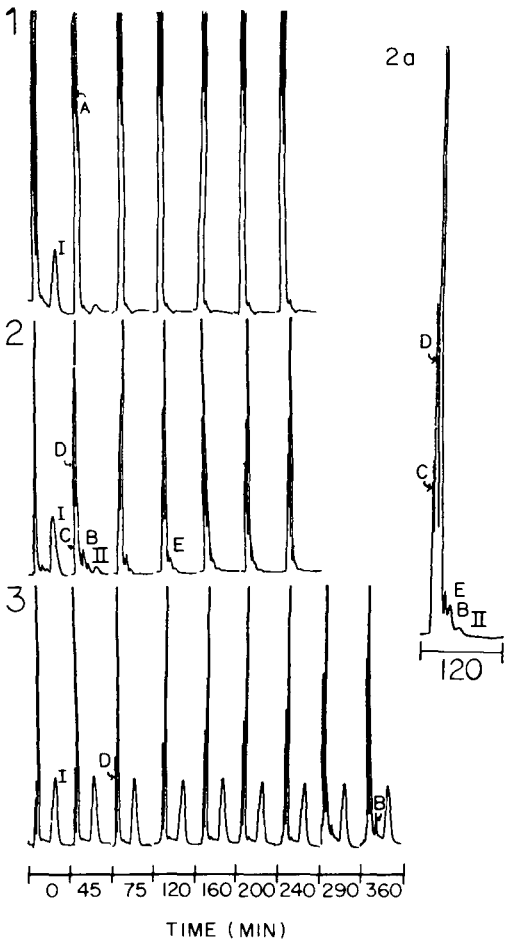
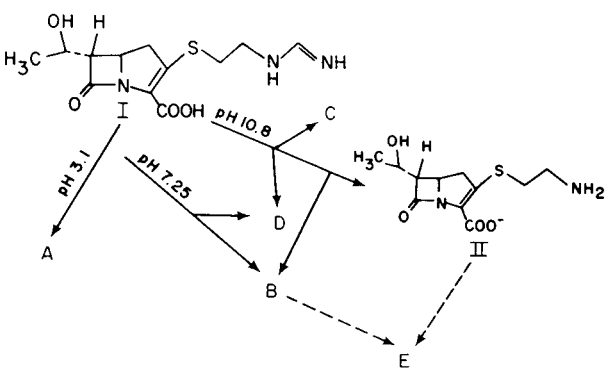


Fig. 4. Reaction of imipenem in (1) citrate—phosphate buffer, pH 3.1, (2) carbonate—bicarbonate buffer, pH 10.8 over a 4-h period, and (3) phosphate buffer, pH 7.25 over 6 h. Inset (2a) is an enlargement of the chromatogram of the reaction products of imipenem in carbonate—bicarbonate after 2 h of reaction time. Imipenem and reaction products are detected at 214 nm after separation on an Altex Ultrasil CX column using the chromatographic conditions described in the text. I = imipenem; II = thienamycin and all other products; A—E = unknowns.

imipenem formulations and this may account for its presence in all solutions at zero hour (Fig. 6). The concentration of thienamycin increases in basic buffer after 45 min of reaction time then slowly degrades.

As indicated in Fig. 5, products A—D and II appear to be directly related to the degradation of imipenem. Product E does not appear in the chromatograms of Fig. 4 until imipenem is no longer detected and as products II and B begin to decrease. It may be possible that product E is a degradation product of II or B or both.



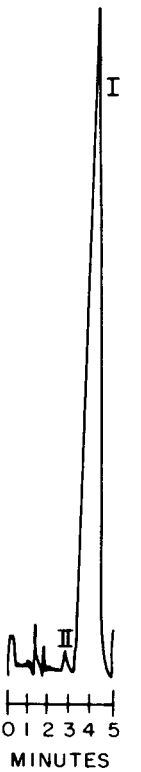


Fig. 5. Possible reaction sequence of imipenem under acidic, neutral and basic conditions. Refer to Fig. 4 for chromatographic peaks of products A—E.

Fig. 6. Chromatogram of imipenem (I) in phosphate buffer detected at 313 nm at zero hour of the reaction study. Thienamycin (II) is also detected.

There appears to be no common products in the acid- and base-catalyzed reaction mixtures. The base-catalyzed reaction of imipenem yields multiple peaks (products B—D and II) and the acid treatment seems to yield only one product (A). The neutral reaction mixture yields two by-products (products D and B) with retention times concurrent with two of the base catalyzed products.

Linearity and reproducibility

The linearity and reproducibility of peak height ratio response with respect to concentration of imipenem was determined by multiple replicate analysis at

each standard concentration. The results were analyzed by linear regression. Both assays were found to be linear, with correlation coefficients (r) of greater than 0.999. Over the linear concentration range, the average coefficient of variation (C.V.) was 4.98 ± 2.3% (n = 3 or 4) for the plasma assay and 6.57 ± 3.0% (n = 5 or 6) for the urine assay.

Sensitivity

The limit of detection of the assay was determined as the lowest detectable concentration above background with a variance of less than 10%. The sensitivity limit of the plasma assay was 0.3 μg/ml (C.V. = 6%) using a 75-μl injection volume, while that of the urine assay was 1.0 μg/ml (C.V. = 9.8%) using a 75-μl injection volume, while that of the urine assay was 1.0 μg/ml (C.V. = 9.8%) using a 30-μl injection volume.

Recovery

The losses of imipenem and internal standard due to adsorption to the ultrafilter or degradation during sample processing were determined by comparing peak heights of authentic standards in stabilizer per se vs. stabilized plasma and stabilized urine standards processed as described. Results from this recovery study are given in Table I.

TABLE I

RECOVERY OF IMIPENEM AND INTERNAL STANDARD FROM PLASMA AND URINE

Compound	Concentration (μg/ml)	Recovery (%)	Standard deviation (%)	n
Plasma				
Imipenem	20	48.7	3.29	5
	80	53.1	4.43	5
Internal standard	136	45.9	6.16	10
Urine				
Imipenem	20	83.8	3.66	5
	80	87.4	4.16	5
Internal standard	136	70.3	2.90	10

Stability

The storage of plasma and urine samples containing imipenem presents stability problems arising from the accelerated inactivation of the antibiotic in the frozen state at conventional temperatures, e.g. −10°C to −20°C, and from its sensitivity to pH extremes both above and below neutrality [7]. Accelerated inactivation in the frozen state has also been reported for penicillins and could be minimized by inclusion of high concentrations of agents disrupting organized water structure (e.g. glycerol or ethylene glycol) [8]. The stability of imipenem has been characterized in a variety of buffers [9, 10]. Stability of imipenem in pH 5—8, non-nucleophilic, inert buffers depends upon the concentration of the drug and upon the concentration of the buffer. Increasing the concentration of a phosphate buffer, pH 7.2 from 0.5 to 1.0 M decreases the half-life of imipenem by a factor of two [9]. The most

effective stabilizing buffers for storage of thienamycin are the zwitterionic substituted morpholines such as 2-(N-morpholino)-ethanesulfonate (MES) and 3-(N-morpholino)propanesulfonate (MOPS), probably because they do not promote cleavage of the beta-lactam [10]. These buffers together with 25% ethylene glycol were used to investigate the stability of imipenem in urine and plasma and to determine the best method for handling and storing the drug.

Aqueous buffer

The N-formimidoyl derivative of thienamycin was developed to circumvent the bimolecular reaction which causes the instability of thienamycin in aqueous solutions. Aminolysis occurs when one molecule of thienamycin undergoes beta-lactam cleavage by the primary amine of another [10]. Beta-lactam cleavage in both thienamycin and imipenem is also promoted by certain aqueous buffers like borate and concentrated (1 *M*) phosphate buffers [9, 10]. Compared to other beta-lactam antibiotics, imipenem has been found to be more susceptible to breakdown in aqueous buffers [9]. Imipenem is less stable than the penicillins and the cephalosporins (which are stable) in phosphate buffer. In dilute phosphate buffer the stability of imipenem is comparable to that found in morpholine buffer—ethylene glycol stabilizer when monitored over a 5-h period at room temperature (Fig. 7). Imipenem reacts more extensively in borate buffer. The degradation of imipenem in 0.2 *M* borate buffer, pH 7.0 is first order with a half-life of 1.82 h.

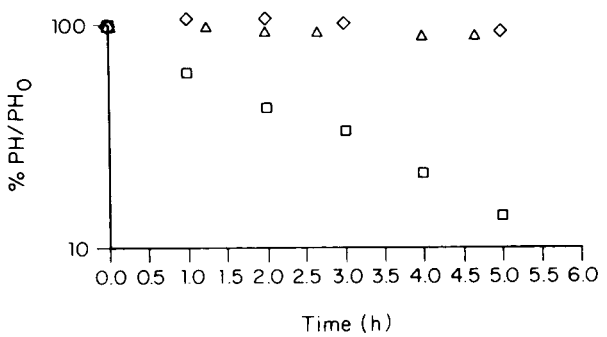


Fig. 7. Stability of 50 μg/ml imipenem in 0.2 *M* borate buffer, pH 7.0 (□), 0.1 *M* phosphate buffer, pH 7.25 (△), and 1.0 *M* MES—ethylene glycol, pH 6.0 (◇) at 24°C.

Short-term plasma and urine stability

The semilogarithmic plot of time vs. percentage of unreacted imipenem (Fig. 8) for deproteinated unstabilized imipenem plasma standards shows first-order breakdown of the drug at 24°C with a half-life of 1.65 h and an 84.1% loss over 5 h. Stabilization of deproteinated plasma standards more than doubles the stability of the drug with only 38.3% lost in 5 h. The results indicate that in handling imipenem plasma samples the greatest degree of stability is achieved by stabilizing the samples upon collection and only deproteinating immediately prior to analysis. Only 5.9% of the drug is lost over 5 h under these optimum conditions, almost fifteen times less than in the case of deproteinated, unstabilized samples.

As in the case of plasma, stabilizing the urine samples and filtering immediate-

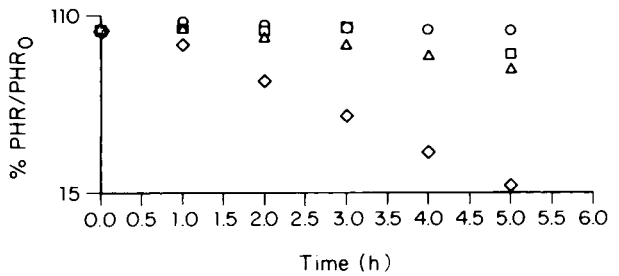


Fig. 8. Stability of imipenem in plasma (□), stabilized plasma (○), deproteinated plasma (◇), and stabilized, deproteinated plasma (△) at 24° C.

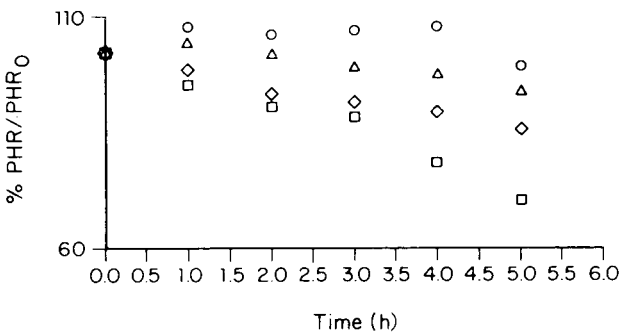


Fig. 9. Stability of imipenem in urine (□), stabilized urine (○), deproteinated urine (◇), and stabilized, deproteinated urine (△), at 24° C.

ly prior to analysis is the most reliable method of handling imipenem samples since only 3.7% of the drug was lost over a 5-h period (Fig. 9). This compared to 32.0% lost from unstabilized urine after 5 h at 24° C. Though there is loss of imipenem from the unstabilized, deproteinated standards in urine, the removal of high-molecular-weight molecules from urine affects imipenem stability in unstabilized urine less adversely than in unstabilized plasma. Only 18.2% of imipenem is lost in urine in this case as compared to 84.1% in plasma. Urine pH was slightly acidic (pH 5.3). It has been found that imipenem breakdown increases as urine pH decreases [4].

Long-term stability

A long-term stability study of imipenem in urine was conducted by storing spiked stabilized urine quality control standards at −70° C and −20° C. The low concentration standard was spiked to contain 19.8 μg/ml while the high concentration standard was spiked to contain 76.9 μg/ml. Results of the analysis of these standards over a 37-day interval are given in Table II. The interassay C.V. for the low concentration standard was 2.88% at −70° C and 7.18% at −20° C. For the high concentration standard the difference in the C.V. value was not as great, i.e. 6.22% at −70° C and 9.62% at −20° C.

The stability of imipenem in stabilized plasma over a 42-day period showed no significant difference between storage at −20° C and storage at −70° C. The mean concentration of a 10 μg/ml imipenem control standard was 9.85 μg/ml (C.V. = 2.59%) at −70° C and 9.82 μg/ml (C.V. = 3.38%) at −20° C. The 40

TABLE II

COMPARISON OF IMIPENEM STABILITY IN STABILIZED URINE AFTER STORAGE AT −70° C AND −20° C

Day	−70° C		−20° C	
	19.8 μg/ml	76.9 μg/ml	19.8 μg/ml	76.9 μg/ml
0	18.2	75.0	18.2	75.0
2	18.5	71.6	17.5	67.9
4	19.6	81.8	17.9	72.9
22	19.1	71.3	15.2	58.0
37	18.8	70.8	18.1	67.7
Mean	18.8	74.1	17.4	68.3

μg/ml control standard stored at −70° C had a mean concentration of 40.0 μg/ml (C.V. = 6.38%). When stored at −20° C, the mean concentration was 38.3 μg/ml (C.V. = 3.00%).

It is recommended, however, that urine and plasma imipenem samples be stabilized and stored at −70° C to −80° C over the long term.

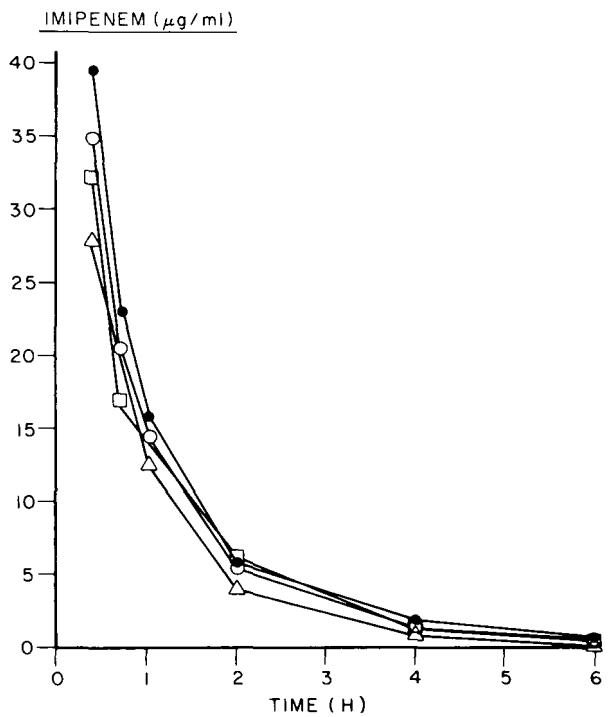


Fig. 10. Plasma concentration of imipenem after intravenous administration of 500 mg of imipenem to subject 1B (△) and subject 5B (○) or of 500 mg of imipenem combined with 250 mg of cilastatin sodium administered to subject 1B (□) and subject 5B (●).

Clinical study assay results

Plasma concentrations of imipenem. The plasma concentration—time curve in Fig. 10 shows the elimination of imipenem from the plasma of two subjects after intravenous infusion of imipenem and imipenem coadministered with cilastatin sodium. Plasma concentrations of the drug after infusion of the imipenem—cilastatin sodium regimen were slightly higher than those following imipenem alone.

Renal excretion of imipenem. The recovery of imipenem from subject urine after administration of imipenem alone is low (Table III). Only 6.45% of the dose is recovered from subject 1B and 20.3% from subject 5B. A dramatic increase in urinary recovery results from the coadministration of cilastatin sodium. The dehydropeptidase inhibitor increased the urinary recovery in both subjects to about 70%.

TABLE III

URINARY EXCRETION OF IMIPENEM ADMINISTERED ALONE (TREATMENT 1) AND WITH CILASTATIN SODIUM (TREATMENT 2)

Collection period (h)	Imipenem (mg)			
	Treatment 1		Treatment 2	
	Subject 1B*	Subject 5B**	Subject 1B***	Subject 5B§
0—1	19.5	54.8	182	168
1—2	5.43	22.5	67.9	59.5
2—3	2.23	9.48	31.7	45.2
3—4	1.35	4.48	11.3	15.3
4—5	0.362	1.83	4.68	6.24
5—6	0.277	0.987	1.92	3.85
6—8	0.355	0.954	1.00	1.39
8—10	0	0.180	0.033	0.492
Total	29.5	95.2	300	300

*Imipenem dose = 457.2 mg.
**Imipenem dose = 469.0 mg.
***Imipenem dose = 422.3 mg (+250 mg cilastatin sodium).
§Imipenem dose = 463.3 mg (+250 mg cilastatin sodium).

Comparison of microbiological and HPLC methods

Good correlation was obtained in comparing the HPLC method to the microbiological method for the analysis of imipenem in urine and plasma samples (Figs. 11 and 12). The linear regression of HPLC results vs. microbiological results gave a correlation coefficient (r) of 0.990 (slope = 1.05) for the plasma assay. The r value for the urine assay was 0.997 (slope = 0.995) over the whole range of values and 0.995 (slope = 1.15) for the lower concentrations (0—70 μg/ml). The close correlation of the two assays indicates that the microbiological assay is specific for imipenem with respect to the HPLC conditions discussed in the text. This suggests that interference due to thienamycin in subject samples is insignificant.

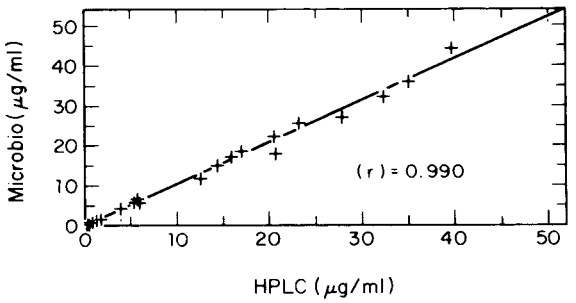


Fig. 11. Comparison of HPLC and microbiological methods for the determination of imipenem in plasma. Individual measurements were plotted for 22 samples.

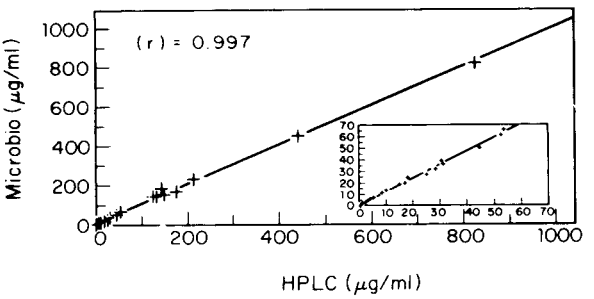


Fig. 12. Comparison of HPLC and microbiological methods for the determination of imipenem in urine. Individual measurements were plotted for 32 samples. The insert is an enlargement of the 0—70 μg/ml area of the correlation plot.

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DIGOXIN AND METABOLITES IN URINE: A DERIVATIZATION—HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD CAPABLE OF QUANTITATING INDIVIDUAL EPIMERS OF DIHYDRODIGOXIN

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of digoxigenin, digoxigenin monodigitoxoside, digoxigenin bis-digitoxoside, digoxin, and dihydrodigoxin as the 3,5-dinitrobenzoyl esters. The method is applied to a 10 ml urine sample by adding digitoxigenin as internal standard, extracting with methylene chloride, derivatizing with 3,5-dinitrobenzoyl chloride in pyridine, chromatographing with a normal-phase system and detecting at 254 nm. Derivatized digoxigenin, digoxigenin mono- and bis-digitoxoside, and digoxin each yielded one symmetrical peak with the limit of sensitivity of the method being approximately 100 ng/ml. Analysis of a commercially obtained sample of dihydrodigoxin resulted in two well-separated, symmetrical peaks that represent the two epimers of derivatized dihydrodigoxin. Data indicate rapid and complete esterification of all primary and secondary alcohol moieties in the various molecules and the derivatives are shown to be stable in chloroform for at least four days. The procedure appears to be suitable for metabolic investigations and as a prototype for future analytical developments.

INTRODUCTION

The known metabolites [1—4] of digoxin (D3) shown in Scheme 1 include digoxigenin (D0), digoxigenin monodigitoxoside (D1), digoxigenin bis-digitoxoside (D2) and dihydrodigoxin (DHD3). The importance of metabolism as an elimination pathway for D3 is underscored by the results of Lukas [5], who used a specific double isotope dilution derivative method and found only 21—55% of an oral dose of digoxin excreted unchanged in urine and feces. Although D0, D1 and D2 usually account for less than 10% of overall urinary recovery, the percentage excreted as DHD3 has been found to vary widely. Dihydrodigoxigenin, the aglycone of DHD3 , was originally detected in the

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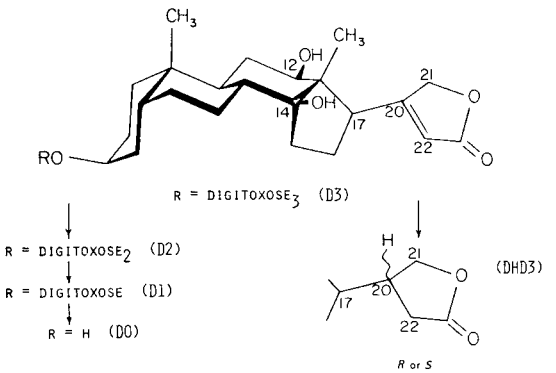

Scheme 1. Abbreviation scheme for digoxin and metabolites.

urine of a patient requiring unusually high doses of D3 [4]. This was followed by the discovery of DHD3 in human plasma [6]. The percentage of the total glycoside isolated as DHD3 has been found to vary widely between patients. Clark and Kalman [3] surveyed 50 patients and found a range of 1—47% of the total glycosides in the methylene chloride extract of urine present as DHD3. Peters and coworkers [7, 8], who investigated 100 patients receiving D3, reported a range of 2—52% of methylene chloride extractable drug plus metabolites present as reduced metabolites, with 53 subjects having over 10% and seven subjects having over 35% as reduced metabolites. This wide inter-subject variability in extent of formation of dihydro metabolites appears to be due to variability in the intestinal microbial flora that are responsible for forming the reduced metabolites [9, 10]. Lindenbaum et al. [10] estimate that about 10% of digoxin patients are substantial dihydro metabolite formers (i.e. > 40% of urinary glycoside excretion attributed to dihydro metabolites).

The major analytical methods that have the selectivity and sensitivity for D3 and its metabolites are gas—liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). A GLC method [3, 6, 11, 12] has been developed which separates reduced metabolites from unreduced metabolites and is sufficiently sensitive for serum or urine samples. However, during the derivatization procedure D3 and DHD3 are converted to the corresponding derivatized aglycone. Recently, Heftmann and Hunter [13] reviewed the HPLC methods for steroids. Both reversed-phase [14—17] and normal-phase [14, 18—20] systems have been reported for digitalis compounds. Excellent specificity was achieved for D0, D1, D2, and D3; however, the molar absorptivities of D3 and its metabolites were inadequate for determinations in biological fluids. The lack of sensitivity has been overcome either by using tritiated digoxin [14, 17] or by collecting appropriate fractions and analyzing by radioimmunoassay [20—22]. The assay of D0, D1, D2, and D3 using derivatization with the ultraviolet chromophore 4-nitrobenzoyl chloride and normal phase HPLC has been reported [23, 24]. There has been no report of the application of this derivatization procedure to dihydro metabolites of digoxin or to determinations in biological samples.

Reduction of the 20,22-unsaturated lactone ring of D3 introduces a center of asymmetry at the 20 position. Brown and Wright [25] reported the separa-

tion and isolation of the epimers of dihydrodigoxigenin (DHD0); however, they were unsuccessful in the separation and isolation of the epimers of DHD3. Watson et al. [6] reported that their GLC method did not separate DHD0 into two peaks. However, quantitation of the sum of the two DHD3 epimers in biological fluids has recently been achieved using radiolabeled drug and either DEAE-Sephadex column chromatography [26], HPLC [14] or a combination of column and thin-layer chromatographic procedures [27]. There have been no methods reported to date that are capable of separating the individual epimers.

This paper describes an HPLC method for the separation and quantitation of D3 and its metabolites and the application of the method to human urine samples. The most significant step in the methodology is the derivatization of D3 and its metabolites with 3,5-dinitrobenzoyl chloride, which facilitates separation and detection. The advantage of the method is the separation, detection, and quantitation of individual epimers of dihydro metabolites without use of radiolabeled drug.

EXPERIMENTAL

Materials

Hexane, methylene chloride, acetonitrile, pyridine, chloroform, and 2-propanol were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). D0, D1, D2, D3, DHD3, and digitoxigenin (DT0) were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The catalyst, 4-dimethylaminopyridine (Purum grade; Fluka, Buchs, Switzerland), was used as supplied, and the derivatizing agent, 3,5-dinitrobenzoyl chloride (DNBCl, Purum grade; Fluka), was recrystallized from petroleum ether and stored in a vacuum desiccator. All other chemicals and reagents were analytical grade or better.

Drug standards

Stock solutions containing D0, D1, D2, and D3 were prepared in 2-propanol at concentrations of 2.5, 5, 10, 12.5, 20, and 25 μg/ml. The internal standard, DT0, 20 μg/ml, was prepared in methylene chloride. The derivatizing agent (DNBCl) was prepared daily by dissolving in pyridine (85 mg/ml) with gentle warming.

Glassware

Glass culture tubes with PTFE-lined screw caps were used for the extraction and derivatization procedures (Corning, Corning, NY, U.S.A.). All glassware was soaked for 24 h in sulfuric acid—nitric acid (4:1), washed, and silanized for 2 min in a 1% solution of Dri Film (Pierce, Rockford, IL, U.S.A.) in toluene. After washing, the glassware was dried in an oven, and glassware for the derivatization procedure was stored in a vacuum desiccator.

Instrumentation and chromatographic conditions

A Model 5000 high-performance liquid chromatograph was equipped with a Model 960 ultraviolet (254 nm) detector (Tracor, Austin, TX, U.S.A.), and a

Rheodyne Model 7105 injection valve containing a 175 μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) Detector output was recorded on a Tracor Model T-11 recorder. A 100 μl aliquot of the derivatized sample was chromatographed at room temperature on a Partisil 10 column (25 cm × 4.6 mm I.D., 10 μm average particle size; Whatman, Clifton, NJ, U.S.A.) and was eluted isocratically with hexane—methylene chloride—acetonitrile (8:3:3 to 12:3:3). The mobile phase was pumped at approximately 1.8 ml/min and the percentage of hexane incorporated into the mobile phase was varied according to the degree of separation desired.

Extraction procedure

A 0.5 ml volume of the internal standard solution and 20 ml of methylene chloride were added to a 45 ml tube containing a 10 ml urine specimen. The tubes were tightly sealed, shaken for 15 min on a mechanical shaker (Eberbach, Ann Arbor, MI, U.S.A.), centrifuged for 20 min, and the aqueous phase was removed and discarded. After the addition of 15 ml of a 5% sodium bicarbonate solution to extract components that interfere with the derivatization procedure, the tubes were recapped, shaken, centrifuged, and the aqueous phase was removed and discarded as before. The organic phase was transferred to a 12 ml tube and gently evaporated to dryness at 50°C under a stream of nitrogen (N-evap®; Organomation Associates, Northborough, MA, U.S.A.). The tubes were tightly sealed and stored at room temperature prior to derivatization.

Standard curves were prepared daily by adding 1 ml of the appropriate stock solution(s) to a 45 ml tube containing drug-free urine and internal standard, and extracting as described. Standard curves were analyzed by unweighted linear least-squares regression.

Derivatization procedure

A 200 μl volume of the derivatizing solution was added to the dried sample and the reaction was carried out for 10 min at room temperature with gentle shaking. The derivatized sample was carefully evaporated to dryness by removing the pyridine with a stream of nitrogen at 50°C. The excess derivatizing agent was hydrolyzed with 2 ml of a 5% sodium bicarbonate solution containing 2 mg/ml 4-dimethylaminopyridine. After shaking for 5 min, 1 ml of chloroform was added to solubilize the derivative and the tubes were rocked on an Aliquot Mixer (Ames, Elkhart, IN, USA). The aqueous layer was discarded and the organic phase was mixed for 2 min with 2 ml of a 5% sodium bicarbonate solution. The aqueous layer was discarded and 3 ml of a 0.05 *M* hydrochloric acid solution containing 5% sodium chloride was mixed with the organic phase for 2 min to remove any residual pyridine. After the organic layer was washed three more times with the acidic solution, the chloroform was ready for chromatographic analysis.

Extent of derivatization

Samples (1 mg) of D0, D1, D2, and D3 were carried through the derivatization procedure and the chloroform solution was evaporated to dryness at 50°C under a nitrogen stream and further dried at room temperature under high

vacuum for 1 h. A sample (200 μg) of DHD3 was derivatized and chromatographed as described except that two columns were used in series and the sample was subdivided into aliquots. The HPLC eluates corresponding to the derivatized *R* and *S* epimers [28] were collected and re-chromatographed several times. After solvent evaporation the samples were dried under vacuum at 80°C for 1 h. Each derivatized sample of D0, D1, D2, D3, *R*-DHD3 or *S*-DHD3 was dissolved in deuterated chloroform, filtered through cotton and analyzed on a Bruker HX-90 nuclear magnetic resonance (NMR) spectrometer.

Stability of derivatized compounds

Samples of DT0, D0, D1 (120 μg each), D2 (180 μg), and D3 (240 μg) were derivatized and a 175 μl aliquot of the final chloroform phase was injected onto the chromatographic column 0, 60, 120, 240, 510, 770, 1400, 2790, and 4260 min after completion of the derivatization procedure. The area of each of the chromatographic peaks was measured in triplicate using a planimeter.

Extraction efficiency

Samples of D0, D1, D2, D3, and DHD3 were added to drug-free urine and prepared according to the extraction and derivatization procedures except that the internal standard solution was added to the methylene chloride after extraction and just prior to evaporation. The extraction efficiency of each compound was calculated by comparing the peak height ratio of extracted samples to the peak height ratio of corresponding unextracted drug standards.

Precision

Drug-free urine specimens were supplemented with D0, D1, D2, and D3 at concentrations of 1 and 0.1 μg/ml and quantitated according to the extraction and derivatization procedures. Six samples at each concentration were analyzed for within-day assay variability and accuracy. In a similar manner, DHD3 was assayed at total (sum of both epimers) added concentrations of 2.5 μg/ml and 0.44 μg/ml (five replicates each).

Molar absorptivity and ratio of the two epimers of dihydrodigoxin

A 50 μg sample of DHD3 was derivatized and three 100 μl aliquots of the final chloroform phase were chromatographed separately. The peak area for each epimer was measured in triplicate using a planimeter. The peak having the larger area was *R*-DHD3 [28]. A ratio of the average area for the two peaks in each chromatogram was used to determine the ratio of *R*-DHD3 to *S*-DHD3.

The molar absorptivities of derivatized *R*-DHD3 and derivatized *S*-DHD3 were determined on a Cary 16 spectrophotometer (Cary Instruments, Monrovia, CA, U.S.A.) at concentrations of $5.58 \cdot 10^{-6}$ *M* and $5.01 \cdot 10^{-6}$ *M*, respectively. The derivatized epimers were purified as described in the previous subsection and weighed samples were dissolved in chloroform.

Application to human urine sample

The assay was used to determine digoxin and metabolites in urine from a 68-year-old female patient taking one 0.125 mg digoxin tablet daily. A column

4.6 mm × 25 cm packed with 5 μm LiChrosorb Si 60 (E. Merck, Darmstadt. F.R.G.) was used for improved efficiency together with a Model 110A pump (Altex, Berkeley, CA, U.S.A.). Other HPLC components were the same as described previously.

RESULTS

The dinitrobenzoate derivatives of DT0, D0, D1, D2 and D3 each yielded a single, symmetrical, well-separated peak upon HPLC (Fig. 1A), whereas derivatized DHD3 yielded two symmetrical, well-separated peaks (Fig. 1B). Other research has shown that these two components of DHD3 are epimers having either the *R* (major) or *S* (minor) configuration at the C-20 position in the lactone moiety [28, 29]. Data on the capacity factor for each peak (DT0 = 4.6, D0 = 7.2, D1 = 9.2, D2 = 12.6, D3 = 16.9, *S*-DHD3 = 16.7, *R*-DHD3 = 21.0, using the 3:1:1 hexane—methylene chloride—acetonitrile mobile phase) together with the data in Fig. 1 indicate that the only incompletely resolved components are the derivatives of D3 and *S*-DHD3.

Reaction of DT0, D0, D1, D2, D3 and DHD3 with an excess of DNBCl in the presence of pyridine was carried out over times ranging from 3 to 60 min

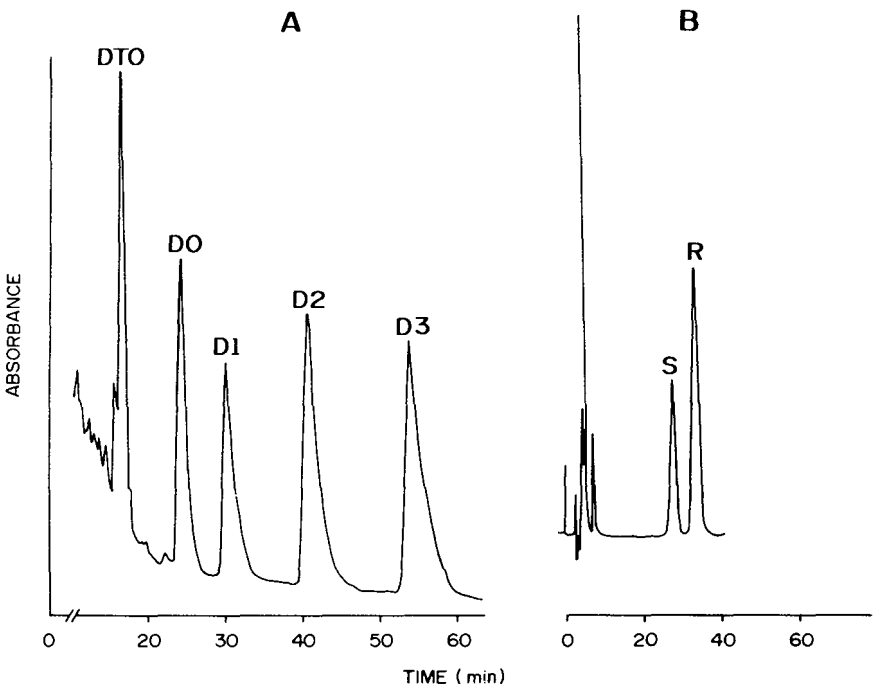

Fig. 1. (A) Representative chromatogram of 3,5-dinitrobenzoate derivatives of the internal standard digitoxigenin (DT0), digoxigenin (D0), digoxigenin monodigitoxoside (D1), digoxigenin bis-digitoxoside (D2), and digoxin (D3). Samples (1 μg/ml of each glycoside) were extracted from urine, derivatized and chromatographed using a mobile phase of hexane—methylene chloride—acetonitrile (3:1:1). (B) Representative chromatogram of 3,5-dinitrobenzoate derivative of dihydrodigoxin (DHD3). The two peaks represent the *S* (minor) and *R* (major) epimers [28, 29]. Samples were derivatized and chromatographed using a mobile phase of hexane—methylene chloride—acetonitrile (8:3:3).

and was found to be complete within 5 min, as evidenced by constancy of peak heights subsequent to this time. Comparison of the integrated NMR for aromatic protons with that for C-18 and C-19 methyl protons yielded the following approximate number of esters per molecule of digitalis compound: D0, 2.1; D1, 3.2; D2, 3.9; D3, 5.1; *S*-DHD3, 5.4; *R*-DHD3, 5.3. Thus, these data indicate that all hydroxyl moieties except one are esterified on each molecule. This is consistent with the findings of Maerten and Haberland [30] who reported five-fold acetylation of digoxin with the tertiary C-14 hydroxyl remaining unesterified. Additional evidence for the five-fold extent of derivatization of the DHD3 epimers was obtained by comparing measured molar absorptivities (254 nm) of 51,560 mol^{-1} cm^{-1} for the derivatized *S* epimer and 51,030 mol^{-1} cm^{-1} for the derivatized *R* epimer with the molar absorptivity of about 10,000 mol^{-1} cm^{-1} at 254 nm for each 3,5-dinitrobenzoate group [31]. Derivatized DT0, D0, D1, D2, and D3 were found to be stable in chloroform for at least three days, as indicated by the constancy of HPLC peak area with respect to time reported for each compound in Table I.

TABLE I

STABILITY OF THE 3,5-DINITROBENZOATE DERIVATIVES OF DT0, D0, D1, D2, AND D3

Time (min)	Peak area as a percentage of the zero time value				
	DT0	D0	D1	D2	D3
0	100	100	100	100	100
60	91	96	96.5	99.4	97.8
120	99.1	101	101	104	102
240	95.5	98.8	99	104	103
510	111	106	112	109	106
770	105	105	112	104	103
1400	100	93.3	108	107	104
2790	103	98.4	109	106	104
4260	103	99.6	104	106	105

TABLE II

EFFICIENCY OF EXTRACTION OF DIGOXIN AND THREE METABOLITES FROM URINE

Values are the mean of 5 determinations, except for digoxin which is 3. Standard deviation in parentheses. The extraction procedure is described in the text.

Concentration (μg/ml)	Extraction efficiency (%)			
	D0	D1	D2	D3
2.5	51.7 (4.4)	38.9 (3.2)	63.3 (6.1)	71.0 (7.8)
1.0	44.5 (5.3)	40.0 (4.2)	65.0 (5.4)	70.9 (6.4)
0.50	46.4 (8.9)	36.6 (3.2)	60.0 (7.4)	71.0 (11.8)
0.25	47.7 (8.0)	33.5 (9.3)	64.2 (5.8)	75.5 (6.0)
0.10	39.5 (9.8)	31.0 (6.6)	53.5 (13.3)	77.8 (43.4)
Mean	46.0 (7.9)	36.0 (6.2)	61.2 (8.4)	73.2 (17.2)

TABLE III

EFFICIENCY OF EXTRACTION OF THE EPIMERS OF DIHYDRODIGOXIN FROM URINE

Values are the mean of 3 determinations; standard deviation in parentheses. The extraction procedure is described in the text.

S-DHD3		*R*-DHD3	
Concentration (μg/ml)	Extraction efficiency (%)	Concentration (μg/ml)	Extraction efficiency (%)
0.625	70.8 (8.8)	1.88	72.4 (4.2)
0.500	70.3 (5.8)	1.50	81.0 (13.3)
0.313	69.8 (6.6)	0.938	75.2 (17.5)
0.250	70.4 (4.1)	0.750	74.7 (4.5)
0.125	78.1 (11.4)	0.375	81.3 (9.1)
		0.188	71.3 (14.5)
Mean	71.9 (7.3)		76.0 (10.3)

Standard curves of peak height ratio (compound/internal standard) vs. concentration (μg/ml) for D3 and the various metabolites were linear and the y-intercept was not significantly different from zero ($P > 0.05$) except for D1. This was consistent with observations from analyses of drug-free urine samples in which the only appreciable chromatographic interference was at the retention time of D1. Slopes and intercepts, respectively, were as follows: D3, 0.560, −0.014; D2, 0.603, 0.002; D1, 0.447, 0.043; D0, 0.657, 0.069; *R*-DHD3, 0.615, −0.015; *S*-DHD3, 0.746, 0.019. Correlation coefficients were > 0.995 except for *R*-DHD3 which was 0.986. The extraction efficiencies for digoxin and metabolites are listed in Tables II and III. There does not appear to be any concentration dependence of the single methylene chloride extraction in the concentration ranges investigated. The extraction efficiencies for the DHD3 epimers and for D2 were comparable to that for D3 whereas the efficiencies of D1 and D0 were only about one-half that of D3. Reproducibility of the method at the low extreme of the assayable concentration range was excellent with a coefficient of variation of 5% or less for D3, D2, D1, D0 and *S*-DHD3 (Table IV). Accuracy for determination of known urinary standards was excellent (< 5% mean deviation from nominal value) in concentrations near 1 μg/ml for all six compounds and in concentrations near 0.1 μg/ml for *S*-DHD3 and *R*-DHD3 (Table IV). The mean deviation for D3 at 0.1 μg/ml was 17% (Table IV).

Since the molar absorptivities for the two derivatized epimers of DHD3 were essentially the same, the relative areas under the chromatographic peaks for the two epimers can be used to determine the epimeric composition of the commercially supplied DHD3, assuming that any on-column loss is the same for both epimers. The ratio found was 3.0 to 1 (*R*/*S*).

Currently this method is being utilized to detect digoxin and dihydrodigoxin in urine from patients. Results from a patient taking oral digoxin are shown in Fig. 2. Chromatographic evidence for digoxin and the *R* epimer of dihydrodigoxin was obtained.

TABLE IV

ACCURACY AND REPRODUCIBILITY OF ASSAY FOR DIGOXIN METABOLITES IN URINE

Compound	Concentration of prepared standards (μg/ml)	Measured concentration (mean of 5 or 6 determinations)	C.V.* (%)
D3	1.0	0.954	1.2
	0.1	0.117	1.7
D2	1.0	1.021	2.4
	0.1	0.124	3.5
D1	1.0	0.985	3.8
	0.1	0.188	4.9
D0	1.0	0.981	2.9
	0.1	0.156	3.1
S-DHD3	0.625	0.620	2.6
	0.0625	0.0606	4.2
R-DHD3	1.875	1.833	4.6
	0.375	0.382	8.5

*Coefficient of variation.

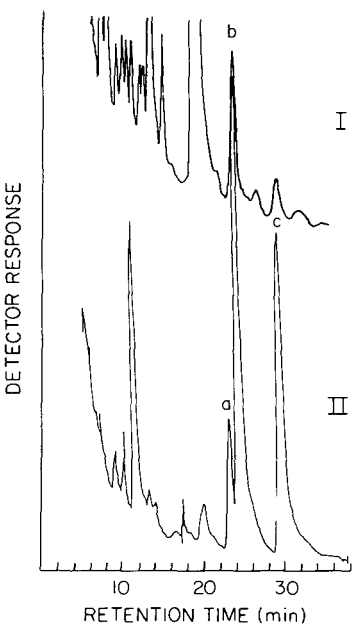

Fig. 2. Chromatogram (HPLC) of derivatized standards (II) and of an extracted and derivatized urine sample (I) from a patient receiving digoxin. Peaks identified as a, b, and c correspond to derivatized *S*-dihydrodigoxin, derivatized digoxin and derivatized *R*-dihydrodigoxin, respectively. For the standards, 10 μg of digoxin and of the dihydrodigoxin epimeric mixture were added to 10 ml of blank urine and assayed (II). The mobile phase consisted of hexane—methylene chloride—acetonitrile (3:1:1).

DISCUSSION

The importance of this analytical procedure lies more in its application to metabolic studies and to future analytical method development for digitalis compounds than in its application to routine determinations of urinary digoxin and metabolites. The procedure described has sufficient sensitivity to quantitate digoxin and the major metabolite, DHD3, in urine. Results of others [26] indicate there would be insufficient sensitivity with this procedure for determining urinary concentrations of the very minor metabolites D2, D1 and D0. More importantly, however, this is the first analytical procedure that has reported separation of the individual epimers of dihydrodigoxin. Use of a column with improved efficiency in studies in patients has also permitted separation of D3 and the minor epimer of DHD3 (Fig. 2), which was not possible under the conditions used for analytical development. Thus, this methodology provides the first opportunity to separate, isolate and identify the individual epimers of DHD3 and to determine which is/are formed in animals and man [28, 29].

The second important aspect of this method is that it points out a potential direction for development of more sensitive methods that would still retain specificity for the individual epimers of DHD3 as well as the other metabolites of digoxin. Two techniques that potentially have sufficient sensitivity for serum concentrations (subnanogram/ml range) are acylation with either fluorescent moieties or with radiolabeled derivatizing agents. If the excellent specificity shown in this investigation with the dinitrobenzoyl derivative (Fig. 1A and B) can also be achieved with derivatives having enhanced detectability, then this direction of research may yield a specific method for serum digoxin and its metabolites. The rapidity and completeness of dinitrobenzoyl derivatization, stability of the derivatives (Table I), linearity of standard curves, and reproducibility of the method (Table IV) all indicate that this type of derivatization is a promising avenue for further research.

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ROUTINE DETERMINATION OF EIGHT COMMON ANTI-EPILEPTIC DRUGS AND METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A COLUMN-SWITCHING SYSTEM FOR DIRECT INJECTION OF SERUM SAMPLES

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SUMMARY

A simple and rapid high-performance liquid chromatographic method for determining eight common anti-epileptic drugs and metabolites in serum is described. A column-switching system including one analytical column and two precolumns for sample enrichment offers the possibility of directly injecting patients' sera without any pretreatment. The two precolumns are alternately switched over to avoid time loss in analysis due to the sample washing step. The samples are flushed with dilute phosphoric acid, as the purge liquid, onto the precolumns which consist of very short cartridges (length 0.5 cm) filled with spherical ODS silica gel (particle size 30 μm). The retained substances are carried over, after purification, onto the analytical column in the same direction of flow as in the flushing step. A mixture of acetonitrile and phosphoric acid—sodium phosphate buffer solution is thereby used as solvent for the gradient elution. The separation was carried out using an analytical column, which was filled with ODS material of particle size 5 μm.

INTRODUCTION

Many authors have in the last decade been concerned with the analysis of anti-epileptic drugs (AEDs) in biological fluids. More than 80 original papers and short communications dealing with the determination of AEDs by high-performance liquid chromatography (HPLC) — valproic acid and the benzodiazepines are not included — in human plasma and serum have been published. For reasons of space not all these papers can be quoted.

In 21 papers protein precipitation using acetonitrile is described [1—4]. In only two laboratories acetone was used for protein precipitation [5, 6]. Extraction of the samples using a solvent which is immiscible with water was

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carried out eleven times with dichloromethane [7—9], twelve times with chloroform [3, 10, 11], nine times with diethyl ether [12—14], seven times with ethyl acetate [15, 16] and eight times using a mixture of different solvents [17, 18]. In ten laboratories the analysed AEDs were first adsorbed onto solid material such as activated charcoal [19, 20], kieselguhr [21] or RP-18 material [22—24] in order to elute them after purification with a suitable purge liquid. One study describes protein precipitation using perchloric acid, but recovery of the analysed AEDs was relatively low [25].

To the best of our knowledge only one of the publications dealing with the HPLC analysis of AEDs describes the direct injection of plasma [26]. However, the separation, using an anion-exchange column, took a very long time and is not comparable with the quality of present-day HPLC separations.

Today on-line column switching techniques offer a better possibility for the direct injection of serum. These have, in the last few years, increasingly been used instead of extraction procedures in sample pretreatment of biological fluids and other complex matrices for HPLC analyses. The substances to be analysed are adsorbed and purified on precolumns, which are filled with ion exchangers [27—29], silica gel [30, 31], CPG (controlled pore glass) [32], styrene—divinylbenzene copolymers [31, 33] and, in the large majority, with reversed-phase material [34—49].

The adaption of the automated column switching technique to the problems encountered in routine drug monitoring in the analytical laboratory of an

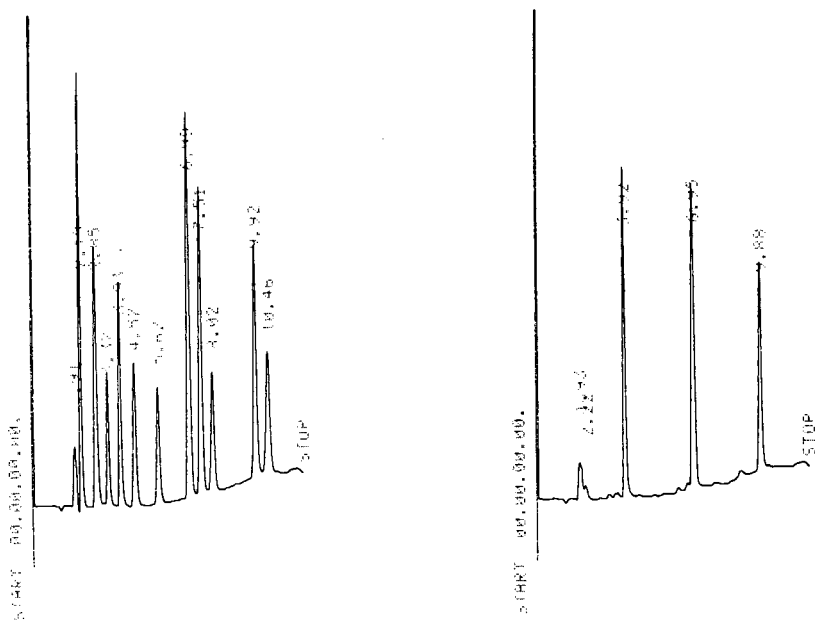

Fig. 1. Chromatogram of a calibration sample. Retention time (min), drug/MB, concentration (μg/ml): 2.19 THE 10, 2.85 CAF 10, 3.37 PE 12.5, 3.93 ET 50, 4.57 PR 12.5, 5.62 DIOL 5, 6.99 PB 25, 7.51 DM 25, 8.02 C-EP 5, 9.92 PT 12.5, 10.46 CBZ 7.5.

Fig. 2. Chromatogram of a serum sample from a patient on ethosuximide, phenobarbital and phenytoin medication. Retention time (min), AED/MB, concentration (μg/ml): 3.92 ET 74.3, 6.95 PB 18.5, 9.88 PT 10.1.

epilepsy centre is discussed in this paper. The following AEDs and their clinically relevant metabolites (MBs) were chromatographically separated: phenobarbital (PB), phenytoin (PT), ethosuximide (ET), carbamazepine (CBZ), carbamazepine-10,11-epoxide (C-EP), primidone (PR), 2-ethyl-2-phenylmalonediamide (PE), and N-desmethylmethsuximide (DM). In addition, the chromatographic conditions must be so chosen that there is no interference in the determination of PE by caffeine (CAF) or theophylline (THE), and of PR by the second main CBZ metabolite 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine (DIOL), (see Figs. 1—4).

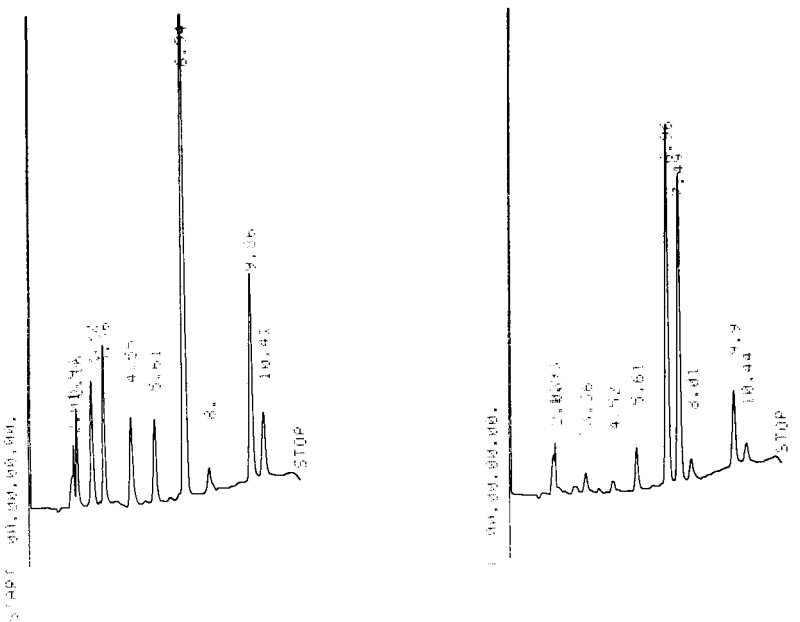

Fig. 3. Chromatogram of a pool serum of patients on primidone medication (inc. other co-medication). Retention time (min), AED/MB, concentration (μg/ml): 2.82 CAF 4.5, 3.36 PE 14.0, 4.55 PR 8.4, 5.61 DIOL 7.1, 6.94 PB 34.6, 8.00 C-EP 2.0, 9.86 PT 10.6, 10.41 CBZ 3.6.

Fig. 4. Chromatogram of a pool serum of patients on methsuximide medication (inc. other co-medications). Retention time (min), AED/MB, concentration (μg/ml): 3.36 PE 2.5, 4.52 PR 1.0, 5.61 DIOL 3.6, 6.96 PB 23.2, 7.49 DM 25.2, 8.01 C-EP 1.9, 9.90 PT 4.0, 10.44 CBZ 1.0.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following firms: PR, ET, PT, CBZ, and C-EP from Desitin-Werk/Carl Klinke, Hamburg (F.R.G.), PE and DM from EGA-Chemie, Steinheim (F.R.G.), PB from Bayer, Leverkusen (F.R.G.). We are grateful to Ciba-Geigy, Basle (Switzerland), for providing DIOL. Acetonitrile and water "for use in HPLC" were from Baker Chemicals, Deventer (The Netherlands). All other chemicals were of analytical reagent grade and were obtained from E. Merck, Darmstadt (F.R.G.).

Apparatus

The equipment was obtained from the following firms: HPLC low-pressure gradient-former 2500, two HPLC constant flow pumps 600/200, column switching module SE-2, spectrophotometer SP-4, Shimadzu printer-plotter integrator C-R 1B from Gynkotek, Munich (F.R.G.), autosampler WISP 710 B from Waters, Koenigstein/Taunus (F.R.G.), HPLC thermostat from Barkey Labortechnik, Bielefeld (F.R.G.), analytical column Shandon-ODS Hypersil (5 μm, 250 mm × 4.6 mm I.D.) and precolumn cartridges (5 mm × 4.6 mm I.D.) filled with Nucleosil 30 C_{18} (30 μm) from Bischoff-Analysentechnik, Leonberg (F.R.G.), on-line filter with 0.5-μm frits from DuPont, Frankfurt/Main (F.R.G.). Materials for the dry filling of the precolumns (40 mm × 4.6 mm I.D.) were: Nucleosil 30 C_{18} (30 μm) from Macherey-Nagel, Düren (F.R.G.), Perisorb RP-18 (30—40 μm) from Merck, and Vydac 201-RP (30—40 μm) from Gynkotek.

Chromatographic parameters

Temperature of the column: 70°C. Injection volume: 50 μl. Detection wavelength: 205 nm. Precolumns: purge liquid 0.01 vol.% phosphoric acid, flow-rate 0.3 ml/min, duration of washing 300 sec. Analytical column: buffer pH 4 (0.01 vol.% phosphoric acid buffered with saturated disodium hydrogen phosphate solution to pH 4). Gradient elution mixtures: A = buffer pH 4—acetonitrile (9:1, v/v), B = buffer pH 4—acetonitrile (4:6, v/v), flow-rate 1.5 ml/min.

The gradient programme is given in Table I.

TABLE I

GRADIENT ELUTION PROGRAMME

Step	A (vol.%)	B (vol.%)	Acetonitrile (vol.%)	Time (min)
1	085	015	17.5	00.00
2	085	015	17.5	01.00
3	050	050	35.0	06.00
4	050	050	35.0	02.00
5	085	015	17.5	00.00
6	085	015	17.5	End

Calibration and control samples

A 10 ml quantity of a stock solution (for calibration) containing 25 mg PE, 100 mg ET, 25 mg PR, 50 mg PB, 50 mg DM, 10 mg C-EP, 25 mg PT, and 15 mg CBZ in 100 ml acetonitrile and another 10 ml quantity of a stock solution with 20 mg THE, 20 mg CAF and 10 mg DIOL (for test purposes) were mixed and made up to 200 ml with HPLC water. The same quantity of this standard solution as for the patient samples (50 μl) was injected, so that the amount of AEDs in μg/ml is given by the integrator on using the external standard mode.

As control sera, lyophilized samples from the firms Biotrol, Fisher and Merck were dissolved in distilled water. Without further pretreatment 50 μl of these solutions were likewise injected.

Lifetime of the columns

At first the method as described here with 4 cm precolumns, filled with Nucleosil 30 C_{18}, was carried out using the back-flush technique. This method has been employed in routine analysis for about half a year. The introduction of very short precolumns (0.5 cm) made it possible to concentrate and purify the drugs, using the purge liquid, as well as subsequently to elute onto the analytical column in the same direction of flow. Thus, blockage which in the back-flush technique occurred mainly at the head of the separation column, could be avoided. As additional protection an on-line filter holder for replaceable filter frits of 0.5 μm pore diameter was built in in front of the analytical column. The frits were replaced after every 200—250 injections of serum. Under the chosen chromatographic and technical conditions the precolumn cartridges had a lifetime of 300—400 serum injections and the analytical column up to about 1400 serum injections.

RESULTS AND DISCUSSION

Recovery of the drugs and metabolites determined

At the very beginning of the development of the routine method described above a suitable precolumn material was sought. This material had to adsorb fully all the analysed AEDs and MBs but not lead to blockage on direct injection of serum. Therefore 4 cm columns were first of all filled with a material consisting of a type of porous layer beads (PLB) such as those used by Roth et al. [42] and Beschke et al. [45]. Unfortunately the recovery rate using both Perisorb RP-18 (30—40 μm) and Vydac 201-RP (30—40 μm) was poor for nearly all the analysed AEDs (see Table II). Both requirements mentioned above (long lifetime and complete recovery) were complied with using Nucleosil 30 C_{18} (30 μm, spherical) which was recommended by Kronbach et al. [47] (see Table II).

TABLE II

RECOVERY FROM THE DIFFERENT PRECOLUMN MATERIALS TESTED

Recovery is expressed as a percentage of the values achieved by direct injection of a calibration sample onto the analytical column.

	Perisorb RP-18 40 mm*	Vydac 201-RP 40 mm*	Nucleosil 30 C_{18}	
			40 mm*	5 mm
THE	3.6	1.5	98.3	99.7
CAF	93.1	29.8	101.1	99.2
PE	0	0	98.1	97.6
ET	0	0	103.1	96.8
PR	7.5	0	100.1	99.0
DIOL	100.6	0	95.9	100.0
PB	24.4	3.7	100.4	103.2
DM	66.5	14.9	100.2	104.1
C-EP	95.6	86.8	100.5	105.3
PT	100.4	62.4	98.7	106.3
CBZ	99.8	103.5	98.9	106.3

*Determination in the back-flush mode.

The recovery rate was determined by repeatedly injecting a calibration sample onto the analytical column and then onto each of the precolumns. The values for direct injection onto the analytical column were taken as 100%. The values obtained following precolumn enrichment were compared with these values and likewise expressed as a percentage (Table II).

A comparison of recovery, as described, could not be carried out with serum samples since the analytical column (5 μm) would already be blocked after one direct injection of serum. For this reason other methods were employed in order to test the quality of the method described here. This was initially checked by analysing commercially obtained control sera. The results using direct injection were in very good agreement with those given by the producers. Because the clinically relevant metabolites PE and DM were not present in the control sera, we carried out a comparative study with some hundred patient samples. These were analysed using gas chromatography (GC) [50] and HPLC with ethyl acetate extraction (HPLC—EE) [51] as sample pretreatment and in addition by direct serum injection, in part as double determinations. Double determination in this case means that each serum was injected once onto precolumn A and once onto precolumn B. For purposes of calibration, the mean values of the double injections of the standard samples were used. The agreement of the double determinations is given in Table III.

TABLE III

REPRODUCIBILITY OF DOUBLE INJECTIONS

n = number of duplicates, $\overline{x}$ = mean value (μg/ml), r = coefficient of correlation, $\overline{d}$ = mean value of the absolute differences of the duplicates $= x_1 - x_2$, $\overline{d}_x$ (%) = mean deviation from the mean value expressed as a percentage $= 100(x_1 - x_2)/(x_1 + x_2)$.

	n	$\overline{x}_1$	$\overline{x}_2$	r	$\overline{d}$	$\overline{d}_x$ (%)
PE	135	8.15	8.17	0.990	0.34	2.25
ET	74	50.11	51.17	0.994	1.69	1.80
PR	135	6.86	6.83	0.991	0.26	1.79
PB	309	26.29	26.30	0.997	0.51	1.07
DM	68	23.06	23.00	0.994	0.55	1.27
PT	244	9.51	9.52	0.998	0.19	1.07
CBZ	203	4.68	4.67	0.995	0.14	1.66

The results in Table III show that for some substances (PB, DM, PT) the agreement is better than for the other compounds. As exactly the same sample had been injected onto both precolumns and as the autosampler used has a good reproducibility, it is apparently — at the time of writing — not possible for the manufacturer to produce two precolumn cartridges which are absolutely identical. As shown in Table III, each substance reacts specifically to the variations in the filling of the cartridges.

Agreement of direct serum injection with the results of the reference methods GC and HPLC—EE

We have recently reported on a study comparing three basic methods of sample pretreatment for HPLC analysis of AEDs [51] with the results obtained from routine GC analysis [50]. GC and HPLC—EE were likewise chosen for

controlling the results obtained from direct serum injection (DI) using a column-switching technique. The results were evaluated, as described in ref. 51, using the statistical procedure of linear regression analysis [52, 53] as given in Table IV.

TABLE IV

STATISTICAL EVALUATION OF THE DIRECT INJECTION HPLC METHOD USING GC AND HPLC—EE AS REFERENCE METHODS

R = reference method, DI = direct injection, n = number of samples, $\overline{x}$ and $\overline{y}$ = mean values (μg/ml), b = slope of the regression line expressing the systematic deviation from the reference values as a percentage, a = intercept of the ordinate by the regression line as a measure of a constant and systematic deviation, r = coefficient of correlation.

	n	$\overline{x}$ (R)	$\overline{y}$ (DI)	b	a	r
PE (GC)	105	7.23	8.58	1.05	1.02	0.972
PE (HPLC—EE)	133	8.07	8.20	1.03	—0.15	0.983
ET (GC)	69	49.71	50.05	0.84	8.47	0.968
ET (HPLC—EE)	73	52.03	50.67	0.88	4.88	0.983
PR (GC)	105	6.59	7.01	0.92	0.98	0.961
PR (HPLC—EE)	133	6.62	6.81	0.99	0.23	0.974
PB (GC)	246	25.97	25.51	0.99	0.85	0.988
PB (HPLC—EE)	298	25.74	26.24	1.01	0.15	0.987
DM (GC)	63	23.26	22.90	0.95	0.79	0.986
DM (HPLC—EE)	67	23.36	22.92	0.98	—0.06	0.988
PT (GC)	193	9.14	9.38	0.98	0.43	0.990
PT (HPLC—EE)	235	9.31	9.44	0.98	0.27	0.991
CBZ (GC)	156	4.56	4.66	0.95	0.30	0.971
CBZ (HPLC—EE)	197	4.66	4.70	1.01	0.01	0.989

As seen from Table IV, the method of direct injection agrees sufficiently with both reference methods (with two exceptions the coefficient of correlation (r) for all AEDs and MBs is greater than 0.970). Whilst the agreement for PB, DM and PT with both reference methods is equally good, the correlation in the case of the other substances between DI and the HPLC—EE is higher than between DI and GC.

Within-day and day-to-day precision

In order to check on the precision of the method described, three pooled sera containing various AEDs and a control serum were analysed several times in a day and daily over the course of several weeks. The results are summarized in Tables V and VI.

The within-day precision is for all AEDs and MBs, with one exception, very much the greater the higher the concentration of the substances concerned in the serum. This was to be expected. Only in the case of ET is there a greater range of fluctuation for the control serum than for the pool serum A. This could be due to a small quantity of interfering compounds which are present in the control serum and which are eluted in the retention range of ET. The coefficients of variation (C.V.) for all AEDs and MBs lie in the within-day control, with one exception, below 5%. Only in pool serum C, CBZ with a very low concentration of 1.1 μg/ml has a C.V. of 7.25% (see Table V).

TABLE V

WITHIN-DAY PRECISION OF DIRECT SERUM INJECTION

x_{max} = maximum value, x_{min} = minimum value, $\bar{x}$ = mean value (μg/ml), m = median, S.D. = standard deviation, C.V. = coefficient of variation.

	n	x_{max}	x_{min}	$\bar{x}$	m	S.D.	C.V. (%)
Control sample							
ET	14	99.5	86.3	93.76	93.45	3.39	3.61
PR	14	19.6	18.1	18.79	18.80	0.41	2.20
PB	14	31.8	30.6	31.00	31.00	0.30	0.96
PT	14	31.7	30.4	30.79	30.75	0.33	1.07
CBZ	14	10.7	10.2	10.35	10.30	0.13	1.24
Pool serum A							
PE	18	6.5	5.6	6.06	6.10	0.26	4.24
PR	18	4.8	4.3	4.50	4.50	0.11	2.53
PB	18	22.1	20.5	21.03	20.95	0.44	2.08
PT	18	5.4	4.9	5.11	5.10	0.13	2.64
CBZ	18	2.2	2.0	2.13	2.10	0.06	2.78
Pool serum B							
ET	18	55.0	51.6	53.53	53.45	1.07	2.00
PB	18	19.2	17.6	18.35	18.35	0.49	2.66
PT	18	2.7	2.5	2.61	2.60	0.08	3.08
CBZ	18	3.3	2.8	3.03	3.00	0.12	4.08
Pool serum C							
PB	18	24.2	23.2	23.72	23.70	0.25	1.04
DM	18	26.8	25.7	26.34	26.30	0.30	1.13
PT	18	4.0	3.7	3.85	3.80	0.08	2.04
CBZ	18	1.3	1.0	1.08	1.10	0.08	7.25

TABLE VI

DAY-TO-DAY PRECISION OF DIRECT SERUM INJECTION

	n	x_{max}	x_{min}	$\bar{x}$	m	S.D.	C.V. (%)
Pool serum A							
PE	20	6.9	5.8	6.32	6.30	0.26	4.13
PR	20	6.1	4.5	5.41	5.45	0.39	7.26
PB	20	23.8	21.4	22.67	22.60	0.65	2.88
PT	20	6.0	5.2	5.72	5.75	0.21	3.65
CBZ	20	2.8	2.2	2.49	2.55	0.14	5.61
Pool serum B							
ET	20	60.1	52.3	55.12	54.85	2.09	3.79
PB	20	19.9	18.1	18.65	18.65	0.42	2.23
PT	20	3.0	2.7	2.76	2.75	0.09	3.38
CBZ	20	3.4	3.1	3.26	3.25	0.10	3.11
Pool serum C							
PB	23	24.4	22.1	23.27	23.25	0.69	2.96
DM	23	27.0	23.2	25.16	25.30	1.03	4.10
PT	23	4.2	3.7	3.90	3.90	0.14	3.48
CBZ	23	1.2	0.9	1.04	1.05	0.08	7.55

The coefficients of variation for the day-to-day control of precision lay, with three exceptions, always below 5% (see Table VI). For the very low CBZ in pool serum C, already mentioned above, the C.V. is 7.55%. Likewise, for the relatively low CBZ concentration (mean value 2.5 μg/ml) of the pool serum A the C.V. is 5.61% and for the PR (mean concentration 5.4 μg/ml) in the same serum is 7.26%.

This shows that PR as well as to a less extent PE and ET, all three of which lie in the early eluted range of the chromatogram, are exposed to disturbing influences to a somewhat greater degree than are the substances that are eluted later.

CONCLUSION

A column-switching technique with purification and enrichment of the samples enables sera of epileptic patients to be injected directly. A large number of possible sources of error in the otherwise necessary extraction, enrichment and evaporation steps can thereby be eliminated in the pretreatment of samples for HPLC analysis. Under the technical and chromatographic conditions described in the experimental section more than 100 samples can be analysed in 24 h. Direct injection of serum is especially suitable for the analysis of small quantities of sample (less than 50 μl). In routine analysis there is normally enough serum to enable 50 μl to be injected. Thereby the reproducibility of the results is improved.

Considering the values given in Table V for within-day precision and those in Table VI for day-to-day precision, the method described is sufficiently suitable for purposes of routine analysis. The avoidance of pretreatment of the samples results in a great saving of costs of reagents and laboratory staff.

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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR MONITORING CAFFEINE AND ITS METABOLITES IN BIOLOGICAL FLUIDS OF MONKEYS CONSUMING CAFFEINE

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SUMMARY

A recently reported high-performance liquid chromatographic procedure, using a 5 μm C_{18} reversed-phase column to separate and quantitate caffeine and seven of its metabolites was modified for use with an automatic sampler to allow the continuous analysis of a large number of samples of various biological fluids obtained from monkeys consuming caffeine. The sensitivity for most metabolites was in the range of 0.1—0.3 μg/ml from a 0.1 ml sample. The repeatability of the method regarding within-day variations was excellent and the absolute retention time for eight standards differed by less than ± 0.03 min. Excellent repeatability in the day-to-day assay, with almost quantitative recoveries, was found for most of the analyzed compounds in various biological fluids. The standard deviation for the quantitation of all standards was in a range of 0.41—2.01 μg/ml, with the standard error less than 0.02. Using this method an analytical chemist could process between 40 and 60 samples of biological fluids in 24 h.

The main metabolite of caffeine in the plasma of the monkey was theophylline, while theophylline and 1,3-dimethyluric acid were the major metabolites in urine. A close correlation was observed for the pattern of metabolites found in plasma and milk.

INTRODUCTION

Coffee is certainly one of the most frequently consumed beverages not only

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in North America but all over the world [1]. Coffee drinking has been associated with a number of pharmacological and physiological effects [2]. Several epidemiological studies have associated coffee consumption with an increased risk for cancers of the bladder, pancreas, prostate, and esophagus or for renal carcinoma. However, most of these reports are controversial and it appears that the association is not causal [3]. The more obvious effects of coffee consumption are physiological in that it causes cardiovascular and psychological disorders [4].

Caffeine is considered to be the most physiologically active component in coffee [5], contributing to pronounced behavioral effects [6]. In addition, caffeine is present in tea, soft drinks, cocoa, chocolate and some medications. Caffeine and its metabolites readily cross the human placenta [7]. The biotransformation of caffeine in the newborn infant is different relative to the adult [8] and the elimination pattern of caffeine and its metabolites during pregnancy is also changed [9]. Due to the fact that the embryo is subjected to the influence of caffeine and its metabolites, it is important to determine the effects of caffeine on the developing nervous system. To study such effects it was proposed that the behavioral consequences of in utero exposure to caffeine be determined in the cynomolgus monkey (*Macaca fascicularis*), both immediately following birth and during the animal's first year of life. In addition, it is important to assess whether pregnant monkeys handle caffeine in a way similar to humans during their pregnancy.

For a long-term study with a large number of samples obtained from monkeys, a simple, uniformly applicable, fast and precise method for the determination of caffeine and its metabolites in biological fluids is required. However, the determination of caffeine metabolites in biological fluids poses analytical problems not encountered in other matrices [10]. There are several combined reasons for this difficulty: first, low dilute concentration and a limitation in the available sample size; secondly, extractions from matrices containing a variety of chemically different compounds; thirdly, the presence of structurally similar metabolites; and fourthly, individual variability in the metabolic pattern. Therefore, a method with high selectivity as well as sensitivity, employing a small sample size is required. Our previously developed method, employing a reversed-phase 5 μm C_{18} column for the high-performance liquid chromatographic (HPLC) separation of caffeine and its metabolites [11], was adapted for use with an automatic sampler for this purpose. This report describes a simple, fast, reliable and accurate method for monitoring caffeine and metabolites in different biological fluids of monkeys, using an automatic sampler, which allows continuous injections of large numbers of samples onto the HPLC column.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph Series 2/2 (Perkin-Elmer, Norwalk, CT, U.S.A.) provided with a Perkin-Elmer high speed 5 μm C_{18} column (15 cm × 4.6 mm I.D.) was used. The ultraviolet (UV) detector (Perkin-Elmer LC-55) connected to a Perkin-Elmer Deuterium Supply (Model

22320) was set at 276 nm. The flow cell had a volume of 8 μl. Samples were injected into the HPLC system using the Perkin-Elmer Intelligent Automatic Sampling System (ISS-100). The automatic sampler can handle up to 100 samples for continuous analysis. The sampler can be pre-programmed for a number of different parameters, such as volumes to be injected, repeated analysis of the same sample, and time between injections.

Data were recorded using a Perkin-Elmer Sigma 10 Data Processing system. The results (the values for the concentrations of the metabolites in μg/ml) were entered into a Data General NOVA computer (Westboro, MA, U.S.A.) for storage, retrieval and statistical manipulation.

All instruments and the column were operated at ambient laboratory temperature (approximately 22° C), although occasional fluctuations within the room (between 18° C and 28° C) were observed.

Chemicals

Sources and the abbreviations for the reference chemicals are: caffeine (1,3,7-trimethylxanthine or 1,3,7-TMX), theobromine (3,7-dimethylxanthine or 3,7-DMX) and theophylline (1,3-dimethylxanthine or 1,3-DMX) from Eastman Kodak, Rochester, NY, U.S.A.; 3,7-dimethyluric acid (3,7-DMU) from Sigma, St. Louis, MO, U.S.A.; paraxanthine (1,7-dimethylxanthine or 1,7-DMX) and 3-methylxanthine (3-MX) from Tridom/Fluka, Toronto, Canada; 1,3-dimethyluric acid (1,3-DMU) from Adams, Round Lake, IL, U.S.A. 8-Chlorotheophylline (8-CT) was purchased from ICN Pharmaceutical. All the standard stock solutions (10 μg/ml) and the internal standard, 8-CT (20 μg/ml) were prepared in 0.9% sodium chloride solution.

For the quality control, a mixture of 100 ml of all standards, at a concentration of 10 μg/ml in 0.9% sodium chloride was prepared. Aliquots of the stock standard solution were placed in several appropriate sample containers (scintillation vials or reagent bottles with glass stopper) and stored in a freezer. Every month (or if required more frequently), a fresh bottle of the standard solution was used and the repeatability for the retention time and peak heights of the "new" and "old" standards was compared.

All solvents used for extractions and for the chromatography were either of HPLC purity or were glass-distilled. All chemicals were analytical grade.

The extracting solvent was chloroform—isopropanol (85:15). The mobile phase consisted of a daily prepared mixture of water—isopropanol—acetonitrile—acetic acid (91:4:4:1), degassed for 2 min under partial vacuum in an ultrasonic bath (Branson Cleaning Equipment, Shelton, CN, U.S.A.).

Biological fluids

Biological fluids (urine, plasma, milk and saliva) for these analyses were obtained from the female monkeys (*Macaca fascicularis*), either from non-treated controls or from animals treated with different concentrations of caffeine in drinking water. Blood was collected from the femoral vein or artery in unheparinized vacutainers containing EDTA (Becton-Dickinson, Rutherford, NJ, U.S.A.), and centrifuged to obtain plasma. Urine was collected over a 24 h period in special metabolism cages. Milk was collected by hand expressing milk from a lactating monkey that did not have an infant.

Saliva was collected as follows: a plastic suction tube, normally used by dentists to remove saliva from a patient's mouth, was inserted into the monkey's mouth. The other end was connected to a small filtering test tube with side arm, which was further connected to a water pump partial vacuum. By applying a low suction pressure, a small sample of saliva (about 200 μl) collected in the plastic tube, was sufficient for the assay, although not every monkey cooperated readily for this type of collection.

Treatment of the monkeys

The monkeys were exposed to caffeine in doses of 0, 0.15 and 0.35 mg/ml in drinking water, seven days a week. Monkeys were given a fixed amount (ten cubes) of Purina monkey chow daily.

Sample preparation

One hundred microliters of the sample to be analyzed (urine, blood, serum or plasma, milk, saliva, or standards) was added to the test tube containing 1.2 g of ammonium bicarbonate and 100 μl of internal standard solution. After adding 8.0 ml of the extraction solvent, the mixture was well agitated on a Vortex mixer for 30 sec. After settling, the clear extracting solvent was filtered through Ottawa sand placed (approx. 1—2 mm depth) on the bottom of a polypropylene disposable mini-column 10 cm long (Alltech Associates, Deerfield, IL, U.S.A.). The extraction was repeated once more, and the pooled extracts were evaporated to dryness using a stream of nitrogen in a water bath (temperature not exceeding 50° C). After adding approximately 500 μl of the mobile solvent, the test tubes were briefly (15—20 sec) warmed (50° C) and sonicated for 10 sec to complete the dissolution. The samples were transferred into automatic HPLC sample vials for the analysis. The vials were not capped. The automatic sampler was set for 80 μl of sample per injection.

Setting the automatic sampler

Vials containing an extracted standard solution were incorporated into the tray of the automatic sampler: the first vial into position No. 1, and then one vial after every ten samples, i.e. in the positions numbered 12, 23, 34, etc.

A reasonable "wash-out" time was allowed between the injections of individual samples. This was important to allow complete elution of some slowly eluting endogenous compounds, which were found in urine. Therefore, urine samples needed an additional 20 min of wash-out time after the elution of the internal standard. There were no visible interference (peaks) with the extracts from plasma, saliva or milk. The wash-out time for the extracts from these samples was set at 5 min. Therefore, when using an automatic sampler it is preferable to group sets of identical fluids in consecutive order and to select the proper "elution" time for each set.

Quality control for the recoveries, repeatability and accuracy

Tests for accuracy were performed regularly using a mixture of standards (10 μg/ml of each) as a means of quality control.

For the purpose of controlling the repeatability of the methodology (extractability, interference) and the performance of the instruments, in addition

to the regular analysis of analytical standards, blank samples of biological fluids were periodically spiked with a mixture of analytical standards, and then analyzed. These tests, indicating the recoveries for different metabolites, were performed by adding 1.0 ml of the mixture of standards (containing 10 μg of each standard) to blood, urine, milk or saliva obtained from control, non-treated monkeys, prior to the analysis.

Additional tests for intra- and interday variability were performed using samples of urine or pooled plasma from animals on test. Samples of plasma were combined in two separate pools: (1) plasma of monkeys treated with low dose of caffeine and (2) plasma from animal treated with high dose of caffeine. These analyses, performed in nine or ten replicates per day, were repeated three times within twelve days. Samples of urine were analyzed in nine or ten replicates each day, and the analysis was repeated three times within one week.

Statistical evaluation

The analytical standards from all runs were pooled and the mean, median, standard deviation and standard errors calculated for each standard.

RESULTS

Very little variation was observed in the elution time for the standards within the day. The results from six injections performed during one working day are presented in Table I. If the room temperature remained constant the differences for the actual retention time differed less than ± 0.03 min for the internal standard, which was the last eluting compound from the column, i.e. peak 9 in Table I. Other metabolites showed even smaller differences. Also very little variation was found in the quantitation (peak heights or area) for the individual standards, when this mixture was repeatedly injected during the same working day.

TABLE I

ACTUAL ELUTION (RETENTION) TIME FROM SIX INDIVIDUAL INJECTIONS OF STANDARDS DURING ONE WORKING DAY

Retention times are given in minutes.

Injection number	Compound/peak number*							
	2	3	4	5	6	7	8	9
1	1.95	2.10	2.41	2.82	3.82	4.13	6.82	8.77
2	1.95	2.09	2.40	2.81	3.80	4.11	6.78	8.73
3	1.94	2.09	2.40	2.81	3.80	4.11	6.75	8.69
4	1.94	2.08	2.39	2.81	3.80	4.12	6.80	8.74
5	1.96	2.10	2.42	2.83	3.82	4.13	6.81	8.76
6	1.96	2.11	2.42	2.84	3.83	4.15	6.82	8.77
Average	1.95	2.095	2.41	2.82	3.81	4.125	6.81	8.74
S.D.	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.03

*For identification of the compound/peak numbers see Fig. 1.

Generally, the results for accuracy and precision in the day-to-day operations were also very good. Table II evaluates the results obtained from 124 injections of the mixture of standards using the same column. The concentration of each added standard was 10 μg/ml. These analyses were performed on 59 different days over an eight-month period. Excellent repeatability with almost quantitative recovery was regularly obtained for most of the analyzed compounds. The compound with the highest variability was 1,3-DMU, with a standard deviation of 2.01 μg/ml, and with a standard error of 0.02. The ranges, standard deviation and standard error values for all other compounds were considerably lower.

The intraday variability for urine samples is presented in the Table III. Similar results (mean, median, standard deviation and standard error) were obtained for the other two sets of analyses performed on different days. The standard error for all metabolites in the interday variability tests was in a range between 0.01 (for 1,7-DMX and 3-MX) and 0.11 (for theophylline).

There were little variations in tests for the intra- and interday variability either for plasma of low or high caffeine treated monkeys. The combined results of the interday variability for serum samples is presented in the Table

TABLE II

REPEATABILITY FOR QUANTITATION OF STANDARDS

Number of analyzed samples of processed mixed standards only = 124. Number of days the analysis was performed = 59. Time period = 8 months.

	Added (μg/ml)	Found (μg/ml)		S.D.	S.E.	Coefficient of variation
		Mean	Median			
Caffeine	10	9.96	10.00	0.84	0.01	0.084
Theophylline	10	10.18	10.15	0.58	0.00	0.057
Paraxanthine	10	10.11	10.10	0.41	0.00	0.041
Theobromine	10	9.96	10.00	0.48	0.00	0.048
1,3-DMU	10	9.66	9.30	2.01	0.02	0.208
3-MX	10	9.91	9.90	1.28	0.01	0.129

TABLE III

INTRADAY VARIABILITY FOR CAFFEINE AND METABOLITES FOUND IN URINE OF TREATED MONKEY

Number of replicates = 9.

	Found (μg/ml)		S.D.	S.E.
	Mean	Median		
Caffeine	9.13	9.30	0.54	0.07
Theophylline	68.49	68.90	1.76	0.22
Paraxanthine	2.01	2.00	0.33	0.04
Theobromine	6.94	6.80	0.59	0.07
1,3-DMU	26.54	26.00	2.23	0.28
3-MX	4.33	4.30	0.28	0.03

TABLE IV

INTERDAY VARIABILITY FOR CAFFEINE AND METABOLITES FOUND IN POOLED PLASMA OF TREATED MONKEY

	Found (μg/ml)		S.D.	S.E.
	Mean	Median		
Low dose of caffeine*				
Caffeine	2.87	2.95	0.31	0.01
Theophylline	8.01	8.00	0.79	0.03
Paraxanthine	0.51	0.50	0.16	0.01
Theobromine	0.60	0.60	0.10	0.00
1,3-DMU	0.17	0.10	0.18	0.01
3-MX	0.09	0.10	0.08	0.00
High dose of caffeine**				
Caffeine	7.57	7.60	0.58	0.02
Theophylline	14.46	14.20	1.09	0.04
Paraxanthine	0.69	0.70	0.20	0.01
Theobromine	1.01	1.00	0.12	0.00
1,3-DIMU	0.51	0.50	0.14	0.01
3-MX	0.18	0.20	0.06	0.00

*Total number of replicates = 27. Tests repeated three times, nine samples/day.
**Total number of replicates = 28. Tests repeated three times, nine or ten samples/day.

IV. The maximum standard error of 0.04 was found for theophylline in the high dose treated samples.

The results of the recovery studies of the mixture of standards added to various biological fluids were the same as previously described [11].

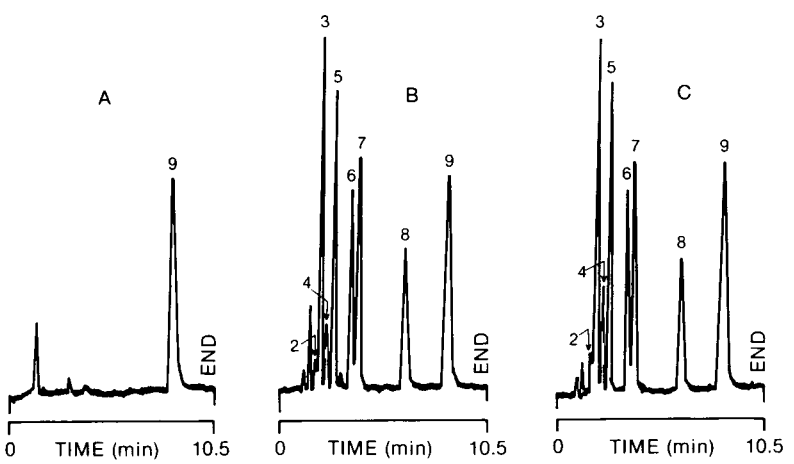

Fig. 1. Recovery profiles of standards (10 μg of each) added to the plasma of a non-treated monkey, before processing for HPLC analysis. (A) non-spiked plasma; (B) spiked plasma; (C) chromatograms of standards (10 μg of each) processed in the same way before the HPLC analysis. Identification of peaks: 2 = 3,7-dimethyluric acid; 3 = 3-methylxanthine; 4 = 1,3-dimethyluric acid; 5 = 3,7-dimethylxanthine; 6 = 1,7-dimethylxanthine; 7 = 1,3-dimethylxanthine; 8 = 1,3,7-trimethylxanthine; and 9 = 8-chlorotheophylline (8-chloro-1,3-dimethylxanthine).

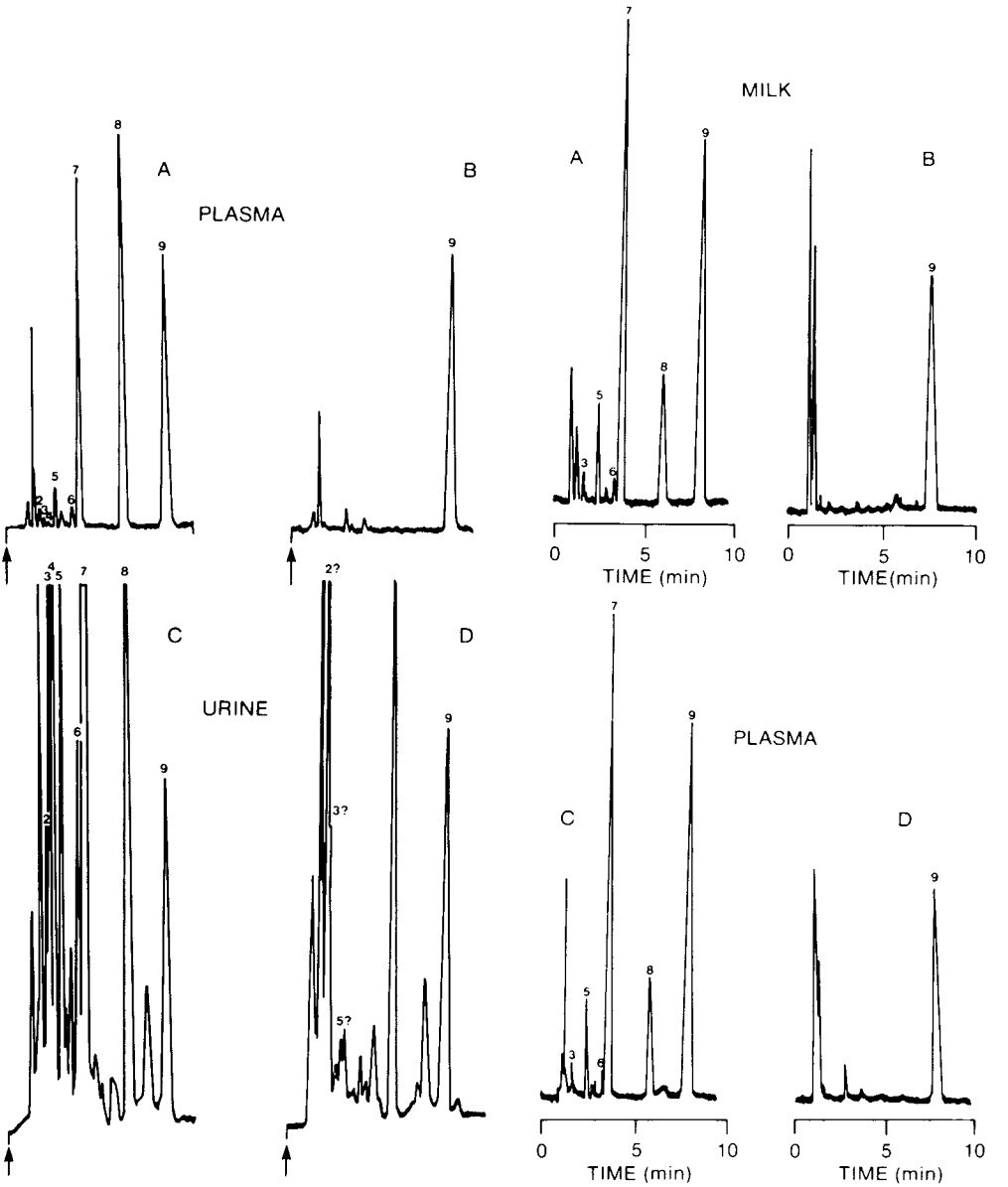

Fig. 2. HPLC profiles of caffeine metabolites in plasma (A) and urine (C) of a monkey consuming caffeine (9 mg/kg) for six months. B and D are comparable chromatograms for plasma and urine from a non-treated control monkey. For the identification of peaks see Fig. 1.

Fig. 3. The correlation between caffeine metabolites in milk (A) and plasma (C) from identical monkey treated with caffeine. B and D are corresponding controls taken from another, non-treated monkey. For peak identification see Fig. 1.

TABLE V

THE AMOUNT OF DIFFERENT METABOLITES FOUND IN PLASMA AND URINE OF A MONKEY RECEIVING 0.35 mg/ml CAFFEINE IN DRINKING WATER, COMPARED WITH A CONTROL

Values are expressed in μg/ml.

Peak number*	Compound	Plasma		Urine	
		A** Treated	B** Control	C** Treated	D** Control
3	3-MX	0.1	0.0	21.6	0.6
4	1,3-DMU	0.5	0.3	67.0	0.0
5	3,7-DMX	0.9	0.0	17.0	1.6
6	1,7-DMX	0.6	0.0	12.6	0.0
7	1,3-DMX	11.4	0.0	128.1	0.0
8	1,3,7-TMX	22.5	0.0	46.5	0.0
9	8-CT	20.0	20.0	20.0	20.0

*For the identification of the peak number see Fig. 1.
**For the chromatograms see Fig. 2.

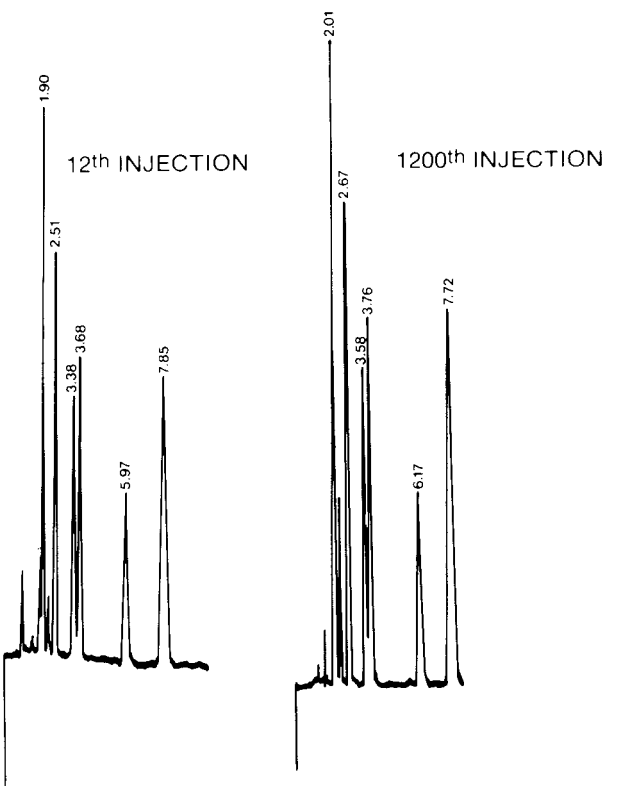

Fig. 4. The HPLC profiles of standards of caffeine metabolites from identical column routinely used for analysis of caffeine metabolites in urine and plasma for five months. Very little differences were observed between the 12th and 1200th injection.

Fig. 1 illustrates the extractability and recovery of metabolites (standards) added to plasma of a non-treated monkey. A is a chromatogram of processed plasma before spiking with standards, B is processed plasma of the same blood after spiking with 10 μg of each standard, and C is processed mixture of 10 μg of each standard. Similarly, very good recoveries for most metabolites, especially for the dimethylxanthines and caffeine, were found for other biological fluids, confirming our previous findings [11].

Typical HPLC profiles of caffeine metabolites in plasma (A) and urine (C) from one monkey consuming approximately 9 mg of caffeine in drinking water daily for six months, are presented in Fig. 2. Chromatogram B and D (Fig. 2) are comparable chromatograms from plasma and urine from another, non-treated control monkey. The concentrations (μg/ml of fluids) of metabolites found in this test are presented in Table V.

Caffeine and its metabolites were found in the milk of a treated monkey. The profiles of these metabolites are presented in Fig. 3A. Fig. 3B is the HPLC profile of milk from a control monkey. Fig. 3C is a chromatogram of metabolites in plasma of the same monkey from whom milk was also analyzed and presented in Fig. 3A.

Fig. 4 gives the HPLC profiles of seven standards using an identical column for 1200 injections of samples from different biological fluids. Very little variation, deterioration or differences were observed in the retention times and separation of peaks with time and usage of the column, when the 12th and the 1200th injection are compared. These two analyses were also incorporated in the above-mentioned evaluation of the repeatability for quantitation of standards, as presented in Table II.

DISCUSSION

The present method has advantages in analysis of caffeine metabolites over some other recently published procedures [12—14] in several aspects. This method avoids the use of elution programming, a procedure [13] that requires more time because of the time necessary for the re-equilibration of the column between runs. The time required for the separation of the caffeine metabolites in our method is less than 10 min, while others [14] need about 30 min. The procedure is equally applicable not only to urine [13, 14] but also to other biological fluids [11]. Using this method we were able to separate and quantitate several metabolites, as theophylline and paraxanthine or caffeine, the separation of which was not possible in some other procedures [12, 14].

From Tables I and II, it is clear that the repeatability, accuracy and the precision when using an automatic sampler was consistently very good. The relative retention time (RRT) for the standards within the day differs less than 0.02. Occasionally, some day-to-day variation in retention time was observed. The absolute retention time was found to fluctuate, up to 1 min, mainly due to changes of room temperature. During some weekends the room temperature was recorded as low as 18°C or as high as 28°C. Using the RRT these variations were easily recognized and corrected by adjusting the acceptable RRT stored in the data system. The difference in the RRT in the day-to-day assay for the standards, analyzed over a period of five months, was less than 0.039 (see Fig. 4).

Intra- and interday variability for the complete procedure, including the extraction from the biological fluids, gave very little variations. Tests with plasma were more consistent than tests with urine (Table III and IV).

The separation/quantitation of 1,7-DMX from 1,3-DMX as well as several other metabolites of caffeine, which was previously described using a Perkin-Elmer high speed 5 μm C_{18} column [11], has been confirmed with two other columns of the same type obtained from the same manufacturer. There were no visible differences in the performance (retention time, response factors) between these individual columns.

The durability of this column (maximum number of injections of samples) has not been reported so far. In our experience, the durability of our first column was excellent. After many months of regular injections of extracts from serum and urine, the separation of peaks, especially those of the 1,7- and 1,3-isomers of DMX was still very good.

The elution pattern of the analyzed caffeine metabolites suffered very little or no interference from certain normal constituents in blood, milk or saliva. However, there were some urine samples (see Fig. 2D) where some normal urinary constituents, such as metabolites from dietary purines (i.e. urates, xanthines), could interfere with the early eluting metabolites. No interferences were found with the major metabolites of caffine, which are eluted later.

The main objective of the feeding study was to find a possible correlation between the levels of caffeine, theophylline, paraxanthine and theobromine in the blood of monkeys during their pregnancy, with a possible neuro-behavioral effect on the infant monkey. For that purpose the method described herein was accurate enough to establish the levels of these metabolites in the blood of individual monkeys before, during and after their pregnancies. In addition, very accurate data have been obtained for the caffeine metabolites in monkey's milk and saliva and, in addition, experimental evidence has been acquired regarding the excretion of the major caffeine metabolites in urine. While this project is still in progress, the experimental data for the metabolism of caffeine during pregnancy will be published elsewhere [15].

Although some expected variabilities in metabolic pattern between individual monkeys were observed, this variation was of a more quantitative than qualitative nature. There were, however, some differences in metabolites found in blood and those excreted in urine. The most significant difference was in the absence (Fig. 3C) or traces only (as in Fig. 2A) of 1,3-DMU in blood (plasma), while there was always a considerable amount of this metabolite in the urine (Fig. 2C). Contrary to this, a very close correlation was regularly observed for the pattern of metabolites found in plasma and in milk (Fig. 3). Similar close resemblances in the content of metabolites was observed between saliva and plasma, which has been also reported for humans [16], and confirmed in our laboratories (results not shown).

Finally, the main difference in the metabolism of caffeine in humans and monkeys seems to be in the reverse ratio of two isomers of dimethylxanthines: while 1,7-DMX is the main metabolite in humans [17] with very little of the 1,3-isomer, the main metabolite in the blood of our monkeys was 1,3-DMX with considerably smaller amounts of the 1,7-isomer (Figs. 2A and 3C).

Once the procedure and the HPLC methodology have been developed, an

automated injection system combined with data system for peak area integration can be used for quantitation of caffeine and its metabolites in biological fluids. This instrumentation increases the analytical precision with additional savings in time, labor and operating cost, with very little supervision from the analyst. Using this method an analytical chemist can handle 40—60 samples of biological fluids in 24 h. The number of samples is determined by the time required for the elution of the other slower moving components between injections, which are found in some biological fluid samples (especially urine). This assay is also applicable to the analysis of caffeine metabolites in biological fluids from other species including humans.

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DETERMINATION OF TIAPAMIL AND OF ITS TWO MAIN METABOLITES IN PLASMA AND IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Selective high-performance liquid chromatographic methods for the determination of tiapamil and its two main metabolites in plasma and urine are described. Tiapamil together with its metabolites is extracted at alkaline pH into dichloromethane. Separation is carried out using normal-phase high-performance liquid chromatography with ultraviolet detection (278 nm). The unchanged drug and the desmethyl metabolite are analysed simultaneously. The second metabolite is analysed separately under more polar conditions. The sensitivity limits are 50 ng/ml for tiapamil, 100 ng/ml for the desmethyl metabolite and 75 ng/ml for the second metabolite, using 0.5 ml of plasma. The sensitivity limits in urine are 100 ng/ml for all three compounds using a 0.5 ml specimen. The method has been applied to the analysis of human plasma and urine after intravenous (70 mg) and oral (400 mg) administration of tiapamil.

INTRODUCTION

Tiapamil, [I], N-(3,4-dimethoxyphenethyl)-2-(3,4-dimethoxyphenyl)-N-methyl-*m*-dithiane-2-propylamine 1,1,3,3-tetraoxide, is a new calcium antagonist, which is undergoing clinical evaluation as an antiarrhythmic and antihypertensive drug [1, 2]. Tiapamil is extensively metabolized in man [3]. The two main plasma and urine metabolites are the N-desmethyl derivative [II] and another secondary amine [III] (Fig. 1). Both metabolites have low pharmacological activity and do not contribute significantly to the effect of the parent drug.

Tiapamil is structurally related to verapamil. Several analytical procedures have been reported for determining verapamil in plasma, including sensitive

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methods such as gas chromatography—mass spectrometry (GC—MS) and high-performance liquid chromatography (HPLC) with fluorescence detection [4, 5]. However, due to its two sulphone groups, tiapamil cannot be determined readily by GC methods and has only poor fluorescence.

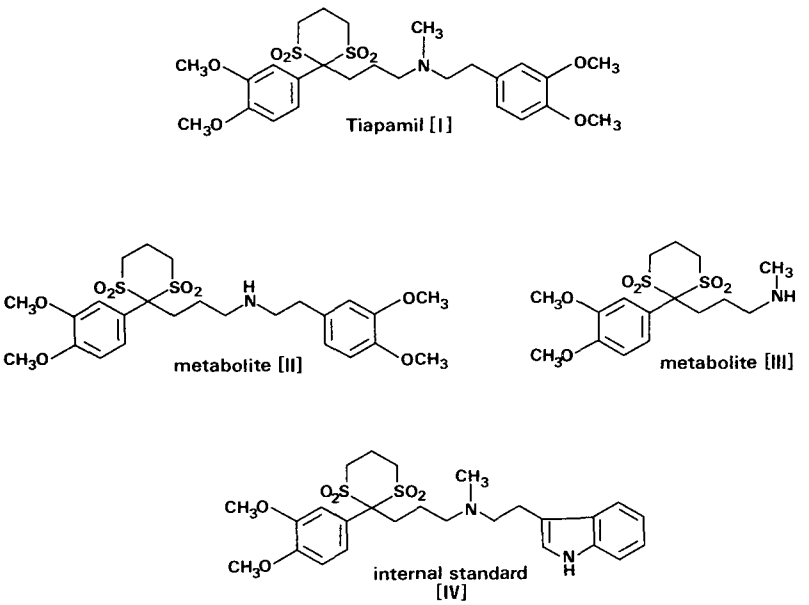

Fig. 1. Chemical structure of tiapamil, its main metabolites and of the internal standard.

In this paper we describe simple and selective normal-phase HPLC methods with ultraviolet (UV) detection for the determination of tiapamil and its two main metabolites in plasma and urine. Tiapamil and metabolite [II] were determined simultaneously. The analogous compound [IV] was used as internal standard. Metabolite [III] was assayed separately by external standardization.

EXPERIMENTAL

Reagents

Chloroform, methanol, dichloromethane, aqueous ammonia (25%), boric acid, and 1 *M* hydrochloric acid were all p.a. grade from E. Merck (F.R.G.). Other reagents were 0.2 mol/l buffer (sodium borate—sodium hydroxide) pH 10, and double-distilled water.

Chromatography

The following modular system was used: Altex Model 110 pump, injector Rheodyne 7125, 10 mV recorder W+W 1100 (all from Kontron Analytik, Switzerland), Knauer UV-detector type 8700 (0.02—0.04 a.u.f.s.) (Knauer, F.R.G.), autosampler ISS-100 (Perkin-Elmer, F.R.G.), computing integrator SP 4100 (Spectra Physics, U.S.A.).

Chromatography of tiapamil and metabolite [II]. A prepacked column (12.5

cm × 4 mm), Hibar RT 125-4, LiChrosorb Si 60, 5 μm, (Merck) was used. The mobile phase was a mixture of chloroform—methanol—25% ammonia (964:35:1) with a flow-rate of 1.2 ml/min at room temperature (90 bars). The monitoring wavelength was 278 nm. Under these conditions, the following retention times (in min) were obtained: t_R [I] = 4.5, t_R [II] = 7.0, internal standard t_R = 10.0.

Chromatography of metabolite [III]. Separations were carried out on a prepacked column Polygosil 60-5, 12.5 cm × 4 mm (Macherey-Nagel, F.R.G.). The mobile phase was a mixture of chloroform—methanol—25% aqueous ammonia (600:400:1) with a flow-rate of 2 ml/min at room temperature. The monitoring wavelength was 278 nm. Under these conditions the compound eluted at 4.5 min.

Solutions

Tiapamil* (10.64 mg HCl salt, corresponding to 10 mg of free base) was dissolved in 10 ml of water (Solution A).

Metabolite [II]* (11.07 mg fumarate salt, corresponding to 10 mg of free base) was weighed into a 10 ml flask and dissolved in water (6—7 ml) with the aid of sonication. The volume was then made up to 10 ml with water (Solution B).

Metabolite [III]* (10 mg free base) was dissolved in 10 ml of methanol (Solution C).

Internal standard* (10.68 mg HCl salt) was dissolved in 10 ml of methanol. This methanolic solution was then further diluted with water to obtain the concentrations suitable for internal standardization.

Calibration standards

Plasma standards for tiapamil and metabolite [II]. A stock plasma was prepared by adding 200 μl of both Solution A and Solution B (Hamilton syringe) to 50 ml of drug-free plasma (4 μg/ml). This stock plasma was then used to prepare lower concentration standards by stepwise dilution with drug-free plasma. The following standards were prepared: 4 μg/ml (stock plasma), 2, 1, 0.5, 0.25, 0.125 and 0.062 μg/ml.

Plasma standards for metabolite [III]. A stock plasma was prepared by adding 50 μl of Solution C (Hamilton syringe) to 25 ml of drug-free plasma (2 μg/ml). By stepwise dilution of this stock plasma, the following standards were prepared: 2 μg/ml (stock plasma), 1, 0.5, 0.25 and 0.125 μg/ml.

Urine standards for tiapamil and metabolite [II]. A stock urine was prepared by pipetting 5 ml of both Solution A and Solution B into a 50 ml flask and making up to the mark with drug-free urine, resulting in a concentration of 100 μg/ml for both compounds. The following standards were prepared by stepwise dilution with drug-free urine: 100 μg/ml (stock urine), 50, 25, 10, 5, 2.5, 1, 0.5 and 0.25 μg/ml.

Urine standards for metabolite [III]. A stock urine was prepared by adding 100 μl of Solution C to 25 ml of drug-free urine, resulting in a concentration

*Tiapamil (base): Ro 11-1781/00. Metabolite [II] (base): Ro 11-5398/00. Metabolite [III] (base): Ro 11-5220/00. Internal standard: Ro 11-6415.

of 4 μg/ml. By stepwise dilution of this stock urine, the following standards were prepared: 4 μg/ml (stock urine) 2, 1, 0.5, 0.25 and 0.125 μg/ml.

Extraction procedures

Extraction of tiapamil and metabolite [II] from plasma. Plasma (0.5 ml) was pipetted into a conical extraction tube (glass, 15 ml); 200 μl of an aqueous solution of the internal standard and buffer pH 10 (0.5 ml) were then added. After the addition of 10 ml of dichloromethane, the sample was extracted (tumbler extractor, 15 min, 20 rpm) and then centrifuged (1000 *g*) at 10°C for 5 min. The upper aqueous phase was then carefully aspirated and discarded; 9 ml of the organic phase were transferred into a new glass tube and evaporated to dryness under a gentle stream of nitrogen (35°C). The dry residue was redissolved in the eluent (200 μl) and injected for analysis (100 μl).

Extraction of metabolite [III] from plasma. Plasma (0.5 ml) was pipetted into a conical extraction tube. Water (0.5 ml) and buffer pH 10 (1 ml) were added and the mixture was briefly shaken by hand. Following the addition of 10 ml of dichloromethane, the subsequent steps were as described above.

Extraction of tiapamil and metabolite [II] from urine. Urine (0.5 ml) was pipetted into a conical tube, to which an aqueous solution of the internal standard and 0.5 ml of buffer pH 10 were then added. After brief mixing on a Vortex-mixer, 10 ml of dichloromethane were added and the sample was extracted and then centrifuged as described above for plasma samples. The upper aqueous layer was carefully withdrawn by aspiration and discarded; 9 ml of the organic phase were transferred into a new tube; 2 ml of 1 *M* hydrochloric acid solution were added, the tube was rotated for 5 min (20 rpm), followed by centrifugation for 5 min (1000 g, 10°C). The aqueous phase was discarded. An aliquot of the organic phase (taking care to avoid carry-over of the remaining acidic phase) was again transferred into a new tube and evaporated to dryness under a gentle stream of nitrogen. The residue of the extract was immediately redissolved in the eluent (200 μl) and injected for analysis (100 μl).

Extraction metabolite [III] from urine. Urine (0.5 ml) was pipetted into a conical extraction tube. Buffer pH 10 (0.5 ml) was added and the mixture briefly shaken by hand. The subsequent steps were as described for plasma samples.

Calibration

Standards (4—5) covering the concentration ranges described below, were processed as described above and analysed alongside the unknown samples. For the determination of tiapamil and [II], the peak height ratios of unchanged drug to the internal standard were measured and the calibration curve was obtained from the least-squares linear regression of the peak height ratio against concentration. This regression line was then used to calculate the concentration of the unchanged drug in unknown samples. Calibration ranges for [I] and [II] were as follows: 0.06—0.5 μg/ml and 0.25—4 μg/ml for plasma; 0.25—5 μg/ml and 5—100 μg/ml for urine. For the determination of [III], external standardization was used. Calibration ranges were 0.125—2 μg/ml (plasma) and 0.125—4 μg/ml (urine).

RESULTS

Recovery

Spiked plasma and urine samples of various concentrations were prepared and extracted as described above, except that the internal standard was not added. Another series of standards was prepared by adding solutions of the free bases of tiapamil, [II] and [III] in the eluent to extracts of drug-free plasma or urine. The peak heights obtained from this latter experiment provided the 100% values, which could be compared with the peak heights obtained from the extracted spiked standards. The mean recoveries were as follows: tiapamil, plasma 72%, urine 101%; metabolite [II], plasma 79%, urine 90%; metabolite [III], plasma 99%, urine 95%.

Linearity

Linear correlations between peak height ratios and the concentrations of tiapamil and [II] (respectively between peak height and the concentration of [III]) were found in the range of the above-mentioned calibration standards.

Reproducibility

Inter-assay reproducibility was calculated from spiked plasma and urine samples of various concentrations, which were analysed as replicates on different days, using a new calibration each day. Tables I and II present the

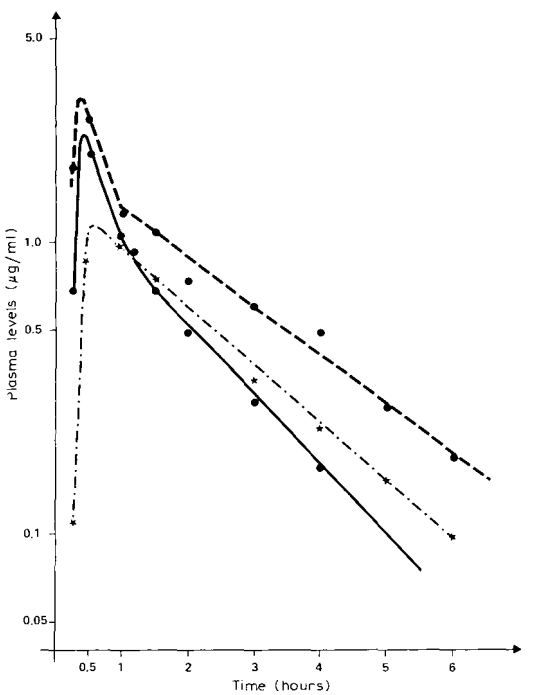

Fig. 2. Plasma levels of tiapamil and metabolites after a single oral administration of 400 mg of tiapamil to a healthy volunteer. (— — —), tiapamil; (———), metabolite [II]; (— • — •), metabolite [III].

TABLE I

INTER-ASSAY REPRODUCIBILITY (C.V.) AND ACCURACY OF THE PLASMA ASSAY

Conc. added (μg/ml)	[I]			[II]			[III]			*n*
	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	
0.12	0.12	13	± 0	0.13	12	+ 8.3				7
0.31	0.32	5.6	+ 3.2	0.33	4.3	+ 6.0				12
0.65	0.61	4.6	− 6.1	0.66	5.7	+ 1.5				6
2.50	2.60	4.5	+ 4.0	2.60	6.2	+ 4.0				11
0.10							0.10	16	± 0	4
0.25							0.26	4.8	+ 4.0	4
0.50							0.51	7.5	+ 2.0	5
1.00							0.99	2.6	− 2.6	5

TABLE II

INTER-ASSAY REPRODUCIBILITY (C.V.) AND ACCURACY OF THE URINE ASSAY

Conc. added (μg/ml)	[I]			[II]			[III]			*n*
	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	Conc. found (μg/ml)	C.V. (%)	Accuracy (%)	
0.25	0.26	10	+ 4	0.25	2.8	± 0				7
1.25	1.23	3.0	− 1.6	1.24	3.0	− 0.8				6
5.0	4.82	2.6	− 3.6	5.23	3.4	+ 4.6				3
25.0	23.6	5.0	− 5.6	24.3	5.0	− 2.8				6
100	96.2	0.5	− 3.8	98.0	1.8	− 2.0				3
0.25							0.24	11.3	− 4.0	4
1.0							1.00	2.6	± 0	5
25.0							24.5	5.4	− 2.0	7
100							98.4	7.2	− 1.6	5

TABLE III

CONTENT OF TIAPAMIL AND OF ITS METABOLITES IN URINE AFTER A SINGLE INTRAVENOUS DOSE OF 70 mg OF TIAPAMIL TO A HEALTHY VOLUNTEER

Time after administration (h)	Urine volume (ml)	Concentration (μg/ml)			Cumulative amount excreted (mg)		
		Tiapamil [I]	[II]	[III]	Tiapamil [I]	[II]	[III]
0—8	1300	11.20	0.30	3.0	14.56	0.39	3.90
8—24	850	1.23	n.d.*	1.55	15.61	0.39	5.05
24—32	525	0.24	n.d.	0.49	15.74	0.39	5.31
32—48	900	n.d.	n.d.	0.21	15.74	0.39	5.50

*n.d. = below limit of detection.

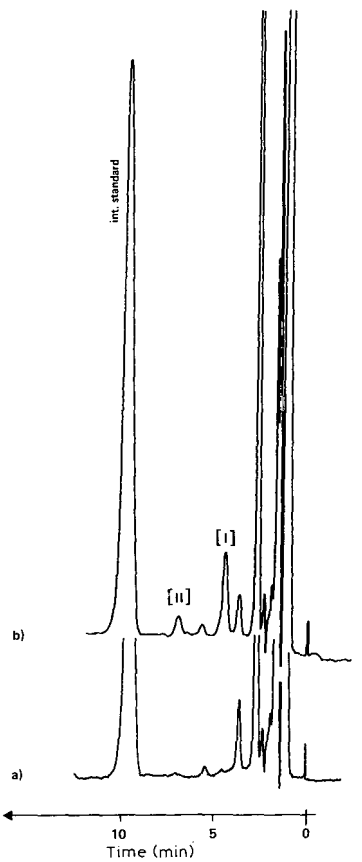

Fig. 3. Determination of tiapamil and [II] in plasma. Chromatograms of plasma extracts of a volunteer having received an intravenous dose of tiapamil (1 mg/kg). Detector range setting: 0.02 a.u.f.s. (a) drug-free plasma; (b) plasma, 45 min after administration. Tiapamil [I] = 210 ng/ml; metabolite [II] = 100 ng/ml.

inter-assay reproducibility (C.V., %) and accuracy (difference between found and added concentration) at different concentrations. The overall precision of the plasma and of the urine assay was about 5%. In the low concentration range (⩽ twice the limit of detection) the coefficient of variation was about 10%. Accuracy was better than two times the standard deviation (S.D.).

Limit of detection

Using 0.5 ml of plasma, the limits of detection were 50 ng/ml for tiapamil, 100 ng/ml for [II] and 75 ng/ml for [III]. For 50 ng/ml tiapamil a 5 mm peak height was obtained, the signal-to-noise ratio being 6:1. Using 1 ml of plasma, a lower detection limit could possibly be obtained. For urine samples, the limit of detection was found to be 100 ng/ml for all three compounds.

Stability of the drug in plasma

Tiapamil and its metabolites were found to be stable in plasma at −18°C for at least six months.

Application of the method to biological samples

The method has been applied to the analysis of tiapamil and its main metabolites in plasma and urine of volunteers and patients after intravenous and oral

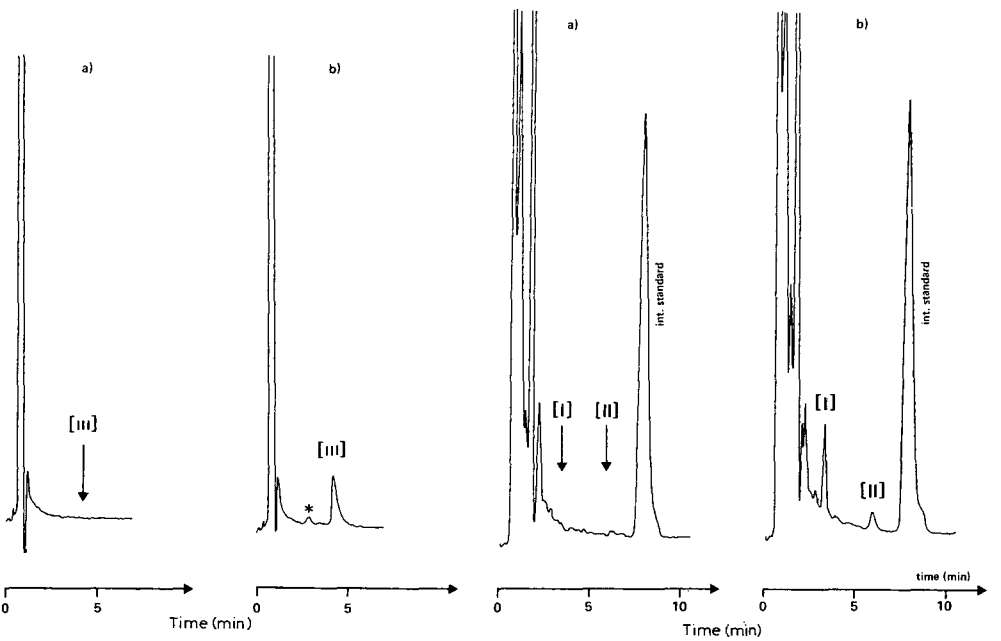

Fig. 4. Determination of [III] in plasma. Chromatograms of plasma extracts of a volunteer having received an intravenous dose of tiapamil (70 mg). Detector range setting: 0.02 a.u.f.s. (a) pre-dose sample, (b) plasma, 40 min after administration. Metabolite [III] = 240 ng/ml; * = minor metabolite.

Fig. 5. Determination of tiapamil and [II] in urine. Chromatograms of urine extracts of a volunteer having received an intravenous dose of tiapamil (70 mg). Detector range setting: 0.02 a.u.f.s.; flow-rate 2.3 ml/min. (a) Pre-dose urine; (b) collection period 24—32 h after administration. Tiapamil [I] = 270 ng/ml; metabolite [II] = 150 ng/ml.

administration. Plasma concentrations of tiapamil and of its metabolites after an oral dose of 400 mg are indicated in Fig. 2. Urine concentrations after an oral dose of 400 mg are shown in Table III. Chromatograms of plasma and urine extracts from volunteers having received a 70 mg intravenous dose are shown in Figs. 3—5.

DISCUSSION

Tiapamil, together with its two metabolites, is extracted at alkaline pH into dichloromethane. However, as the polarity of [III] is rather different from that of the two other compounds, a simultaneous determination of these three compounds could not be achieved under isocratic conditions. Therefore, [III] had to be analysed separately under more polar conditions.

The chromatogram illustrated by Fig. 3 represents the normal separation of tiapamil from endogenous peaks, that could be obtained in routine analysis. In the case of less efficient columns or in the presence of interfering comedication, adequate separation could be achieved by reducing the methanol and/or ammonia content of the mobile phase (for instance, to 974:25:1 chloroform—methanol—ammonia).

The eluent proportions 964:35:1 were also used for the analysis of urine samples from healthy volunteers. For the analysis of urine samples from patients, it was sometimes advantageous to reduce the content of methanol or ammonia. In this way, the separation from comedicated drugs could be improved.

For the determination of unchanged drug and [II] in urine, especially in the low concentration range (below 250 ng/ml), it was necessary in most cases to wash the organic extract with 1 *M* hydrochloric acid. Tiapamil and [II] remained in the organic phase, whereas interfering compounds and [III] were extracted into the acidic phase. Some of the urine samples, especially in the higher concentration range, could be analysed without this clean-up step.

In addition, this clean-up was sometimes necessary for the analysis of plasma samples from patients.

The recovery for tiapamil and [II] was lower for plasma samples than for urine samples, whereas the recovery of [III] was not affected by the change in sample matrix. This result may be explained by strong binding of these two compounds to protein materials in the plasma.

ACKNOWLEDGEMENT

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CHROMBIO. 2183

DETERMINATION OF BROMAZEPAM IN PLASMA AND OF ITS MAIN METABOLITES IN URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of bromazepam in plasma and of its main metabolites in urine is described. The unchanged drug is extracted from plasma with dichloromethane, using Extrelut 1 extraction tubes. The residue from this extract is subsequently analysed by reversed-phase high-performance liquid chromatography with ultraviolet detection (230 nm). The limit of detection is 6 ng/ml of plasma, using a 1-ml specimen. For the determination of the metabolites, the urine samples are incubated to effect enzymatic deconjugation and are then extracted with dichloromethane. Following two clean-up steps (back extractions), the final residue is analysed on the same reversed-phase system as the plasma samples. The limit of detection for the two metabolites is 200 ng/ml.

INTRODUCTION

Bromazepam (I) is a member of the 1,4-benzodiazepine class of compounds and is in clinical use as an antianxiety agent. After both oral and intravenous administration the compound is almost completely metabolized [1, 2]. The major urinary metabolites are the conjugates of 3-hydroxybromazepam (II) and of 2-(2-amino-5-bromo-3-hydroxybenzoyl)pyridine (III). The parent drug (I) and 2-(2-amino-5-bromobenzoyl)pyridine (ABBP) (IV) are excreted only in small amounts (Fig. 1).

Several methods have been described for the determination of the parent drug. Gas—liquid chromatographic (GLC) determinations include the direct measurement of the unchanged drug [3], hydrolysis followed by quantification as 2-(2-amino-5-bromobenzoyl)pyridine (ABBP) [4, 5], and determination as the N^1-methyl derivative [6]. With thin-layer chromatography (TLC),

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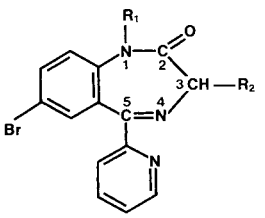
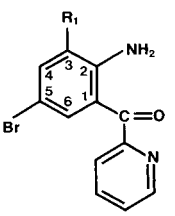
1,4-benzodiazepin-2-one



Compound	R_1	R_2
Bromazepam [I]	H	H
3-Hydroxy-Bromazepam [II]	H	OH
N_1-Methyl-Bromazepam [V]	CH_3	H

benzoyl-pyridine



Compound	R_1
ABBP [IV]	H
3-Hydroxy-ABBP [III]	OH

Fig. 1. Chemical structure of bromazepam, its metabolites, and the internal standards.

bromazepam can be determined either directly or after derivatization to an azo dye on the plate [7].

It was the aim of this work to develop a high-performance liquid chromatographic (HPLC) method for the determination of the unchanged drug in plasma and of the main metabolites in urine. Methylbromazepam (V) was used as internal standard.

EXPERIMENTAL

Reagents and materials

Dichloromethane, methanol, 3 *M* and 1 *M* sodium hydroxide and 3 *M* hydrochloric acid were all p.a. grade from E. Merck (F.R.G.). Phosphate buffers (Sörensen) were 0.067 *M* pH 7.5, 0.5 *M* pH 5, and 1 *M* pH 6; sodium acetate buffer was 0.2 *M* pH 5. Enzymes were Suc d'*Helix pomatia*, 100,000 units (Fishman) β-glucuronidase, 1,000,000 units (Roy) sulphatase (Pharmindustrie, France), and β-glucuronidase from bovine liver, 170,000 Fishman units/g (Serva, F.R.G.).

Solid extraction columns (glass) Extrelut 1 (Art. No. 15371, Merck) were used.

Chromatography

The following modular system was used: Waters M6000 HPLC pump (Waters Assoc., U.S.A.); Knauer Model 8700 UV—HPLC detector (Knauer, F.R.G.); automated sampling system ISS-100 (Perkin-Elmer, F.R.G.); Model 1100 W+W recorder (Kontron, Switzerland).

The column was a prepacked HPLC column Supelcosil LC 18, 5 μm, 15 cm × 4.6 mm (Supelco, Switzerland).

The mobile phase consisted of a mixture of methanol—0.067 *M* phosphate buffer pH 7.5 (47:53) at a flow-rate of 1 ml/min (room temperature, 70 bars). The monitoring wavelength was 230 nm, with a detector range setting of 0.04 a.u.f.s.

The following retention times (min) were obtained: 3-hydroxybromazepam

(II), 6.2; 3-hydroxy-ABBP (III), 9.0; bromazepam (I), 10.0; internal standard (V), 11.5; ABBP (IV), 19.5.

Solutions

Methanolic solutions (0.5 mg/ml) of compounds I, II, III and V (internal standard) were prepared. The bromazepam solution (I) was further diluted with water to a concentration of 0.05 mg/ml. From the solution of the internal standard (V), further dilutions in water were made to be used for internal standardization.

Plasma standards. A 200-μl volume of the aqueous solution of bromazepam (I) was added to drug-free plasma (50 ml). Starting from this stock plasma (200 ng/ml), the standards 100, 50, 25 and 12.5 ng/ml were prepared by stepwise dilutions with drug-free plasma.

Urine standards. A 200-μl volume of each of the methanolic solutions of II and III was added to drug-free urine (50 ml). Further dilutions from this stock urine (2000 ng/ml) with drug-free urine were made to obtain the concentrations 1500, 1000, 500 and 250 ng/ml.

Extraction of plasma samples

The extraction was carried out using solid extraction columns Extrelut 1. First, 1 *M* sodium hydroxide (100 μl), plasma (1 ml) and 100 μl of the aqueous solution of the internal standard, in that order, were applied to the column. Then the mixture was allowed to stand for 10 min and the drug was then extracted twice, each time with a 5-ml portion of dichloromethane. The extracts were combined in a conical glass tube and then evaporated to dryness under a gentle stream of nitrogen. The residue of the extract was redissolved in the eluent (200 μl) and injected for analysis (100 μl).

Extraction of urine samples

Urine (1 ml), buffer (1 ml), internal standard (100 μl of an aqueous solution) and enzyme were mixed and then allowed to incubate overnight at 37°C in a shaking water bath. The buffer was either acetate (0.2 *M*, pH 5) for Suc d'*Helix pomatia* (20 μl of enzyme solution per ml of urine) or phosphate (0.5 *M*, pH 5) for the Serva enzyme (1500 units/ml of urine). The pH of the mixture was then adjusted to 6 by dropwise addition of 1 *M* sodium hydroxide. After the addition of dichloromethane (10 ml), the sample was extracted by shaking on a rotating shaker (10 min, 40 rpm) and then centrifuged (1000 *g*) at 10°C for 5 min. The upper aqueous layer was carefully aspirated and discarded. An aliquot of the organic phase (8 ml) was transferred into a new conical tube. Then 3 *M* hydrochloric acid (1 ml) was added and the mixture again shaken on a rotating shaker (10 min). After centrifuging (5 min), the organic phase was carefully aspirated and discarded. Then 3 *M* sodium hydroxide (1 ml) and 1 *M* phosphate buffer pH 6 (1 ml) were added to the remaining aqueous phase. The resulting aqueous solution (pH 6) was allowed to stand for 20 min and was then extracted with dichloromethane (10 ml). The aqueous phase was again aspirated and discarded. A 9-ml volume of the remaining organic phase was transferred into a new conical glass tube and evaporated to dryness under a gentle stream of nitrogen (35°C). The residue of the extract was redissolved in the eluent (300 μl) and injected for analysis (50 μl) (Fig. 2).

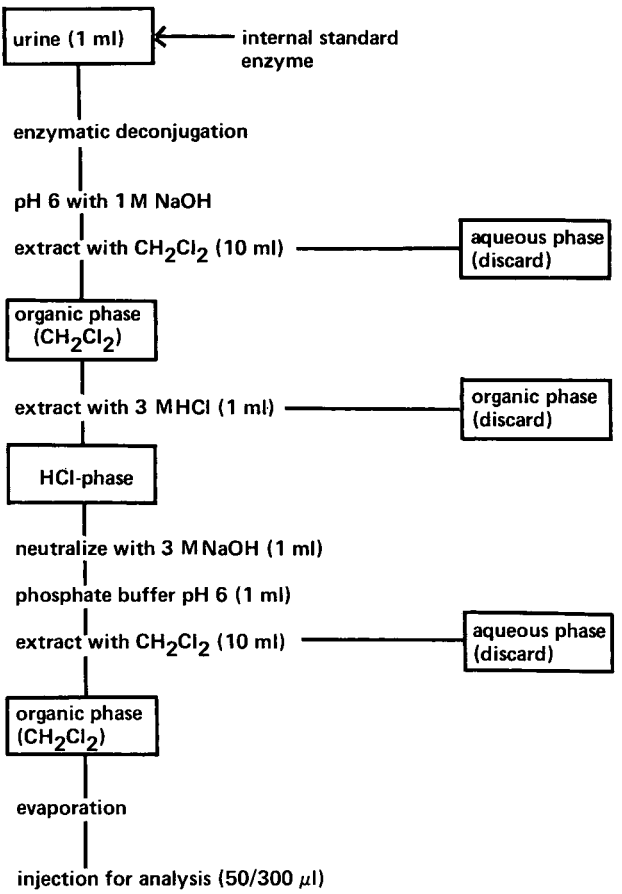


Fig. 2. Scheme for urine analysis.

Calibration

Plasma or urine standards (4—5) were processed as described above and analysed as calibration samples alongside the unknown samples. Peak height ratios of unchanged drug to internal standard were measured and the calibration curve was obtained from a least-squares linear regression. This regression line was then used to calculate the concentration of the drug in the unknown samples.

RESULTS

Recovery

Spiked plasma and urine standards of known concentrations were extracted as described above, except that the internal standard was not added. Another series of standards was prepared by adding solutions of I, II and III in the eluent to extracts of drug-free plasma (I) or urine (II, III). The peak heights obtained from this latter experiment provided the 100% values which could be compared with the peak heights obtained from the extracted spiked standards. The recovery of I from plasma was between 87% and 95% (Table I). The

TABLE I

RECOVERY OF BROMAZEPAM (I) FROM SPIKED PLASMA SAMPLES

Concentration (ng/ml)	Replicates (*n*)	Recovery (%)	C.V.* (%)
12.5	4	87	10.5
50.0	4	91	8.4
200	4	95	3.2

*C.V. = coefficient of variation.

TABLE II

RECOVERY OF 3-HYDROXYBROMAZEPAM (II) AND 3-HYDROXY-ABBP (III) FROM SPIKED URINE SAMPLES

Concentration (ng/ml)	Replicates (*n*)	II		III	
		Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
250	4	53	2.8	49	10.8
400	4	62	4.8	51	7.6
1000	4	60	9.0	58	9.6
2000	4	56	4.6	59	5.4

recovery of II and III from urine was between 53% and 62%, and between 49% and 59%, respectively (Table II).

Linearity

A linear correlation between peak height ratios and the concentration of I in plasma or II and III in urine was found in the range of the calibration standards used (12.5—200 ng/ml in plasma, 250—2000 ng/ml in urine).

TABLE III

INTER-ASSAY PRECISION OF BROMAZEPAM (I) IN PLASMA

Experiment was performed with volunteer plasma samples.

Sample	Replicates (*n*)	Mean concentration found (ng/ml)	C.V. (%)
101	4	88	4.0
103	3	77	2.6
104	3	72	2.8
105	4	71	3.3
107	3	74	1.6
110	4	61	2.3
112	3	50	3.1
113	4	26	7.8
114	3	16	9.4
115	3	8	8.3

TABLE IV

INTER-ASSAY PRECISION OF 3-HYDROXYBROMAZEPAM (II) AND OF 3-HYDROXY-ABBP (III) IN URINE

Experiment was performed with volunteer urine samples.

II		III		*n*
Mean concentration found (ng/ml)	C.V. (%)	Mean concentration found (ng/ml)	C.V. (%)	
213	8.2	266	8.3	3
338	7.0	560	5.9	4
429	1.6	797	6.5	3
648	8.9	897	6.5	3
864	6.5	1170	3.9	3
1080	7.9	2090	6.8	3
1360	4.8	6330	8.2	3

Reproducibility

Inter-assay precision was calculated from volunteer plasma or urine samples, which were analysed as replicates on different days using a new calibration each day. For plasma samples, the mean coefficient of variation was found to be ± 2.8% (concentrations > 25 ng/ml) and ± 8.5% (concentrations < 25 ng/ml) (Table III). For urine samples, the mean coefficient of variation was found to be ± 6.4% (II) and 6.6% (III) (Table IV).

Detection limit

Under the conditions described, the limit of detection for I in plasma was

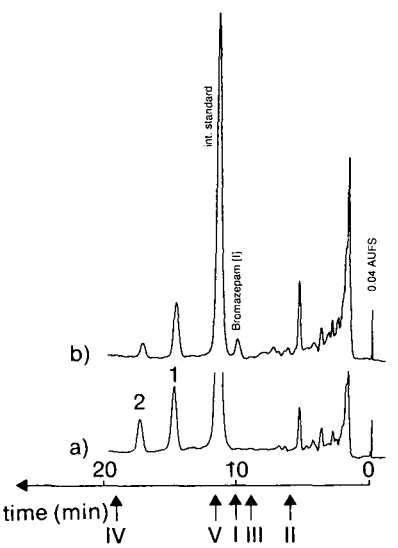


Fig. 3. Chromatograms of plasma extracts after a 6-mg oral dose of bromazepam to a volunteer. The arrows indicate the retention times of the compounds from Fig. 1. Peak 1 = impurity from plasma; peak 2 = impurity from the Extrelut material. (a) Pre-dose plasma, (b) plasma, 48 h after administration. Bromazepam concentration = 11 ng/ml.

6 ng/ml plasma. At this concentration a signal-to-noise ratio of 5:1 was observed. The detection limit for compounds II and III in urine was 200 ng/ml.

Application of the method

Plasma and urine samples from volunteers receiving 6 mg oral and intravenous administrations of bromazepam were analysed by this method. Fig. 3 shows chromatograms of plasma extracts from a volunteer. Plasma levels of the parent drug after oral administration of bromazepam to a volunteer are presented in Fig. 4. In this case the elimination half-life $t_{½\beta}$ was found to be 15 h. Fig. 5 shows chromatograms of urine extracts of a volunteer.

The absolute bioavailability of an oral solution of bromazepam is lower than that of the conventional formulation. As a possible explanation for this, ring opening of bromazepam at the low pH which obtains in the stomach, followed

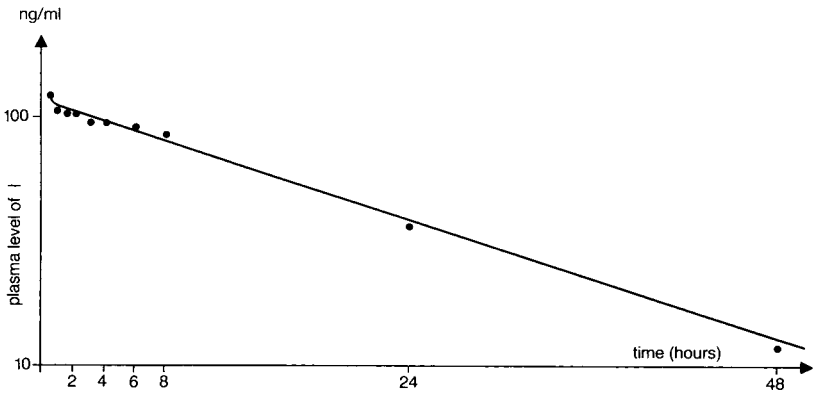


Fig. 4. Plasma levels of bromazepam after a 6-mg oral dose of bromazepam to a volunteer. Elimination half-life $t_{½\beta}$ = 15 h.

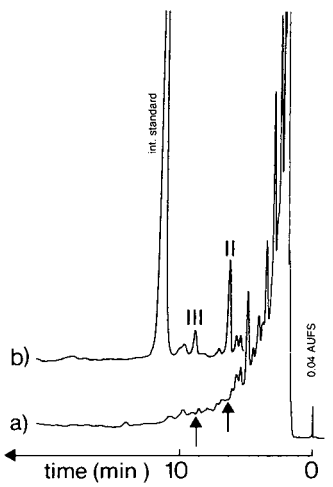


Fig. 5. Chromatograms of urine extracts after a 6-mg intravenous dose of bromazepam to a volunteer. Enzyme: β-glucuronidase from bovine liver, 3 h incubation. (a) Pre-dose urine, (b) urine fraction 12—24 h after administration. Concentration of II = 340 ng/ml, concentration of III = 150 ng/ml.

TABLE V

EXCRETION OF 3-HYDROXYBROMAZEPAM (II) AND OF 3-HYDROXY-ABBP (III) IN URINE FOLLOWING ORAL OR INTRAVENOUS APPLICATION OF 6 mg OF BROMAZEPAM TO A VOLUNTEER

Urine fraction (h)	II				III			
	Oral		Intravenous		Oral		Intravenous	
	ng/ml	Percentage dose cumulative	ng/ml	Percentage dose cumulative	ng/ml	Percentage dose cumulative	ng/ml	Percentage dose cumulative
0—12	200	2.1	614	9.8	1880	20.3	324	5.1
12—24	334	7.1	975	16.6	226	23.6	210	7.1
24—36	338	13.5	292	20.1	130	26.0	—	—

by formation of metabolite III from this open form, was invoked. This hypothesis was proved by determination of the corresponding metabolites in urine [8]. More of metabolite III than metabolite II was found following oral administration of bromazepam. After intravenous administration, more of metabolite II was found (Table V).

DISCUSSION

In our hands, the direct GLC measurement of underivatized bromazepam suffered from strong adsorption of bromazepam to the stationary phase. The two hydrolytic GLC assays for the determination of I in plasma were time-consuming and not specific, since metabolites II and IV, if present in plasma, would yield the same hydrolysis product. The present HPLC assay for the determination of bromazepam in plasma does not require any derivatization procedure or time-consuming clean-up step. The method is specific, since all known metabolites are separated from the parent drug.

Classical liquid—liquid extraction, using a 10-ml portion of dichloromethane, gave adequate recovery and clean extracts as did solid extraction columns. However, extraction with Extrelut 1 columns proved to be the most convenient and most rapid technique for this purpose. The columns were used without exit cannulae to avoid an interfering compound from this plastic material. Under the conditions described, the C_{18} material from Supelco was the most stable of those used by us.

Slight variations in the eluent composition had a marked influence on the retention times. For instance, by increasing the methanol content from 47% to 50% retention times were reduced for about 20%. Reducing the molarity of the buffer solution resulted in a poor peak shape for both I and V.

The determination of II and III in urine, following direct extraction, was not possible due to endogenous interferences. A clean-up step (back-extraction) was necessary.

The overnight incubation was carried out for practical reasons, though a 3-h incubation was sufficient for quantitative deconjugation [1, 3]. Incubation with the Serva enzyme resulted in a cleaner extract, and we therefore recommend the use of this enzyme or any other purified β-glucuronidase for this purpose.

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CHROMBIO. 2185

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF CADRALAZINE FROM ITS POTENTIAL METABOLITES AND DEGRADATION PRODUCTS. QUANTITATION OF THE DRUG IN HUMAN PLASMA AND URINE

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SUMMARY

The chromatographic behaviour of cadralazine and its potential metabolites and degradation products with respect to pH, buffer molarity and composition of eluent is described. A selective method with an adequate sensitivity for the determination of the drug in human plasma and urine is also reported. The method includes extraction of biological fluids with chloroform and the analysis of extracts on a reversed-phase column with isocratic elution and detection at 254 nm. The method has been applied to the analysis of plasma and urine of a patient administered a single oral dose of 30 mg of cadralazine.

INTRODUCTION

Cadralazine (Fig. 1), ethyl 2-{6-[ethyl-(2-hydroxypropyl)amino]-3-pyridazinyl}hydrazinecarboxylate, is a new antihypertensive agent [1] which has shown its long-lasting activity in animals and man at very low dosages [2—6]. Studies of degradation in aqueous solutions [7] at different conditions of pH, temperature, concentration and in the presence or absence of light and oxygen, showed the formation of the following main degradation products: the pyridazine (PY), the pyridazinone (PYO) and the triazolone (TZO) derivatives (Fig. 1).

Metabolic investigations in rat and dog have shown that the drug is transformed into several metabolites [8, 9]. The main metabolites besides PY, PYO and TZO, are methyltriazole (MTZ), triazole (TZ), the N-deethyl derivative (PPY), the N-deisopropanol derivative (EPY), the oxopropyl derivative (OPY) and the deethoxycarbonyl derivative (HPY) (Fig. 1).

This paper describes a high-performance liquid chromatographic (HPLC)

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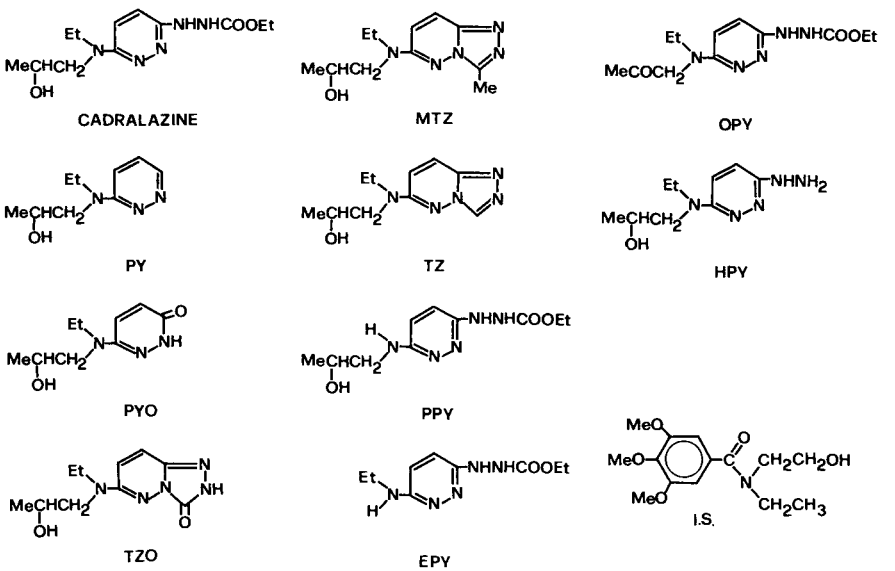

Fig. 1. Chemical structures of cadralazine, its degradation products, metabolites, and internal standard (I.S.).

separation of the parent drug from its main degradation products and metabolites, and its quantitation in human plasma and urine. An example from a pharmacokinetic study is also shown.

EXPERIMENTAL

Chemicals and glassware

Cadralazine, PY, PYO, TZO, MTZ, TZ, PPY, EPY, OPY, HPY and the internal standard (I.S., Fig. 1) were synthesized in our laboratories. Chloroform, methanol and acetonitrile (LiChrosolv) were obtained from Merck (Darmstadt, F.R.G.); double-distilled water, 0.05 *M* sulphuric acid, sodium dihydrogen phosphate, phosphate buffer pH 7.4, 0.1 *M* hydrochloric acid, and sodium bicarbonate were from Carlo Erba (Milan, Italy). Separating phase filters (Whatman, Maidstone, U.K.) were used after extraction with chloroform.

The test tubes were cleaned with sulphochromic mixture and then silanized with a 10% toluene solution of Surfasil (Pierce, Rockford, IL, U.S.A.).

Cadralazine and internal standard were dissolved in pH 7.4 buffer for the calibration curves as reported under assay procedure and under calibration curves.

Chromatographic conditions

The high-performance liquid chromatograph consisted of a Model 6000A pump, a Model Lambda Max 480 ultraviolet (UV) absorbance detector operating at 254 nm and a U6K injector, all from Waters Assoc. (Milford, MA, U.S.A.). The signal of the detector was recorded with an Omniscribe recorder from Houston Instruments (Austin, TX, U.S.A.) and integrated with an M2 integrator from Perkin-Elmer (Norwalk, CT, U.S.A.). A stainless-steel column,

Hibar RP-8, 10 μm (25 cm × 4 mm I.D., Merck) connected with a precolumn dry packed with Perisorb RP-8 30—40 μm (Merck) was used. The mobile phase consisted of acetonitrile—0.1 *M* sodium dihydrogen phosphate in double-distilled water brought to pH 6.0 with 0.1 *M* sodium hydroxide (15:85); the flow-rate was 2.5 ml/min for urine and 2.7 ml/min for plasma analysis.

Assay procedure in plasma

To 2-ml aliquots of human plasma, 1 ml of internal standard solution (621 ng/ml) was added and the mixture was brought to 5 ml with pH 7.4 phosphate buffer. Extraction was performed with 6 ml of chloroform by mechanical shaking for 15 min. After centrifugation at 2900 *g* for 5 min, the organic phase was filtered through a phase separator filter. The aqueous phase was extracted again with 6 ml of chloroform with the same procedure. The organic layers were collected and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 2 ml of methanol, vortexed, and then dried under nitrogen. Methanol (100 μl) and 0.05 *M* sulphuric acid (50 μl) were added and 50 μl were injected.

Assay procedure in urine

Aliquots of 1 ml of human urine were brought to 4 ml with water, added with 0.5 ml of 0.1 *M* hydrochloric acid and then extracted with 10 ml of chloroform by mechanical shaking for 20 min. After centrifugation at 2900 *g* for 3 min, the organic layer was discarded, the aqueous phase was added with 1 ml of a saturated solution of sodium bicarbonate and 1 ml of internal standard (12.43 μg/ml) and then extracted with 10 ml of chloroform as previously described. After centrifugation the organic layer was filtered through a phase separator filter, evaporated to dryness in the usual manner and reconstituted with 50 μl of 0.05 *M* sulphuric acid and 50 μl of methanol. Then 25 μl of the mixture were injected.

Calibration curves

Drug solutions in pH 7.4 phosphate buffer ranging from 800 to 60 ng/ml were used for plasma (S-1) and from 35 to 1 μg/ml for urine (S-2). Aliquots of 1 ml of S-1 were added to 2 ml of human plasma to obtain amounts of cadralazine in the range 400 to 30 ng/ml of plasma and aliquots of 1 ml of S-2 were added to 1 ml of human urine to obtain amounts from 35 to 1 μg/ml of urine. Then the samples were treated according to the assay procedures.

Study in man

A male patient (with essential hypertension) was orally given a single dose of 30 mg of cadralazine as a tablet. Blood samples collected at 0, 0.5, 1, 3, 6, 9 and 12 h after administration were mixed with heparin and centrifuged. The separated plasma was immediately frozen and stored at −20°C until the analysis. Urine was collected at 0, 0—3, 3—6, 6—9, 9—12, 12—24 and 24—30 h after administration; the volumes were recorded and aliquots of 50 ml of each sample were immediately frozen and stored at −20°C until analysis.

RESULTS AND DISCUSSION

Determination of chromatographic conditions

The goal of this study was to achieve separation between cadralazine and its potential metabolites and degradation products in order to selectively quantify the drug in biological fluids. Thus we have used C_8 reversed-phase hydrophobic chromatography and systematically studied different factors which control the retention of the examined compounds.

Effect of pH

Fig. 2 shows the pH dependence of the capacity factor k' for the examined compounds calculated in the usual way as the mean of at least three determinations. The range of pH values explored was between 2 and 7. It can be seen that for cadralazine, HPY, PY and MTZ the capacity ratios are sensitive to pH and this dependence is sigmoidal (with inflection points between pH 4 and 7).

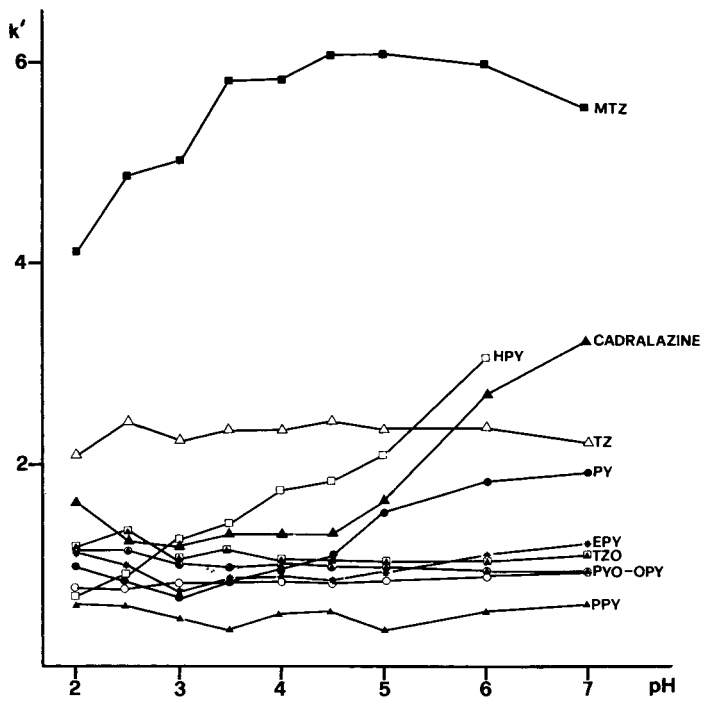

Fig. 2. Effect of pH on capacity ratio (0.1 *M* buffer—acetonitrile, 85:15).

This behaviour reflects the fact that, in general, the retention of weak bases from aqueous solutions, as a function of pH, follows their dissociation curves [10]. Consequently, the inflection points are located at $pH_m = pK_{am}$, i.e. when the pH of the mobile phase and the pK_a of the solute are equal. In Table I the pK_a values of all the compounds are reported. The best separations of the drug from its potential metabolites and degradation products were obtained at pH values higher than 5. Thus pH 6 was selected as the best compromise between selectivity and column stability.

TABLE I

pK_a VALUES OF CADRALAZINE AND ITS POTENTIAL METABOLITES AND DEGRADATION PRODUCTS IN AQUEOUS SOLUTION

Compound	pK_a*
Cadralazine	6.0
PYO	0.6
TZ	2.5
HPY	3.0 and 7.1
MTZ	4.5
PY	5.5
EPY	6.7
PPY	6.7
OPY	9.0
TZO	9.25

*Values were calculated by photometric titration.

Buffer molarity

The screening of the buffer molarity (Fig. 3) showed that the retention of all the considered products is largely influenced when the molarity is below 0.05, while it is practically insensitive above that value. Nevertheless at higher molarity the peaks sharpen, so the value of 0.1 *M* was selected in order to have the greatest sensitivity.

Effect of eluent composition

The composition of the eluent was explored between 10% and 20% of acetonitrile (Fig. 4). As expected, an increase in acetonitrile decreases the

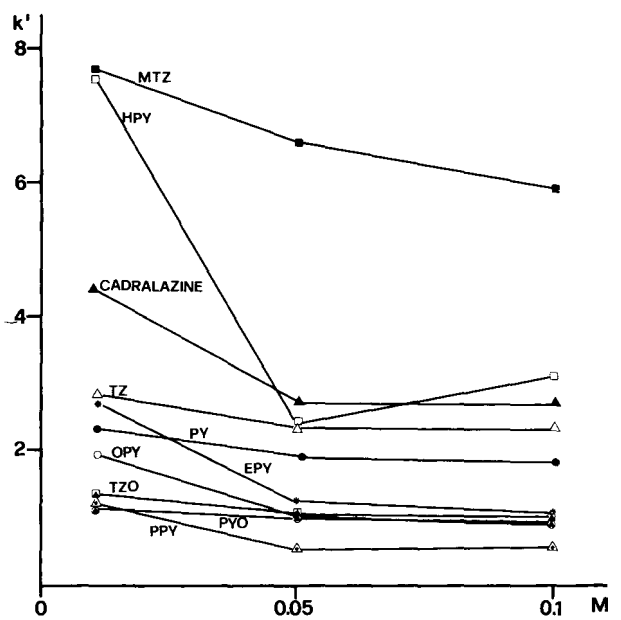

Fig. 3. Effect of buffer molarity on capacity ratio (buffer pH 6.0—acetonitrile, 85:15).

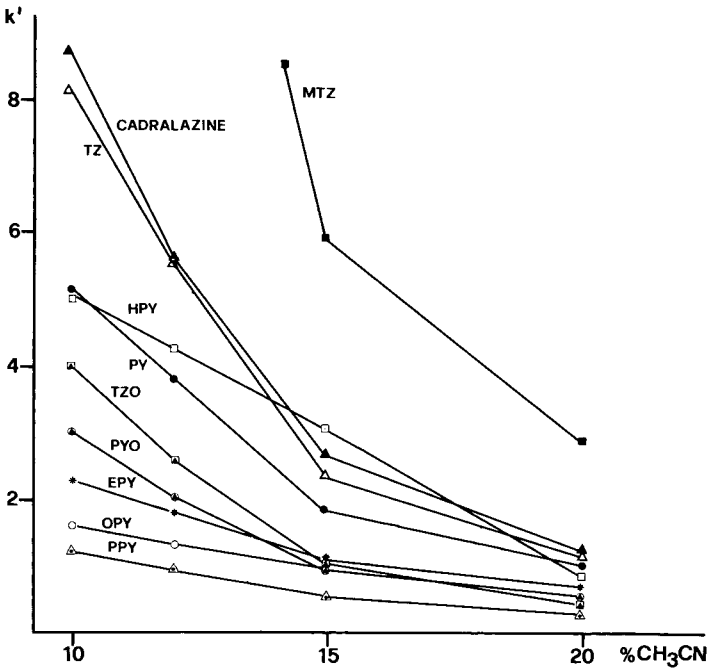

Fig. 4. Effect of eluent composition on capacity ratio (phosphate buffer 0.1 *M*, pH 6.0).

capacity ratios of all the compounds. Acetonitrile of 15% gives the best selectivity, particularly between cadralazine and TZ.

Chromatographic selectivity

On the basis of the foregoing, the chromatographic conditions reported in the experimental section were chosen. Under these conditions cadralazine elutes as a sharp peak and it is well separated from the other products (Fig. 5). No interference from naturally occurring endogenous compounds (either in plasma or urine) was observed. The chromatograms of plasma and urine blanks spiked with cadralazine and internal standard and of plasma and urine samples obtained from the patient after the oral administration of 30 mg of cadralazine are illustrated in Figs. 6 and 7, respectively.

Recovery

The recovery at different concentrations (extraction yield) was determined from the difference between the peak area ratio when cadralazine was added to plasma and urine (the internal standard being added to the final extracts) and the peak area ratio when both were added to the final extracts of blank plasma and urine. For the plasma the recovery was 87%, while for urine it was 65%; although the latter value was relatively low, due principally to the preextraction, it was nevertheless accepted because of its reproducibility over the whole calibration range. However, for the pharmacokinetic studies, single calibration curves were constructed for each subject using the individual urine blanks.

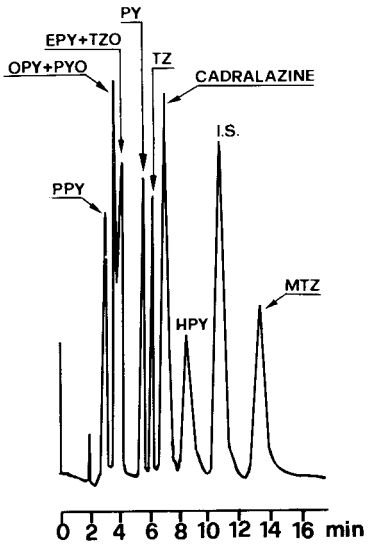

Fig. 5. HPLC separation of cadralazine from its degradation products, metabolites and internal standard (I.S.).

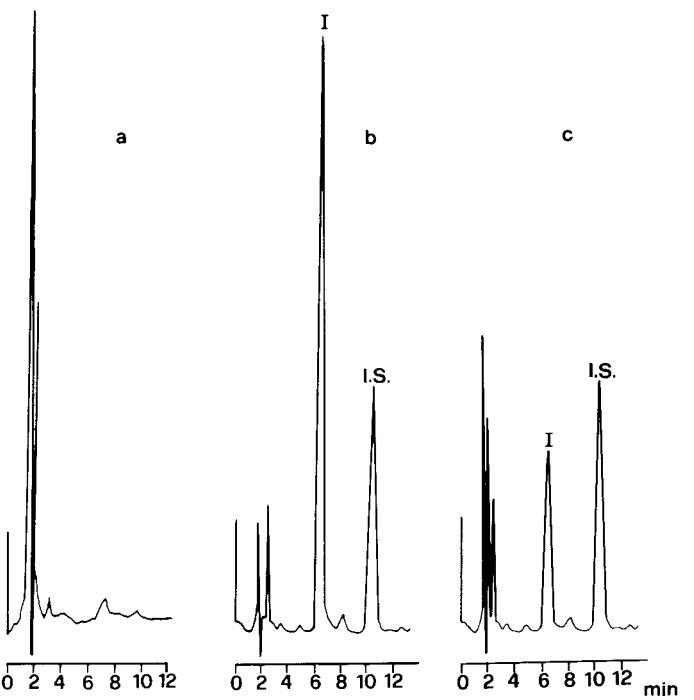

Fig. 6. HPLC profiles of (a) plasma blank, (b) plasma blank spiked with cadralazine (263 ng/ml) and internal standard (I.S., 310.5 ng/ml), and (c) a plasma sample 12 h after the administration of 30 mg of cadralazine to the patient.

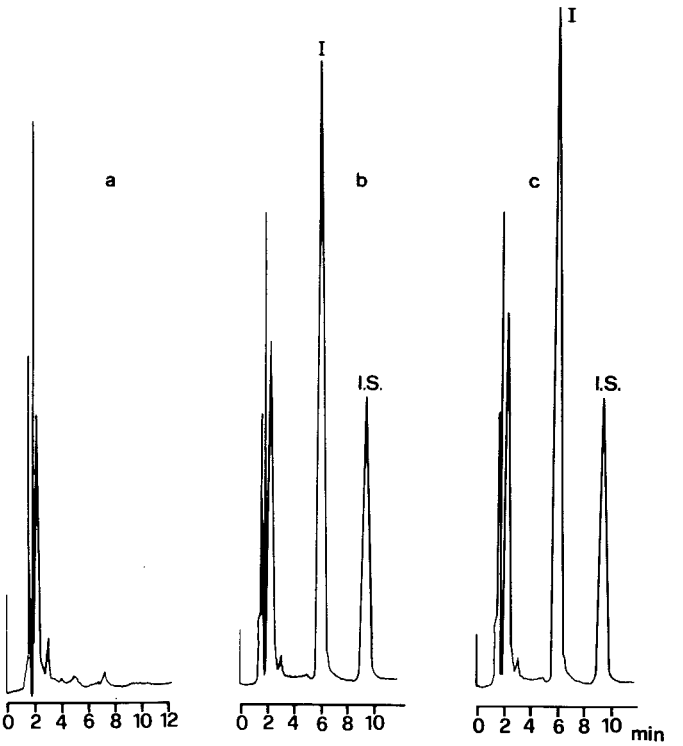

Fig. 7. HPLC profiles of (a) urine blank, (b) a urine blank spiked with cadralazine (7.02 μg/ml) and internal standard (I.S., 12.43 μg/ml), and (c) a sample of the patient's urine after administration of 30 mg of cadralazine.

TABLE II

VARIANCE ANALYSIS OF CADRALAZINE CALIBRATION CURVES IN BOTH PLASMA AND URINE

Data processed as described in ref. 11.

Source	Deviance	Degree of freedom	Variance	F calculated	F ($P < 0.05$) tabulated
Plasma					
Regression	16.07652	1	16.07652	2860.59	6.94
Residual	0.05616	10	0.00562		
Total	16.13268	11			
Lack of fit	0.02076	2	0.01038	2.35	6.06
Error	0.03540	8	0.00442		
Urine					
Regression	5.09171	1	5.09171	765.67	6.94
Residual	0.06652	10	0.00665		
Total	5.15823	11			
Lack of fit	0.01011	2	0.00506	0.72	6.06
Error	0.05641	8	0.00705		

Linearity of response and detection limits

The calibration curves (in the ranges mentioned above) were calculated using the peak area ratios versus drug concentrations by the method of least squares. The straight lines were: $Y = 0.07731 + 0.00720X$ for plasma; and $Y = -0.02410 + 0.18762X$ for urine. The goodness of fit of linear models was verified by analysis of variance [11] (Table II).

The detection limit for the drug in spiked plasma samples, based on a reproducibility of about 20%, was 10 ng/ml.

Reproducibility

The reproducibility of the method was evaluated over a concentration range of 50—330 ng/ml of plasma and over a concentration range of 1—35 μg/ml of urine. Each plasma specimen was analysed in triplicate over nine days within

TABLE III

PRECISION AND ACCURACY IN THE DETERMINATION OF CADRALAZINE IN PLASMA AND URINE

Conc. added (plasma ng/ml; urine μg/ml)	Conc. found (plasma ng/ml; urine μg/ml)	C.V. (%)	Difference between found and added conc. (%)
Plasma (n = 3)			
99.7	97.3	3.45	−2.41
303.2	285.6	2.92	−5.80
404.4	391.9	0.84	−3.09
49.7	48.4	8.66	−2.62
187.0	187.0	0.75	0
331.0	338.2	2.31	+2.18
196.0	198.7	2.77	+1.38
216.0	217.7	1.29	+0.79
263.0	257.2	1.52	−2.21
Urine (n = 6)			
71.0	70.4	3.46	−0.8
28.2	27.9	2.42	−1.1
17.7	17.1	2.16	−3.4
1.4	1.3	4.21	−7.1

a period of about twelve weeks, while each urine specimen was analysed six times within a period of one week.

The data for precision (C.V. of replicate analyses) and accuracy (difference between found and expected concentrations) are presented in Table III.

Application

The described method was used for the determination of cadralazine in both plasma and urine of a subject administered the drug. The cumulative urinary excretion and the plasma levels found are shown in Fig. 8.

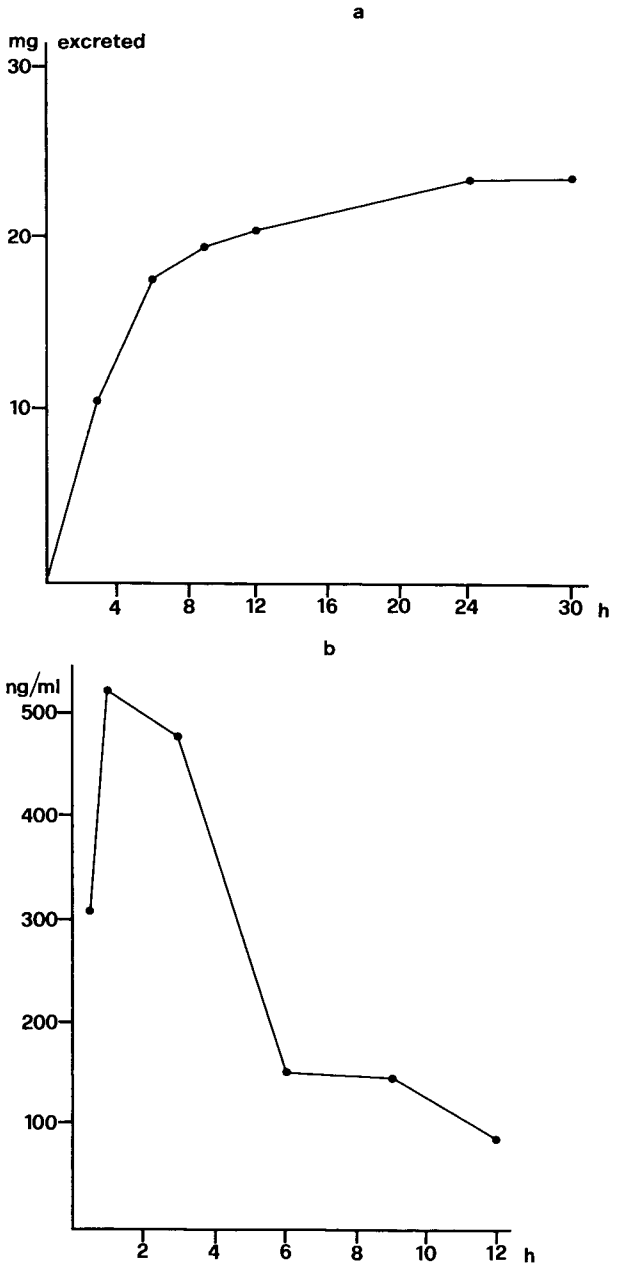

Fig. 8. Urinary cumulative excretion (a) and plasma levels (b) of cadralazine (30 mg, single oral dose).

CONCLUSIONS

A simple, sensitive, precise and accurate HPLC technique for the determination of cadralazine in human plasma and urine has been developed and it can be applied to routine analysis. The drug is well separated from its

metabolites and potential degradation products. The lower limit of sensitivity is adequate to follow the absorption and distribution of cadralazine in plasma and urine of human subjects administered a therapeutic dose of the drug, which lies between 15 and 30 mg, once a day.

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FLUORESCENCE DETERMINATION OF 5-FLUOROURACIL AND 1-(TETRAHYDRO-2-FURANYL)-5-FLUOROURACIL IN BLOOD SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Fluorescence derivatization of 5-fluorouracil (5-FU) and 1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur, FT) with 4-bromomethyl-7-methoxycoumarin using 18-crown-6 as a catalyst is studied with aim of developing a sensitive and selective liquid chromatographic method. 5-FU and FT form virtually substituted derivatives which possess maxima in their fluorescence emission spectra near 400 nm. These derivatives are separated by thin-layer chromatography and high-performance liquid chromatography to confirm the completion of reaction. For the determination of 5-FU and FT in serum, the reversed-phase high-performance liquid chromatographic separation of the derivatives is studied with a C_{18} column. This chromatography is of importance for the accurate determination of 5-FU and FT, which are, respectively, an important antitumour agent for the treatment of solid tumours in clinical medicine and a masked form of 5-FU generated in vivo.

INTRODUCTION

5-Fluorouracil (5-FU), an antimetabolite of uracil, has been in clinical use for many years and plays an important role in the chemotherapy of certain forms of cancer [1]. It may undergo anabolism to form ribo- and deoxyribonucleosides, and their mono-, di- and triphosphates. Among these, 5-fluorodeoxyuridine monophosphate which inhibits thymidylate synthetase and DNA synthesis, and 5-fluorouridine triphosphate which is incorporated to form fraudulent RNA, are believed to be responsible for the cytotoxicity of 5-FU [2].

1-(Tetrahydro-2-furanyl)-5-fluorouracil (ftorafur, FT), a masked form of 5-FU, is an effective antitumour agent. FT can be given orally and has a lower toxicity than 5-FU [3]. The mechanism of conversion of FT to 5-FU remains unknown. Several reports support the hypothesis that 5-FU formation may not

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be the only mechanism of FT activation and that a microbiologically active metabolite fraction of FT in addition to 5-FU may be present [4, 5].

For elucidation of their mechanism of action, it seemed important to establish a quantitative method for the determination of FT and 5-FU in serum. Several methods are reported for measuring FT and 5-FU in serum: bioassay to determine FT and 5-FU separately [6] and high-performance liquid chromatography (HPLC) with a reversed-phase chromatographic system for the simultaneous determination of FT and 5-FU [7, 8]. Most frequently in these HPLC separations detection of the eluate is achieved using an ultraviolet (UV) detector. However, the direct analysis of serum samples has been especially difficult with low concentrations of FT and 5-FU.

Fluorescence labelling in conjunction with HPLC is attractive since the detection limits are quite low. Fluorescence labelling of FT, 5-FU, pyrimidine bases and nucleosides has not been used extensively. An important example is presented by Dünges and Seiler [9]. They reported the use of 4-bromomethyl-7-methoxycoumarin (Br-Mmc) as fluorescence labelling reagent in the HPLC analysis of imide compounds.

The purpose of this paper is to demonstrate the applicability and scope of Br-Mmc labelling in the trace analysis of FT and 5-FU. A rapid, simple and virtually quantitative esterification method yields stable derivatives that are amenable to HPLC using a reversed-phase system.

EXPERIMENTAL

Reagents and chemicals

All organic solvents (Nakarai Chemicals, Kyoto, Japan) were commercial analytical-reagent grade materials. The distilled water used for the mobile phase was passed through an ion-exchange column (Millipore, type ZD20-11583). Br-Mmc, 18-crown-6 and *n*-valeric acid were purchased from Nakarai Chemicals and were used without purification. 5-FU and FT were obtained from commercial sources.

Preparation of derivatives for chromatographic studies

A ten-fold excess of Br-Mmc, a two-fold excess of 18-crown-6 and 25 mg of crystalline water-free potassium carbonate were added to 0.5 mg each of 5-FU and FT in 20 ml of acetone—acetonitrile mixture (1:2, v/v) (protected from light by wrapping the flask in aluminium foil). The mixture was allowed to reflux for 45 min in a water bath. After cooling, 0.2 ml of *n*-valeric acid was added to the mixture for the esterification of excess Br-Mmc. The mixture was refluxed again for 5 min. About 10 μl of this solution were applied to a thin-layer chromatography (TLC) plate. For HPLC injection, the mixture was diluted 10^2- to 10^5-fold with acetone.

Preparation of derivatives for serum analysis

Serum samples (0.5 ml) were adjusted with physiological saline to a total volume of 1 ml, and 0.1 ml of 0.5 *M* sodium dihydrogen phosphate buffer and 8 ml of ethyl acetate were added. After extraction and centrifugation, the organic layer was removed [4] and evaporated by aspirator vacuum. To the

residue, 1 ml of acetone—acetonitrile mixture containing 0.5 mg/ml Br-Mmc, 0.1 mg/ml 18-crown-6 and 1 mg of potassium carbonate were added and the mixture was refluxed as above. The resulting Mmc esters were utilized for HPLC.

Thin-layer chromatography

A 5—10 μl sample solution was spotted on a Kieselgel 60 plate, F-254 (Merck). Chromatography was performed in the solvent system ethyl acetate—methanol—10% (v/v) formic acid in water (9:1:1). The chromatogram was developed by the ascending technique for 15 cm and dried in a stream of cold air for a few minutes. The separated Mmc esters were identified by viewing the plate under a UV lamp giving maximum transmission at about 254 nm and 365 nm.

HPLC analysis

Analysis was performed using a Shimadzu (Kyoto, Japan) Model LC-4A chromatograph equipped with a Model SIL-1A injector. Separations were achieved with a column (20 cm × 4 mm I.D.) with C_{18}-brushes on a 5-μm silica core (Nucleosil 5, C_{18}; Macherey, Nagel & Co., Düren, F.R.G.). The column was packed using a balanced density slurry packing procedure similar to that described by Majors [10]. All chromatography was done at ambient temperature. The separation of 5-FU and FT was carried out with the eluent 70% methanol in water.

The column effluent was monitored by fluorescence detection with excitation and emission wavelengths of 346 nm and 395 nm, respectively. The fluorescence intensity was measured by a Shimadzu Model RF-530 fluorescence spectrophotometer.

RESULTS AND DISCUSSION

Reactivity of Br-Mmc

The reactivity of Br-Mmc in non-aqueous media was investigated in series of experiments using comparative TLC [11]. Lam and Grushka have studied various derivatization schemes of monocarboxylic [12] and dicarboxylic acids [13] with Br-Mmc. They have shown that the use of potassium carbonate to form the salt prior to the phase transfer yields an excellent compromise between the ease of the procedure and the rate of derivatives formation, and that the use of crown ether as a catalyst drastically reduces the reaction time [14].

In the procedures for the 5-FU-Mmc and FT-Mmc derivatives, the acetone—aetonitrile (1:2) reacted rapidly and efficiently as well as the crown ether. Fig. 1 shows the amount of 5-FU-Mmc derivative formed versus the reaction time for three different derivatization procedures. The rate of FT-Mmc derivative formation in the optimum conditions is also shown in Fig. 1.

The reaction rates were dependent on the 18-crown-6 concentration, the volume ratio of acetonitrile in acetone and the temperature. With 18-crown-6 as the catalyst in the presence of powdered potassium carbonate, formation of the 5-FU-Mmc derivative proceeded to completion at 75°C in 45 min. More

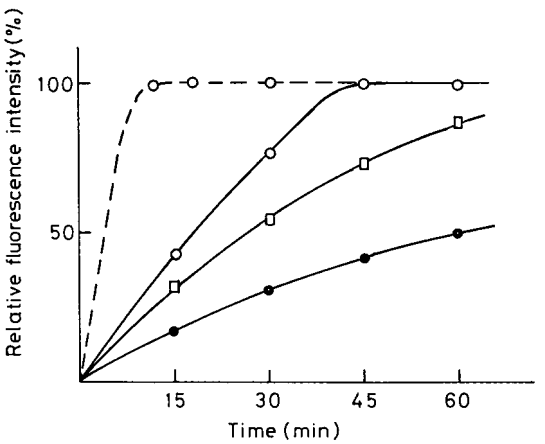


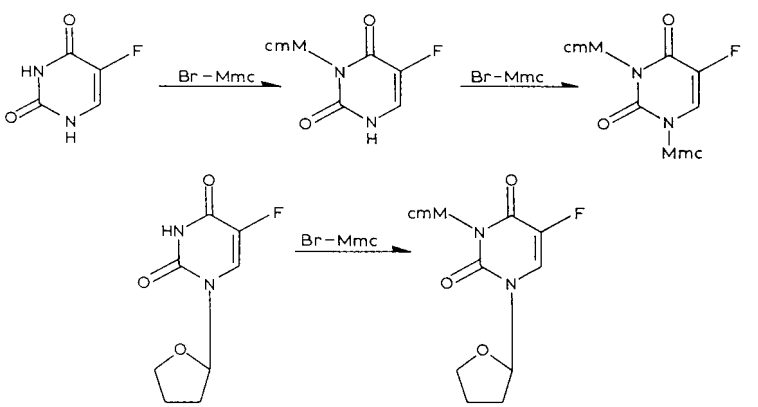
Fig. 1. Study of the rate of derivative formation. •, 0.5 mg of 5-FU in 20 ml of acetone is derivatized with 5 mg of Br-Mmc and 1 mg of 18-crown-6 in the presence of potassium carbonate at room temperature. □, 0.5 mg of 5-FU in 20 ml of acetone is derivatized in the above conditions at 75°C. ○—○, 0.5 mg of 5-FU in 20 ml of the solvent mixture acetone—acetonitrile (1:2) is derivatized in the above conditions, ○ - - ○, 0.5 mg of FT is derivatized in the above optimal conditions.

than 60 min were needed when the mixture was reacted in acetone at 75°C, and at room temperature the reaction was not complete after 2 h. The reaction of Br-Mmc with FT proceeded rapidly compared with 5-FU and only 10 min were needed when FT was derivatized under the optimal conditions for 5-FU.

The yield of the reaction was not investigated. However, as will be discussed in a later section, a linear relationship exists between the reaction yield and the amount of 5-FU and FT present in blood serum. Therefore, the present procedure is suitable for purposes of quantitation.

Structure of the derivatives

The structure of the alkylated derivatives has been confirmed by many workers [15—18], and it can be concluded that the possible equations for the labelling reaction of 5-FU and FT are



The separation of N-mono-Mmc and N,N′-bis-Mmc derivatives of 5-FU is shown in Fig. 2. The fluorescence intensity differs markedly between the mono and the bis derivative; i.e. the quantum yield of the bis derivative is higher than that of the mono derivative. The chromatogram of the Mmc-FT derivative showed a peak not containing other undesirable components. These labelling reactions were also studied in detail in conjunction with TLC.

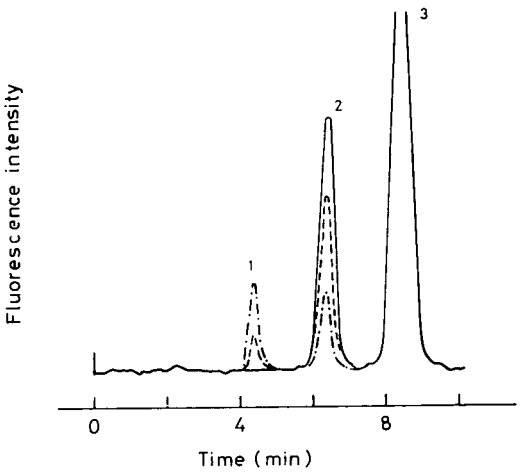


Fig. 2. Separation and formation process of N-mono-Mmc and N,N′-bis-Mmc derivatives of 5-FU. [Br-Mmc]/[5-FU] = 10. 1 = N-mono-Mmc derivative of 5-FU, 2 = N,N′-bis-Mmc derivative of 5-FU, 3 = Mmc derivative of *n*-valeric acid. — • — • —, after 15 min reaction time; - - - - -, after 30 min reaction time; ——, after 45 min reaction time. After each reaction time, 0.2 ml of *n*-valeric acid was added to the reaction mixture and the solution refluxed for 5 min at 75°C. Mobile phase = 70% methanol; flow-rate = 0.8 ml/min.

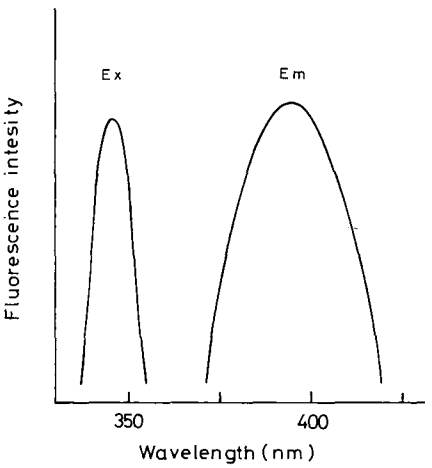
Separation by TLC

With the solvent system ethyl acetate—methanol—10% formic acid, a satisfactory separation was obtained with R_F values of 0.89 for the N,N′-bis-Mmc derivative of 5-FU, 0.87 for the Mmc derivative of FT and 0.65 for the N-mono-derivative of 5-FU. The Mmc derivative of *n*-valeric acid (esterification of excess Br-Mmc) had an R_F value of 0.93. The spot of the N-mono-Mmc derivative of 5-FU gradually disappeared according to the progress of reaction and was not seen 45 min after the start of the reaction. Other fluorescent spots, which could represent by-products of Br-Mmc and other undesirable components, were well separated in both systems.

The spots of the Mmc derivatives on the silica gel plates were stable without spraying or other special protective procedures for several days. By repeated chromatography of the same sample solution it could be shown that the Mmc derivatives are stable for at least several weeks in the reaction mixtures.

Fluorescence properties

The fluorescence spectrum of the FT-Mmc derivative was obtained in a solvent system of 70% methanol (Fig. 3). The excitation maximum appeared at 346 nm and the emission maximum at 395 nm. The fluorescence spectrum of the 5-FU-Mmc derivative was similar to that of the FT-Mmc derivative.



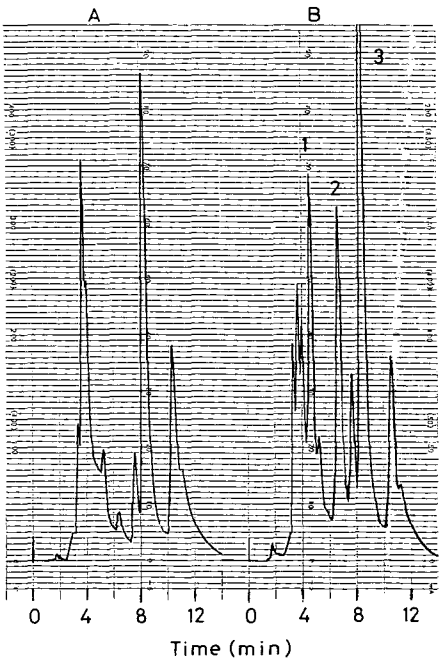


Fig. 3. Fluorescence spectra of FT-Mmc derivative. Fluorescence spectra are not corrected. Ex = excitation, Em = emission. Solvent = 70% methanol.

Fig. 4. Separation of 5-FU-Mmc and FT-Mmc derivatives in a serum sample. A, Serum blank. B, Serum spiked with 10 μg each of 5-FU and FT per ml. Mobile phase = 70% methanol; flow-rate = 0. 8 ml/min. 1 = Ft-Mmc derivative, 2 = 5-FU-Mmc derivative, 3 = *n*-valeric-Mmc derivative.

The fluorescence properties of the Mmc derivatives are very favourable for reversed-phase work as a signal can be observed in an aqueous mobile phase.

Separation by HPLC

Fig. 4B shows a chromatogram of the 5-FU-Mmc and FT-Mmc derivatives in serum and Fig. 4A shows the separation of a serum extract free from 5-FU and FT (blank sample). The blank sample shows that the serum constituents do not interfere with the separation of the 5-FU and FT derivatives. The identification of each compound was made by cochromatography with pure derivatives and comparing retention times. The blank peaks in Fig. 4 increased when using Br-Mmc stock solution that was exposed continuously to light. When the derivatizing reagent was protected from light, the 5-FU-Mmc and FT-Mmc derivatives obtained were almost completely separated from blank peaks as can be seen in Fig. 4A and B. It should be emphasized that the derivatization reaction was carried out under protection from light.

Calibration curves and detection limit

To be useful in quantitative analysis, the amount of ester formed in the derivatization reaction should be related to the amount of FT and 5-FU. Serum samples were spiked with increasing amounts of FT and 5-FU (final concentrations 0.02, 0.05, 0.1—10 μg of each drug per ml of serum). The samples

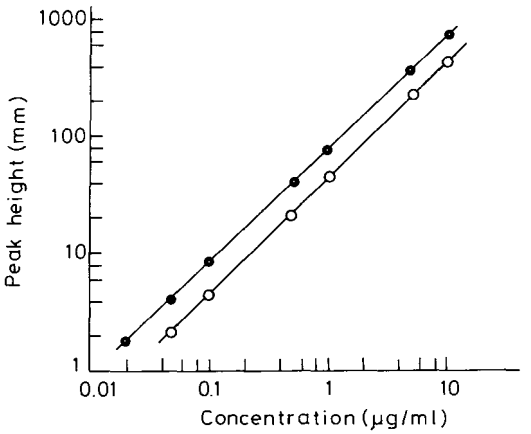


Fig. 5. Calibration curves of Mmc-derivatized 5-FU (•) and FT (○) extracted from serum.

were submitted to the extraction procedure described and standard curves were generated for each series of determination by plotting peak height against known drug concentration (Fig. 5). The linear relationships indicate that the procedure described here can be used to quantitate FT and 5-FU in serum. Recoveries for the method including extraction, Mmc derivatization and quantitation averaged 85 ± 5% for FT and 55 ± 5% for 5-FU.

The detection limits were found to be a function of the column condition, the mobile phase and the flow-rate. Under the present separation conditions, typical detection limits were 384 fmol for FT and 100 fmol for 5-FU.

Since the derivatization reaction can be scaled down to a reaction volume of 10 μl [19], it is possible to determine a few fmoles of the derivatized compounds.

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CHROMBIO. 2188

DETERMINATION OF MISONIDAZOLE AND DESMETHYLMISONIDAZOLE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH REDUCTIVE ELECTROCHEMICAL DETECTION

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SUMMARY

A reversed-phase high-performance liquid chromatographic method with reductive mode electrochemical detection was developed for the determination of misonidazole and desmethylmisonidazole in plasma. A thin-layer amperometric detector with glassy carbon working electrode was used to detect the nitroimidazoles at a potential of —0.60 V. The calibration curves were linear. The within-day and day-to-day coefficients of variation were below 3% for plasma misonidazole concentrations of 6—60 mg/l and 1—15 mg/l for desmethylmisonidazole. Electrochemical detection limits were between 2 and 4 pg, which is about 10—20 times lower than that obtained by detection at 323 nm. Limits of quantitation of the nitroimidazoles in plasma were in the order of 1—2 μg/l. Under the described conditions other nitroimidazoles and nitro compounds can also be detected with ultimate sensitivity by reductive mode electrochemical detection.

INTRODUCTION

Misonidazole, 1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol, has been shown to increase the sensitivity of hypoxic cells to the lethal effects of ionizing radiation [1, 2]. Hypoxic cells present in animal and human tumours [3, 4] are more resistant to radiotherapy than oxygenated cells [5, 6], and misonidazole has been widely investigated as a radiosensitizing drug in the radiation therapy of tumours [1, 2, 7—10]. However, several toxic effects have been observed after the administration of misonidazole to man [7, 9—12]. Determination of misonidazole and its metabolites in biological materials might help to determine a therapeutic range that is non-toxic.

Various methods of determination of misonidazole and its metabolites have

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been reported. Spectrophotometric [9, 13] and polarographic [11, 13] procedures used in animal and human studies do not differentiate between misonidazole and its major metabolite desmethylmisonidazole [14]. Polarographic [15, 16] and spectrophotometric [15] procedures with an extra thin-layer chromatography (TLC) separation step and gas—liquid chromatographic (GLC) methods with electron-capture detection [14, 15] are selective, but these methods are too lengthy for routine use. Normal-phase [14, 17] and reversed-phase [18] TLC techniques, although selective, lack sensitivity. High-performance liquid chromatographic (HPLC) methods with ultraviolet (UV) detection [19—21] are available for the separate quantitation of misonidazole and desmethylmisonidazole. However, HPLC technology with UV detection is not able to detect the amine metabolite of misonidazole [14], which does not have a typical UV spectrum [22]. Because metabolic alteration of misonidazole at the nitro group might be an important factor for the development of the neurotoxic [23], cytotoxic [24] and mutagenic [25] effects of the drug, it was felt necessary to develop more specific methodology.

This paper describes a fast, selective and sensitive procedure for the determination of misonidazole and desmethylmisonidazole in plasma by means of liquid chromatography with reductive electrochemical detection.

EXPERIMENTAL

Chemicals and reagents

Misonidazole, 1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol (Ro 07-0582), desmethylmisonidazole, 1-(2-nitroimidazol-1-yl)-2,3-propandiol (Ro 05-9963), and the internal standard, 1-(2-nitroimidazol-1-yl)-3-ethoxypropan-2-ol (Ro 07-913), were kindly provided by Hoffmann-La Roche (Mijdrecht, The Netherlands). Metronidazole and dimetridazole were donated by Rhône-Poulenc (Amstelveen, The Netherlands). Tinidazole was a gift from Pfizer (Rotterdam, The Netherlands). Nimorazole was provided by Farmitalia Carlo Erba (Brussels, Belgium). Nitrofurantoin and 2-nitroimidazole were purchased from Ega-Chemie (Steinheim, F.R.G.). Analytical grade acetic acid, sodium acetate, ethylenediamine tetraacetic acid disodium salt (Na_2EDTA) and HPLC grade methanol were obtained from Merck (Darmstadt, F.R.G.). Water was twice distilled in glass.

Apparatus

The high-performance liquid chromatograph consisted of a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 syringe loading sample injector with 50-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) and a Model LC-4 amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.). All tubings were constructed of stainless steel. A Bourdon tube pulse damper filled with nitrogen gas was fixed off-line in a T-union between pump and injector. Separations were performed on an octadecylsilane reversed-phase column (μBondapak C_{18}, 30 cm × 3.9 mm, mean particle size 10 μm) (Waters Assoc.). The amperometric detector used a TL-5 thin-layer detector cell with a glassy carbon working electrode.

A Model 970 A variable-wavelength UV—VIS detector (Tracor Instruments,

Austin, TX, U.S.A.) was also used in some experiments. Detector output was registered by a 3353 A Laboratory Automation System (Hewlett-Packard, Avondale, PA, U.S.A.). Other equipment included a Vortex type mixer, reciprocating shaker, centrifuge and 100 × 16.25 mm disposable glass tubes (Renes, Zeist, The Netherlands) with polypropylene caps (Greiner, Alphen a/d Rijn, The Netherlands).

Chromatographic conditions and instrumental settings

Chromatography was performed at ambient temperature. The mobile phase, which consisted of a mixture (20:80, v/v) of methanol and 0.15 *M* acetate buffer (pH 4.0) containing 1.25 m*M* Na_2EDTA, was continuously stirred and delivered isocratically at a constant flow-rate of 1.5 ml/min and a pressure of about 11.0 MPa. The mobile phase was deaerated by passing nitrogen via a 2-μm mobile phase filter through the solution at a flow-rate of 20 ml/min. The sample solution was also deaerated before injection by passing nitrogen through the solution via a Pasteur pipette for about 7 min at a flow-rate of about 20 ml/min.

The column effluent was monitored at a working electrode potential of −0.60 V versus the Ag/AgCl reference electrode. Further instrumental settings of the electrochemical detector were a sensitivity of the controller output of 50 or 100 nA full scale deflection and a time constant of 2 sec unless otherwise stated. The electrochemical detector output was recorded at 10 mV with a chart speed of 20 cm/h. Instrumental settings of the UV detector were a detection wavelength of 323 nm and a detection sensitivity of 0.005 absorbance unit full scale deflection (a.u.f.s.). The UV detector output was recorded at 1 mV.

Standard solutions

A methanolic standard solution containing 6.3 mg/l of the internal standard and an aqueous additional buffer solution containing 0.16 *M* acetate buffer pH 4.0 and 1.33 m*M* Na_2EDTA were prepared. A standard solution in mobile phase was made, containing desmethylmisonidazole, misonidazole and internal standard at respective concentrations of 75, 290 and 1190 μg/l. Five plasma standards were prepared, containing misonidazole in the range 6—60 mg/l as well as desmethylmisonidazole in the range 1—15 mg/l. Aliquots of 1 ml of the plasma standards were stored frozen at −20° C until analysis.

Precision, recovery and linearity

Precision, recovery and linearity of the described method were determined using the five plasma standards containing both misonidazole and desmethylmisonidazole.

Within-day precision was established by processing ten aliquots of each plasma standard and day-to-day precision was determined by processing one aliquot of a plasma standard on each of ten days. Precision was expressed as coefficient of variation of the peak area ratio of misonidazole or desmethylmisonidazole to the internal standard. Recovery data were the means of four determinations at each concentration. Linearity of the calibration curves was studied with one aliquot of each plasma standard.

Procedure

Add 4 ml of the methanolic internal standard solution to 1 ml of plasma in a stoppered glass tube. Vortex the tube for 10 sec, extract for 5 min on a reciprocating shaker and centrifuge at 2000 *g* for 10 min. After centrifugation transfer 1 ml of the supernatant to a second tube, add 3 ml of additional buffer solution, mix and deoxygenate the solution with nitrogen. Fill the sample loop completely by suction and inject the deoxygenated solution once.

Quantitation

Plasma samples were processed according to the described procedure. Concentrations of misonidazole and desmethylmisonidazole in plasma samples were always calculated from calibration curves obtained after analysis of the five plasma standards. For both drugs calibration curves were constructed by plotting peak area ratio of the drug to the internal standard versus drug concentration in plasma and finding the best fitting line after least-squares linear regression analysis.

RESULTS AND DISCUSSION

Chromatographic system

Chromatographic systems consisting of an octadecylsilane reversed-phase column and a mobile phase of methanol—water (20:80, v/v) have shown a good separation between the nitroimidazoles [19, 20]. As the electrochemical reduction of the aromatic nitro group proceeds more easily at lower pH [26], the mobile phase should preferably be acid. A methanol—acetate buffer (pH 4.0) (20:80, v/v) with a buffer acetate concentration of 0.15 *M* gave a good separation and an acceptable compromise between an adequate detector response and a low background current. Addition of 0.001 *M* Na_2EDTA to the mobile phase reduced noise and baseline drift of the detector signal. This may be due to complexation of traces of metal ions, which are easily reduced at the glassy carbon electrode. Chromatograms of a plasma blank (A), plasma standard (B) and a standard solution in mobile phase (C) are shown in Fig. 1.

Removal of oxygen

For optimum performance of the electrochemical detector in the reductive mode oxygen should be removed from the mobile phase and from the injected solution [27]. The described method of deoxygenation with nitrogen, which was presaturated with mobile phase, prevented the appearance of an interfering oxygen peak with a retention time of 4.2 min almost completely. Background currents normally ranged between 10 and 20 nA.

Electrochemical detection

A glassy carbon electrode was chosen as a working electrode because of its known chemical resistance to organic solvents used in reversed-phase liquid chromatography, its large overpotential of oxygen, its mechanical stability and its simple repolishing procedure. The voltammetric behaviour of the three nitroimidazoles at the glassy carbon electrode was studied by obtaining multiple voltammograms of the drugs under the conditions described. At

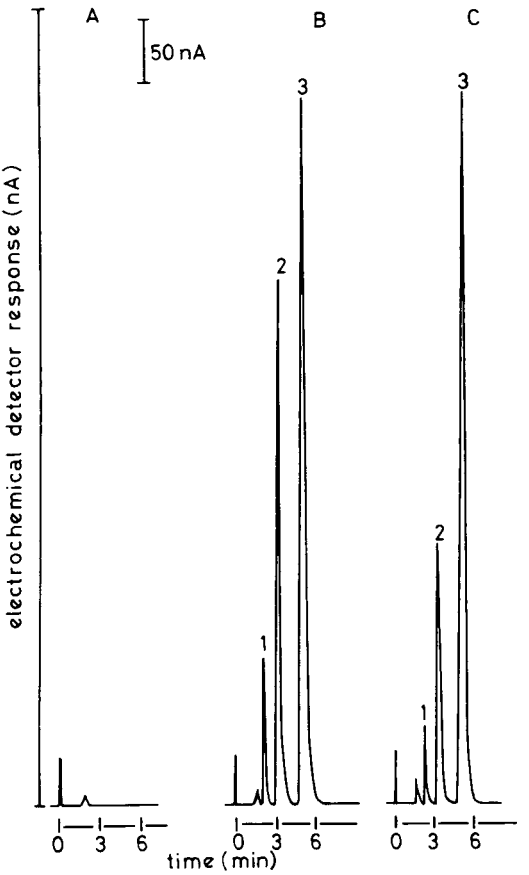

Fig. 1. Chromatograms of (A) blank plasma, (B) plasma standard containing 3.1 mg/l desmethylmisonidazole and 12.4 mg/l misonidazole, and (C) standard solution in mobile phase containing 75 μg/l desmethylmisonidazole, 290 μg/l misonidazole and 1190 μg/l internal standard. Peaks: 1 = desmethylmisonidazole (t_R = 2.5 min); 2 = misonidazole (t_R = 3.7 min); 3 = internal standard (t_R = 5.5 min). t_R = retention time. Injection volume: 50 μl.

each potential desmethylmisonidazole, misonidazole and internal standard were injected three times at respective amounts of 536, 542 and 488 pmol and peak areas were measured. The hydrodynamic voltammograms (Fig. 2) demonstrate a very similar voltammetric behaviour of the three nitroimidazoles showing only one reductive wave. Hydrodynamic voltammograms of the nitroimidazoles were made in the potential range of 0 to —0.9 V, because sudden changes in detector sensitivity and non-reproducible voltammetric behaviour were noticed at working electrode potentials below —0.9 V. The choice of a detector potential of —0.60 V seemed to be a good compromise between an adequate current response to nitroimidazoles and low background current.

Precision, recovery and linearity

Precision at the studied concentrations was good with coefficients of variation of 0.5—1.8% for the within-day precision and 1.9—3.1% for the day-to-day precision. The recoveries of 98.8—99.7% found for misonidazole and

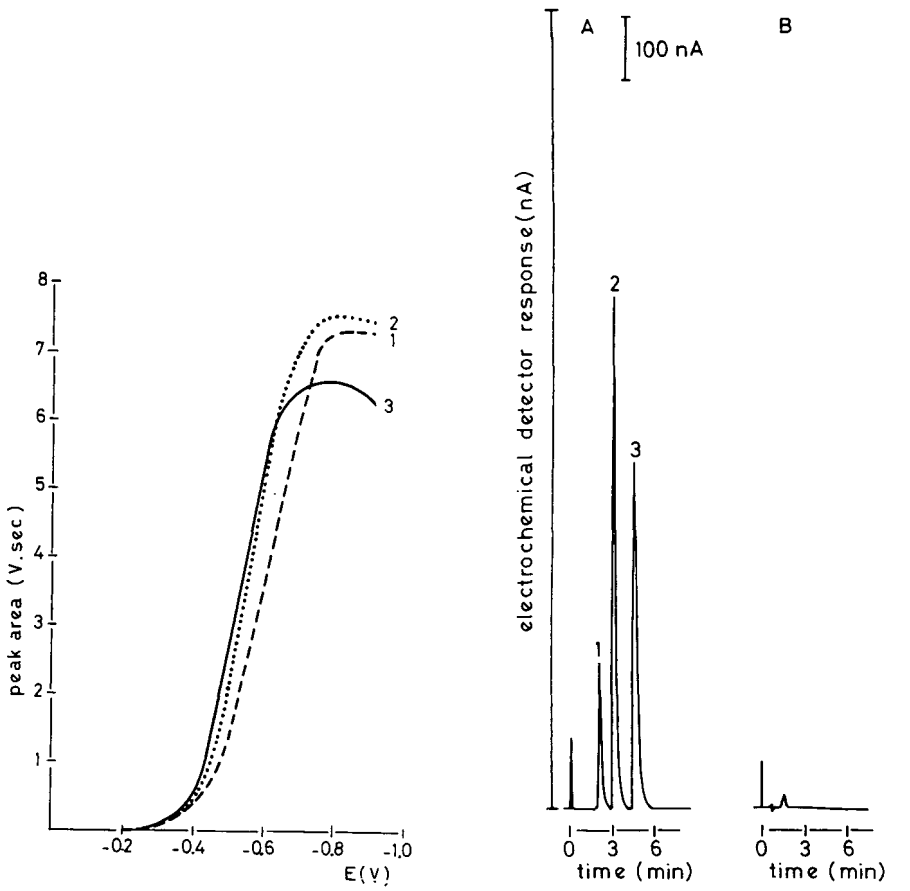

Fig. 2. Peak area—potential curves for desmethylmisonidazole (1), misonidazole (2) and internal standard (3).

Fig. 3. Chromatograms of a plasma sample of a patient 24 h after oral intake of 2.85 g/m^2 misonidazole (A) and of the control plasma sample before drug intake (B). Concentrations of desmethylmisonidazole and misonidazole were 6.8 mg/l and 27.5 mg/l, respectively. Injection volume: 50 µl. Peaks: 1 = desmethylmisonidazole; 2 = misonidazole; 3 = internal standard.

97.8—99.0% obtained for desmethylmisonidazole indicate a complete recovery of both nitroimidazoles from plasma. Calibration curves constructed from the five plasma standards demonstrated good linearity. Equations of the linear regression lines of typical calibration curves are for desmethylmisonidazole $Y = 0.034X + 0.005$ ($r = 0.9999$) and for misonidazole $Y = 0.037X + 0.062$ ($r = 0.9997$), where Y is the peak area ratio of drug to internal standard, X is the plasma drug concentration in mg/l and r is the correlation coefficient.

Sensitivity and detection limit

Allowing a signal-to-noise ratio of 2 and measuring peak heights the absolute limits of detection for desmethylmisonidazole, misonidazole and internal

standard are 2, 3 and 4 pg, respectively. Detection limits were determined at a detector sensitivity of 2 nA full scale deflection, a time constant of 0.5 sec, a noise equal to 60 pA and an injection volume of 5 μl. At a signal-to-noise ratio of 2 the limits of quantitation are 1.0 μg/l for desmethylmisonidazole and 1.6 μg/l for misonidazole. If necessary these limits may be lowered by increasing the injection volume or by choosing another procedure, which makes use of solvent extraction. Such extraction procedures have been described for spectrophotometric [15], polarographic [15, 16], TLC [17] and LC—UV [28] procedures for misonidazole and desmethylmisonidazole in biological fluids.

Electrochemical detection versus UV absorbance detection

Comparative detection limits for electrochemical detection at —0.60 V and UV absorbance detection at 323 nm are given in Table I. For the studied nitroimidazoles the detection limits determined at a signal-to-noise ratio of 2 were 10—20 times lower using electrochemical detection at —0.60V than those obtained with UV detection at 323 nm being the wavelength of maximal absorbance.

TABLE I

DETECTION LIMITS OF NITROIMIDAZOLES WITH ELECTROCHEMICAL AND UV ABSORBANCE DETECTION

Nitroimidazole	Detection limit* (pg)	
	Electrochemical detection at —0.60 V	UV absorbance detection at 323 nm
Desmethylmisonidazole	2	35
Misonidazole	3	50
Internal standard	4	55

*Determined at a signal-to-noise ratio of 2.

Clinical application

Chromatograms of plasma samples of a patient before and 24 h after oral intake of 2.85 g/m^2 misonidazole are shown in Fig. 3. Using the described procedure we were able to study the pharmacokinetics of nitroimidazoles in patients undergoing radiation therapy of tumours. Results of these studies will be published soon.

Other nitro compounds

This technique also proved to be applicable to the sensitive electrochemical detection of other nitroimidazoles such as 2-nitroimidazole, metronidazole, nimorazole and tinidazole and nitro compounds such as nitrofurantoin. Under the conditions of this method these compounds eluted as sharp and symmetrical peaks with retention times between 2.7 and 5.9 min. For 100 pmol injected compound the current response at —0.60 V ranged between 8 and 10 nA for the 5-nitroimidazole drugs and nitrofurantoin, while the response for the 2-nitroimidazole compounds varied from 16 to 28 nA.

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α-ALKYL-α-ARYLACETIC ACID DERIVATIVES AS FLUORESCENCE MARKERS FOR THIN-LAYER CHROMATOGRAPHIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF AMINES AND ALCOHOLS

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SUMMARY

Activated *R,S*-benoxaprofen is described as a new reagent for fluorescent derivatization of drugs with primary or secondary amino groups or with hydroxyl groups. Separation of the reaction products is demonstrated by thin-layer chromatography and high-performance liquid chromatography. The sensitivity of the detection is in the picomole range. Derivatization procedures can be easily and rapidly performed.

INTRODUCTION

The determination of drugs in biological materials usually requires very sensitive assay methods. Because of the low detection limit, the high specificity and the linearity in a large concentration range, measuring the fluorescence of the drugs has proved to be advantageous. Insufficiently fluorescent compounds with reactive groups can be converted to highly fluorescent derivatives by fluorescence markers [1]. The reagent should fulfil several requirements. First, it has to exhibit a high intensity of fluorescence; furthermore, it must react quantitatively and specifically with defined functional groups. The resulting products should be stable and possess chromatographic properties that make

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the separation from the excess of the reagent and from by-products possible. In general, the fluorescence markers described in the literature and used up to now have several disadvantages; for example, instability, such as photoinstability, and formation of by-products or insufficient fluorescence yield [2].

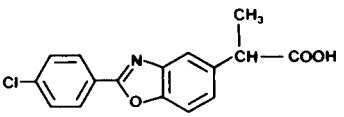


Fig. 1. Structural formula of benoxaprofen.

There are compounds in the chemical group of α-alkyl-α-arylacetic acids that are distinguished by pronounced absorption qualities or strong intrinsic fluorescence or both. For the investigations described in this paper, benoxaprofen [(*R,S*)-2-(*p*-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid, Fig. 1), an antiinflammatory drug, which shows an intense fluorescence, was chosen from this group. Activation of benoxaprofen to a fluorescence marker (e.g. the corresponding acid chloride or imidazolide) can easily be performed. The agents obtained are characterized by a good reactivity leading to extraordinarily stable products when allowed to react with amines and alcohols.

MATERIALS AND METHODS

Reagents and chemicals

Solvents (analytical grade), thionyl chloride and thin-layer chromatography (TLC) plates (coated with silica gel 60 and reversed-phase material, RP-8 and RP-18) were obtained from E. Merck (Darmstadt, F.R.G.). Benoxaprofen was made available by Eli Lilly (Bad Homburg, F.R.G.), and tranylcypromine by Röhm Pharma (Weiterstadt, F.R.G.). Carbonyldiimidazole was purchased from Serva (Heidelberg, F.R.G.), and α-methylbenzylamine from EGA (Steinheim, F.R.G.). Amphetamine, methamphetamine, β-phenylethylamine, benzylamine, methanol, ethanol, propanol, butanol, isoamyl alcohol, choline (all from Merck), maprotiline (Ciba-Geigy, Basel, Switzerland), tolylethylamine, phenylbutylamine (both from EGA), procaine (Hoechst, Frankfurt/M., F.R.G.) and scopolamine N-butylbromide (Boehringer Ingelheim, Ingelheim, F.R.G.) were also used as substrates for derivatization.

Apparatus

Melting points were obtained with a Büchi apparatus and are uncorrected. Infrared (IR) spectra were recorded in potassium bromide discs with a Beckman Acculab 2 spectrophotometer.

Elemental analyses were performed by the Department of Organic Chemistry, University of Frankfurt (Prof. Dr. Ried) and analytical results are within 0.3% of the theoretical values.

TLC plates were scanned with a chromatogram-spectrophotometer KM3 (Carl Zeiss, Oberkochen, F.R.G.) and a recorder 56 (Perkin Elmer, Überlingen, F.R.G.).

Solutions were applied onto TLC plates using a Linomat III (Camag, Muttenz, Switzerland).

HPLC was performed using a chromatograph LC 601 and a fluorescence detector 650-10 S (Perkin Elmer).

Activation of benoxaprofen

Formation of the acid chloride. Benoxaprofen (600 mg, about 2 mmol) was dissolved in 50 ml of dried toluene and, after slowly adding 5 ml of thionyl chloride (ca. a 20-fold amount, freshly distilled over linseed oil), the mixture was refluxed for 30 min. The solution was evaporated to dryness and the crystalline residue was recrystallized from dichloromethane if necessary.

Formation of the imidazolide. A solution of 500 mg of carbonyldiimidazole in 50 ml of dried dichloromethane was added to 100 mg of benoxaprofen. The solution was allowed to stand at room temperature for 30 min and was shaken occasionally. Then 5 ml of glacial acetic acid were added to destroy the excess of carbonyldiimidazole. The reagent solution obtained was used immediately.

Characterization of the physical and spectral properties of benoxaprofen (BOP) and benoxaprofen chloride (BOP-Cl)

m.p.: BOP = 191°C, BOP-Cl = 91.5°C. IR (cm^{-1}): BOP, 1700 (> C=O, acid); BOP-Cl, 1775 (>C=O, acid chloride).

Fluorescence spectra were scanned after chromatography on the TLC plate using the chromatogram-spectrophotometer KM 3. Excitation spectra: excitation, deuterium lamp; emission, monochromatic filter M 365. Emission spectra: excitation, mercury lamp (313 nm line).

HPLC fluorescence spectra were registered during chromatography (stop flow) using a fluorescence detector 650-10 S with a xenon lamp. Excitation spectra: emission wavelength = 365 nm. Emission spectra: excitation wavelength = 312 nm.

Synthesis of reference compounds (derivatives of amines and alcohols)

α-Methylbenzylamine. (Modified from Bopp et al. [3].) Benoxaprofen chloride (600 mg) was dissolved in 50 ml of dried dichloromethane. Then 10 ml of a solution of α-methylbenzylamine in dichloromethane (1.5 ml of α-methylbenzylamine and 8.5 ml of dichloromethane) were added slowly with stirring at room temperature. After refluxing for 3 h this solution was washed first with 0.2 *M* hydrochloric acid, then with water and dried over sodium sulphate. The solvent was evaporated and a white crystalline solid was recovered: m.p.: 169°C, yield: 85%.

The solid gave one fluorescent spot when examined by TLC. Solvent system [3]: chloroform—methanol—water—ammonia (70:30:5:1, v/v). R_F values: 0.89 (amide), 0.35 (BOP). IR (cm^{-1}): 1640 (> C=O, amide).

Tranylcypromine. The amide of tranylcypromine was synthesized with benoxaprofen chloride as described for the amide of α-methylbenzylamine. Because of the product's insolubility in dichloromethane it was easily isolated from the reaction mixture.

m.p.: 247°C, yield: ≈98%. TLC solvent system: cyclohexane—ethyl acetate—methanol (7:3:2, v/v), ammonia atmosphere, R_F values: 0.43 (amide), 0.10 (BOP). IR (cm^{-1}): 1640 (>C=O, amide).

Choline. Benoxaprofen chloride (300 mg, about 1 mmol) and 420 mg of

choline chloride (about 4 mmol) in acetonitrile were refluxed for 1 h. The excess of choline chloride crystallized nearly quantitatively after cooling the reaction mixture. After centrifugation the solution was evaporated to dryness, and the residue was dissolved in water. This solution was shaken with toluene to remove benoxaprofen and evaporated to dryness again. The resulting ester was recrystallized from acetonitrile.

m.p.: 205°C, yield: ≈50%. TLC solvent system: acetone—methanol—formic acid (2:2:1, v/v), atmosphere of the glass tank saturated for 48 h. R_F values: 0.36 (ester), 0.86 (BOP). IR (cm^{-1}): 1725 (>C=O, ester).

Methanol. Two drops of concentrated sulphuric acid were added to 200 mg of benoxaprofen chloride in 4 ml of methanol, and the mixture was heated to 80°C for 10 min. After cooling the ester crystallized from methanol. After centrifugation, methanol was decanted, the residue was washed with a mixture of water and methanol several times and then dried over phosphoric anhydride.

m.p.: 100.5°C, yield: ≈92%. TLC solvent system: toluene—dichloromethane—tetrahydrofuran (5:1:1, v/v). R_F values: 0.78 (ester), 0.29 (BOP). IR (cm^{-1}): 1730 (>C=O, ester).

Quantitative determination of primary and secondary amines

Reaction conditions for the derivatization with benoxaprofen chloride (e.g. α-methylbenzylamine). To 10 μg of amine, 1 ml of a solution of benoxaprofen chloride (1 mg/ml) was added. The mixture was allowed to stand at room temperature. The investigation was performed with and without the addition of sodium and potassium carbonate (20 mg) and triethylamine (10 μl, freshly distilled) to the reaction mixture. The concentration of amide in the mixtures was assayed every 10 min after the reaction had been started. The TLC plates (silica gel, 5 × 10 cm) were developed immediately after application of the solutions using the synthesized amides as reference compounds.

General procedure for the formation of derivatives with benoxaprofen chloride. Up to 0.5 μmol of amine (or the residue after extraction from biological material) and 500 μl of benoxaprofen chloride in dried dichloromethane (1 mg/ml) were allowed to stand at room temperature for 60 min or heated to 50°C for 30 min.

Formation of derivatives using the benoxaprofen imidazolide. Up to 0.5 μmol of amine and 500 μl of a solution of benoxaprofen imidazolide (freshly prepared as described above) were allowed to stand at room temperature for 0.5 h.

Extraction procedures from biological material. Example 1: β-phenylethylamine from plasma. Plasma (1 ml), 1 ml of 0.1 *M* sodium hydroxide solution and 2.5 ml of *n*-hexane were mixed in a centrifuge tube. After shaking (20 min), the tubes were centrifuged briefly to separate the layers. Then 2 ml of the organic phase were transferred into another tube and evaporated to dryness using a vacuum centrifuge. The benoxaprofen chloride solution was added to the resulting residue. The derivatization was performed at room temperature.

Example 2: maprotiline from plasma. To 1 ml of plasma, 0.5 ml of potassium carbonate solution (10%) and 3 ml of *n*-hexane (freshly distilled) were added. After shaking (30 min) and centrifuging, 2 ml of the organic phase were transferred into another tube and evaporated to dryness using a vacuum

centrifuge. The benoxaprofen chloride solution was added. The mixture was heated to 50° C (30 min).

TLC conditions. Volume applied = 10 µl, band width = 5 mm. Solvent systems: (I) toluene—dichloromethane—tetrahydrofuran (5:1:1, v/v), ammonia atmosphere; (II) toluene—chloroform—tetrahydrofuran (5:4:1, v/v), ammonia atmosphere; (III) chloroform—methanol—formic acid—tetrahydrofuran (110:20:5:2, v/v); (IV) acetone—methanol—formic acid (2:2:1, v/v); (V) aqueous solution of sodium heptane sulphonate (0.01 mol/l)—acetonitrile—phosphoric acid (40:60:0.15, v/v); (VI) cyclohexane—ethyl acetate—methanol (7:3:2, v/v), ammonia atmosphere; (VII) methanol—water (9:1, v/v). Detection was by densitometric measurement of the intensity of fluorescence using a chromatogram-spectrophotometer KM3. Excitation wavelength was the 313 nm line of a mercury medium pressure lamp ST 41; slit 0.1 × 6 mm. Emission: M 365 monochromatic filter. Amplification: 1—10.

HPLC conditions. Injection volume: 10 µl System a: analytical column (Dupont); 250 mm × 4.6 mm; stationary phase Zorbax-sil (7 µm) (Dupont); mobile phase cyclohexane-dichloromethane-tetrahydrofuran (5:1:1, v/v); ambient temperature; flow-rate 1 ml/min (at a pressure of 8.5 MPa). System b: analytical column (Knauer); 120 mm × 4.6 mm; stationary phase LiChrosorb RP-8 (5 µm) (Merck); mobile phase sodium heptane sulphonate (0.01 mol/l)—acetone—phosphoric acid (40:60:0.15, v/v); temperature 55° C; flow-rate 2 ml/min (at a pressure of 7.7 MPa). Detection was by fluorescence measurement; excitation wavelength 312 nm; emission wavelength 365 nm.

Quantitative determination of alcohols

General procedure for the formation of derivatives with benoxaprofen chloride. Up to 0.5 µmol of alcohol and 500 µl of a solution of benoxaprofen chloride (1 mg/ml) in acetonitrile, toluene or dichloromethane are heated to 60—80° C for 1 h. Sometimes higher temperatures (> 100° C) are necessary. The derivatives were examined by TLC, the conditions being the same as those described for amines.

Example for application. Detection of ethanol in chloroform using benoxaprofen chloride. Chloroform (100 µl) was mixed with 500 µl of a solution of benoxaprofen chloride in dichloromethane (1 mg/ml). The mixture was heated to 60° C for 60 min. Then 10 µl of the reaction mixture were applied onto a TLC plate (silica gel 60) which was developed in solvent system II (without ammonia).

Influences on the fluorescene intensity of benoxaprofen derivatives

Solvents, acids and bases. Benoxaprofen-α-methylbenzylamide was applied onto TLC plates (100 ng/spot; at least two spots on each plate). The plates were developed in solvent system II (without ammonia) and dried completely. Each plate was half dipped into a tank containing one of the solvent mixtures A—K (see Table II for composition of solvents). Then the plates were dried in the air. The fluorescence intensity was measured at various times after dipping. The benoxaprofen-α-methylbenzylamide spots on the untreated part of the plates were used as references.

Light and air. The fluorescence of benoxaprofen-α-methylbenzylamide on

silica plates (100 ng/spot) was measured immediately after development and drying of the plate. Then one plate was exposed to light and air and a second plate was kept as a reference in vacuum and darkness (ambient temperature). The fluorescence intensity was measured twice a day over a period of four days.

RESULTS AND DISCUSSION

Reaction products

Numerous primary and secondary amines and aliphatic alcohols can easily be converted to well-detectable compounds by activated benoxaprofen.

TABLE I

R_F VALUES AND RETENTION TIMES OF SEVERAL DERIVATIVES USING DIFFERENT STATIONARY AND MOBILE PHASES

Chromatographic conditions are described in the text. For composition of solvent systems I—V and VII, see section *TLC conditions*. For composition of HPLC solvent systems, see section *HPLC conditions*.

Substance	R_F values on TLC plates							Retention times in HPLC (min)	
	Silica gel 60			RP-18 VII					
	I	II	III					System a (silica gel)	
α-Methylbenzylamine	0.16	0.45	0.81	0.31				6.7	
	0.28	0.56						10.8	
Tranylcypromine	0.16	0.39	0.76	0.32				9.0	
	0.21	0.45						10.7	
Amphetamine	0.14	0.53	0.81	0.29				8.0	
	0.21	0.59						9.5	
Methamphetamine	0.27	0.65	0.79	0.23				10.5	
	0.33							11.5	
β-Phenylethylamine	0.16	0.49	0.79	0.33				11.8	
Tolylethylamine	0.18	0.49	0.79	0.30				10.9	
Phenylbutylamine	0.18	0.48	0.80	0.26				11.3	
Benzylamine	0.19	0.44	0.77	0.37				10.3	
Maprotiline	0.25	0.69	0.82	0.13				8.6	
Procaine	0.08	0.14	0.30	0.04				8.5	
Benoxaprofen	0.09	0.05	0.68	0.43				4.3	
Benoxaprofen chloride	0.64	0.84	0.85	—				4.3	
	Silica gel 60			RP-8 V	RP-18 VII	System a (silica gel)	System b (RP-8)		
	I	II	IV						
Methanol	0.69	0.44			0.32	11.4			
Ethanol	0.73	0.48			0.29	9.9			
Propanol	0.79	0.51			0.25	8.5			
Butanol	0.81	0.53			0.22	7.3			
Isoamyl alcohol	0.83	0.54			0.20	6.7			
Choline			0.36	0.21			7.5		
Scopolamine N-butylbromide				0.09			8.3		
Benoxaprofen	0.09	0.05	0.86	0.45	0.48	4.3			
Benoxaprofen chloride	0.64	0.84	0.86	0.20		4.3			

Benoxaprofen chloride and benoxaprofen imidazolide show good reactivity. The derivatives of amines can be prepared at room temperature in various dried solvents (e.g. dichloromethane, chloroform, acetonitrile, toluene).

Experiments were performed to optimize the reaction conditions. In the case of α-methylbenzylamine, the influence of bases on the reaction was investigated. Without bases the yield of reaction product with α-methylbenzylamine was about 91% after 1 h. If sodium or potassium carbonate or triethylamine (freshly distilled) was added, the yield was nearly 100%. After 12 h identical yields of reaction product (~100%) were obtained in all cases. If triethylamine was used as proton acceptor in the reaction mixture even trace amounts of contaminating ethylamine and diethylamine were detected and interfered in the determination of the substrates. Addition of carbonates increased the standard deviation and sometimes had an unfavourable influence on the TLC behaviour of the reaction mixtures. Therefore, in the case of α-methylbenzylamine and tranylcypromine, it is preferable to perform the derivatization without the addition of bases.

Reaction times can be shortened by heating the reaction mixture to 50—60°C. For the reaction with some drugs which contain OH groups, higher temperatures (e.g. 110°C) are sometimes necessary. The optimal reaction conditions (reaction time and temperature, addition of bases, solvent) for the derivatization with activated benoxaprofen have to be investigated for each substrate.

Examples of the formation of derivatives with benoxaprofen chloride and the chromatographic properties of the derivatives are given in Table I. Thin-layer chromatograms of the reaction mixtures of benoxaprofen chloride with five different alcohols (C_1—C_5) are shown in Fig. 2A and B.

For the development of analytical methods it is advantageous if highly purified reference substances are available in a sufficient amount. In general, derivatives of amines and alcohols with benoxaprofen can easily be synthesized

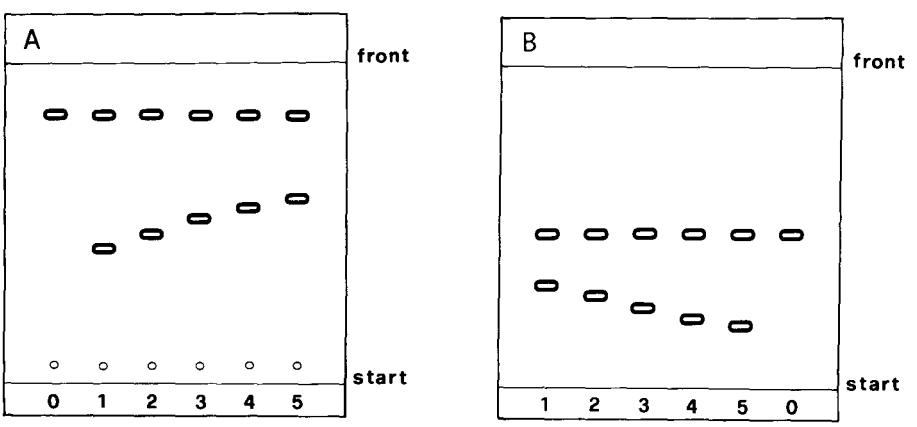


Fig. 2. (A) Chromatogram of benoxaprofen chloride reaction mixtures of five different alcohols from C_1 to C_5, directly applied to a silica gel plate (mobile phase: cyclohexane—dichloromethane—tetrahydrofuran (5:4:1, v/v), ammonia atmosphere). 0 = without alcohol (blank sample), 1 = methanol, 2 = ethanol, 3 = propanol, 4 = butanol, 5 = isoamyl alcohol. (B) Chromatogram of reaction mixtures of the five alcohols (C_1—C_5) on an RP-18 TLC plate (mobile phase: methanol—water (9:1, v/v)).

in adequate quantities. The benoxaprofen amides and esters of all the primary and secondary amines and alcohols investigated are strongly fluorescent. Amino acids and similar substances can also be detected in very low concentrations [4]. Even problematical substances such as, for example, quaternary compounds with reactive functional groups (e.g. choline, scopolamine N-butylbromide) can easily be changed into fluorescing, well-detectable compounds [5] which can be assayed by TLC and HPLC on silica gel or reversed-phase stationary phases.

Fluorescence properties

The fluorescence spectra of benoxaprofen after TLC or HPLC are shown in Fig. 3A and B. On TLC plates the excitation maximum is localized at 310 nm, the emission maximum at 365 nm. The spectrum registered during HPLC

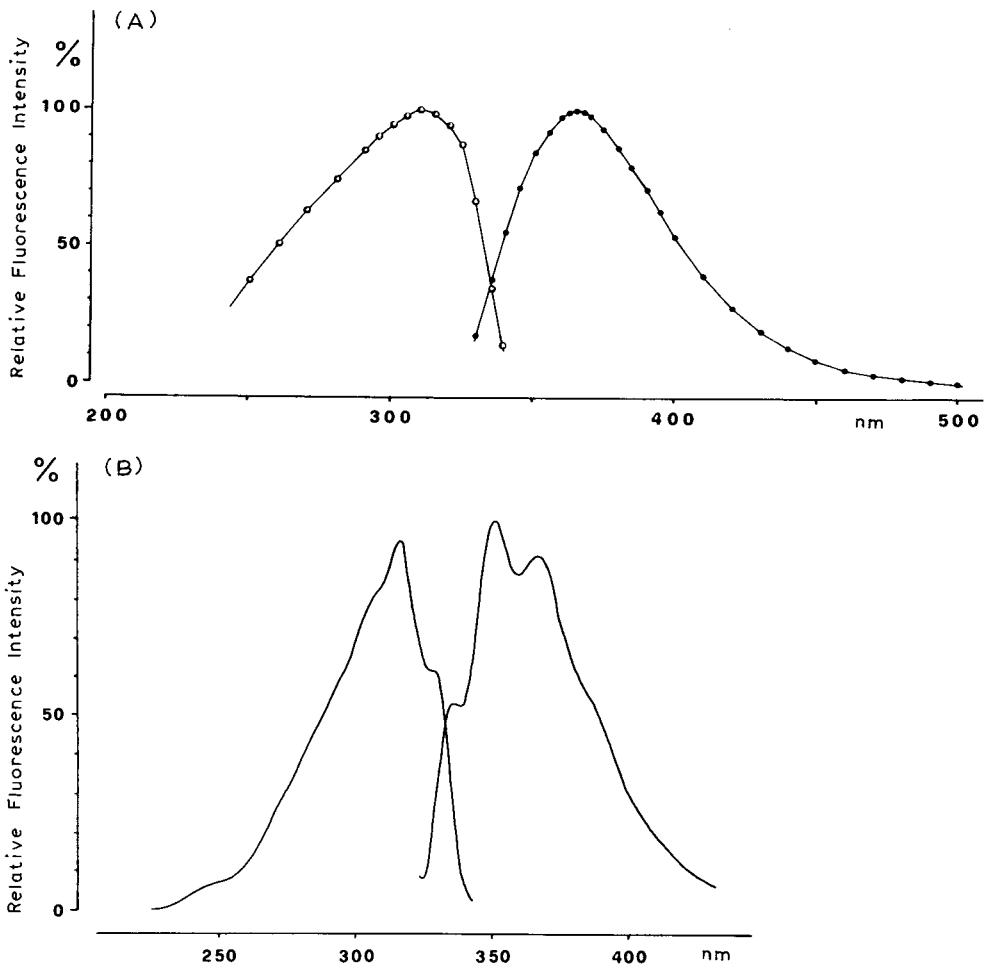


Fig. 3. (A) Fluorescence excitation and emission spectra of benoxaprofen on silica gel TLC plates. Excitation maximum = 310 nm, emission maximum = 365 nm. (B) Fluorescence excitation and emission spectra of benoxaprofen measured during HPLC. Excitation maximum = 312 nm, emission maxima = 350 and 365 nm.

shows an excitation maximum at 312 nm; there are two maxima in the emission spectrum, at 350 nm and at 365 nm. The spectra of benoxaprofen chloride are identical with the benoxaprofen spectra.

The quantum fluorescence yield for the benoxaprofen molecule was not determined. However, it may be supposed that it is in the same range as for related molecules, e.g. 2-(4-fluorophenyl)-5-phenyloxazole (quantum yield 0.95) [6].

All derivatives investigated exhibit fluorescence properties that are similar to or even identical with those of benoxaprofen or benoxaprofen chloride. In solution, i.e. during HPLC, the excitation and emission spectra of all these derivatives are nearly congruent with those of benoxaprofen. On TLC plates several but only insignificant hypsochrome or bathochrome shifts were found, which were obviously not due to different pH values of the solvent systems. Because of the high fluorescence intensity of benoxaprofen the detection limit on TLC plates is very low, namely 50—100 pg benoxaprofen per spot, i.e. 1/6 up to 1/3 pmol of benoxaprofen or benoxaprofen derivative.

Detection of alcohols in organic solvents

With benoxaprofen chloride it is possible to detect even small amounts of alcohols in organic solvents. Fig. 4 shows the TLC scans of benoxaprofen ethyl ester after the addition of benoxaprofen chloride to chloroform of different grades of purification.

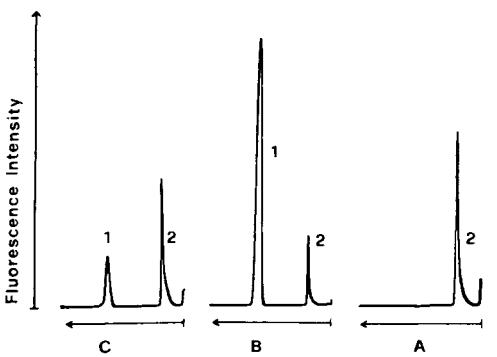


Fig. 4. TLC detection of ethanol in chloroform as benoxaprofen ethyl ester (= 1; benoxaprofen = 2): (A) blank sample; (B) chloroform p.a. (stabilized with ethanol); (C) chloroform for chromatography, stabilized with amylene.

Derivatization after extraction from biological materials

After derivatization with activated benoxaprofen it is possible to quantify, also in biological materials, very low concentrations of substances that in the underivatized state or after derivatization with other agents can be determined not at all or only in a high concentration range. Fig. 5 shows the TLC separation of maprotiline and one of its metabolites after extraction from plasma and derivatization. The calibration curve for maprotiline after derivatization with benoxaprofen chloride shows a linear relationship within the range 25—1000 ng/ml. Fig. 6 illustrates the HPLC separation of β-phenylethylamine extracted from plasma and derivatized to the corresponding amide.

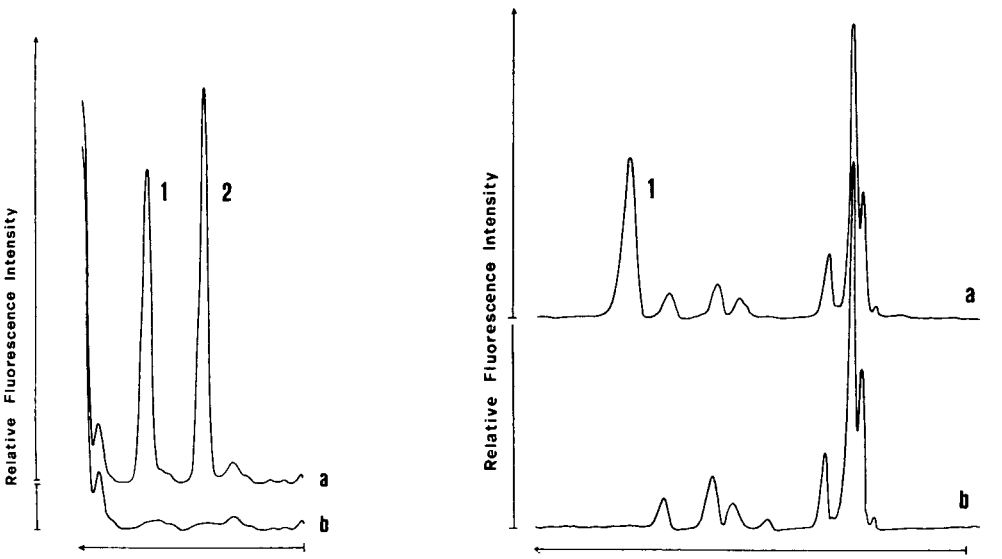


Fig. 5. Thin-layer chromatogram of maprotiline (1) and desmethylmaprotiline (2) after extraction from plasma (150 ng/ml) and derivatization with benoxaprofen chloride (= a; blank plasma = b). TLC separation was performed on silica gel plates using toluene—dichloromethane—tetrahydrofuran (5:2:1, v/v/v; ammonia atmosphere) as mobile phase.

Fig. 6. HPLC separation (system a) of β-phenylethylamine (1) after extraction from plasma (100 ng/ml) and derivatization with benoxaprofen chloride (= a; blank plasma = b).

Influence on the fluorescence intensity of benoxaprofen derivatives (e.g. benoxaprofen-α-methylbenzylamide)

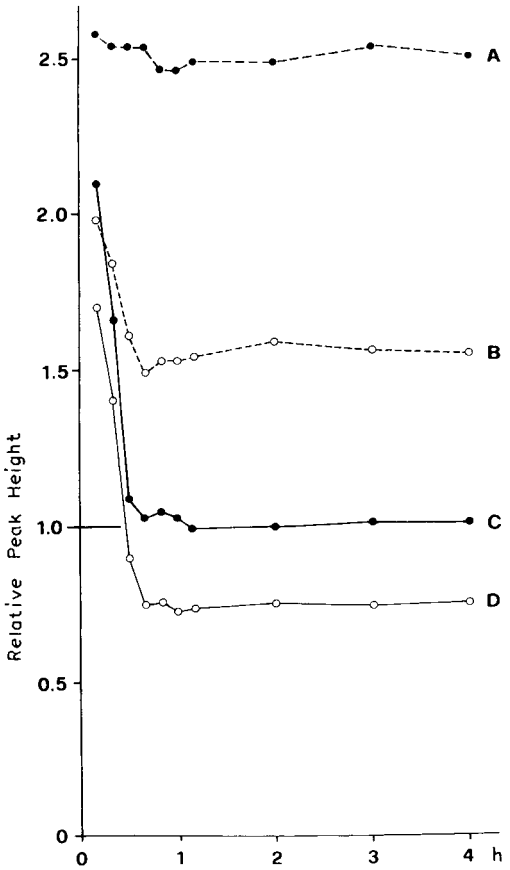
Solvents, acids and bases. The detection limit can even be lowered by spraying the plates with viscous organic solvents [7]. Table II and Fig. 7 show the influence of dipping the dried plates into various solvents (neutral, acidic and basic). Mixtures containing paraffin as a component enhanced the fluorescence intensity markedly. The investigations also demonstrate that the plates must be dried completely before measuring, as volatile organic solvents also increase the fluorescence intensity.

TABLE II

INFLUENCE OF SOLVENTS, ACIDS AND BASES ON THE FLUORESCENCE OF BENOXAPROFEN-α-METHYLBENZYLAMIDE

The peak heights are related to the peak height on untreated plates, which is 100% or 1. The fluorescence intensity was measured 2 h after dipping.

Solvent system	Relative peak height
Untreated plate	1.00
A. Paraffin—chloroform—isopropanol (1:1:4, v/v)	2.49
B. Paraffin—chloroform—isopropanol—concentrated ammonia (5:5:20:2, v/v)	1.58
C. Isopropanol	1.00
D. Isopropanol—concentrated ammonia (12:1, v/v)	0.75
E. Solution of sodium hydroxide (10%) in methanol—water (1:1, v/v)	0.58
F. Triethanolamine—chloroform—isopropanol (1:1:4, v/v)	0.01
G. Solution of citric acid (2%) in ethylene glycol—water—methanol (2:5:100, v/v)	0.90
H. Ethylene glycol—water—methanol (2:5:100, v/v)	0.89
I. Solution of citric acid (2%) in water—methanol (1:20, v/v)	0.82
K. Paraffin—chloroform—isopropanol—glacial acetic acid (5:5:20:2, v/v)	2.89



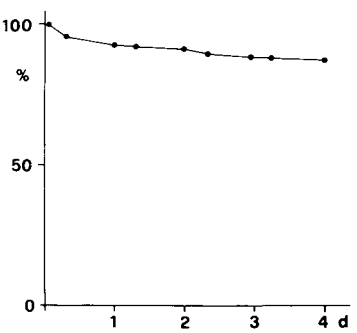


Fig. 7. Relative peak heights of benoxaprofen-α-methylbenzylamide on TLC plates within 4 h after dipping in solvent systems A—D, respectively. (The peak height on the untreated plates is 1.)

Fig. 8. Relative fluorescence intensity of benoxaprofen-α-methylbenzylamide on TLC plates exposed to light and air within 96 h. The values are means of four separate determinations. The standard deviations range from 0.9 to 2.5%.

Light and air. Fig. 8 shows the decrease of the fluorescence intensity within four days, if the plates are exposed to daylight and air. After 96 h the initial fluorescence intensity was diminished only by 13.5%.

Chemical stability

A special advantage of the fluorescence marker benoxaprofen chloride and benoxaprofen imidazolide is the chemical stability of the fluorophor. By-products have not been observed. Our experiments demonstrate that even at high temperatures (e.g. 110° C) the reaction takes place without any decomposition. The addition of potassium carbonate or acids to the reaction mixture does not influence the stability of the reagent or the products. In solution the derivatives are so stable that they are quantifiable even if stored for several days. Chromatography (TLC, HPLC) can be carried out in neutral as well as in acidic or basic eluents without decomposition.

Conclusions

In summary, it can be stated that benoxaprofen can easily be activated and that activated benoxaprofen is a reactive and an intensely fluorescent marker. The derivatives formed are stable. The spectral properties and chemical stability of the esters and amides on thin-layer plates as well as after HPLC separation allow their qualitative and quantitative analysis in the lower picomole range. This fluorescent label seems to be applicable for the development of sensitive assay methods for toxicological and pharmacological investigations. Furthermore, it was observed that after reaction of the activated racemic benoxaprofen with optically active compounds (e.g. α-methylbenzylamine, tranylcypromine), two peaks or spots with different retention times and R_F values are obtained in several chromatographic systems. These peaks or spots could be identified as the respective diastereoisomers. In a previous paper the application of (+)- and (—)-benoxaprofen as reagents for the TLC and HPLC determination of chiral amines has already been described [8].

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MASS SPECTROMETRIC DETERMINATION OF N-HYDROXYPHENACETIN IN URINE USING MULTIPLE METASTABLE PEAK MONITORING FOLLOWING THIN-LAYER CHROMATOGRAPHY

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SUMMARY

This work describes a method for the quantitative determination of the labile, toxic N-hydroxy metabolite of phenacetin in urine. A thin-layer chromatography step was used for the preliminary purification of extracts, and the specificity of the assay was based on the monitoring of specific metastable decompositions in a forward geometry double-focussing mass spectrometer, in a manner analogous to conventional tandem mass spectrometry. This precluded the need for a gas chromatographic separation, thus minimizing thermal decomposition which can occur with these compounds, as well as enabling very rapid analyses.

INTRODUCTION

The toxicity of the antipyretic—analgesic drug, phenacetin, is considered to be at least partly mediated by its N-hydroxy metabolite [1]. The formation of N-hydroxyphenacetin (NHP), and its biochemical reactivity, have been studied extensively in vitro, but little is known of the extent of N-hydroxylation of phenacetin in vivo. The problem has been to measure NHP, which is only formed in small amounts from phenacetin, in the presence of other phenacetin metabolites and endogenous interfering substances in urine. A variety of assays have been reported for NHP and similar N-arylacetylhydroxamic acids. At first, colorimetric methods were used for urinary analysis [2], but these lack specificity [3]. NHP and other N-arylacetylhydroxamic acids formed in biochemical reactions in vitro have been assayed by high-performance liquid chromatography (HPLC) [4, 5], gas chromatography—mass spectrometry (GC—MS) [6], and thin-layer chromatography (TLC) using radiolabelled phenacetin [7]. We have previously reported the assay of urinary NHP by GC

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of its methylated derivative, N-methoxyphenacetin (NMP), using flame-ionization detection [8]. However, as mentioned in that report [8], NMP can undergo decomposition under GC conditions, so we sought a more reliable assay of NHP.

This paper describes the determination of NHP, as NMP, using TLC to separate NMP from most interfering substances, followed by specific detection by MS using metastable peak monitoring. This MS technique deserves some introductory remarks.

Increasing use is being made of mass spectrometers with more than one analysing region to act as specific and sensitive detectors for target compounds in relatively impure samples. Much of this work has been carried out with reversed geometry [9, 10] or tandem quadrupole [11, 12] instruments. In these cases, the two sectors are used sequentially, the first to isolate an ion of interest and the second to examine its metastable or collisionally induced decomposition products (MS—MS). Full daughter ion spectra [13, 14] or selected daughter ion monitoring [15—17] have been used. Double-focussing mass spectrometers of normal geometry have also been used, in which the metastable or collisionally induced decompositions occur in the first field-free region, just after the ion source. This was used originally to increase the specificity of GC—MS [18, 19], and more recently has been applied to direct probe analyses [20, 21]. Although the sectors are not used sequentially in normal geometry instruments, since the products of specific decompositions are being monitored, it nevertheless constitutes a form of MS—MS.

EXPERIMENTAL

Materials

NHP was prepared and characterised as previously described [8]. As internal standard, the deuterated analogue of N-hydroxyphenacetin was synthesized from 4-nitro[2,3,5,6-$^2H_4$]phenol (KOR Isotopes, Cambridge, MA, U.S.A.). The deuterated 4-nitrophenol was first ethylated, using ethyl iodide—potassium carbonate in acetone, after the method of Vogel [22]. The resulting 4-nitro-[$^2H_4$]phenetole was reduced and selectively acetylated to give N-hydroxy-[2,3,5,6-$^2H_4$]phenacetin (DNHP), by the method used for the synthesis of unlabelled NHP [8]. DNHP was identical to NHP in m.p. (104°C) and TLC mobility, and on reduction with titanous chloride it yielded a single product which co-chromatographed with phenacetin on TLC [8]. The mass spectrum of DNHP was similar to that of NHP [23] except the major fragments were four a.m.u. higher. The isotopic purity was 98% $^2H_4$.

N-Butyryl-4-aminobenzoic acid (4-BABA) was prepared by reacting 4-aminobenzoic acid with butyric anhydride, and was recrystallized from 95% ethanol, m.p. 228—231°C. 4-BABA ran as a single substance on TLC, and its methylated derivative, N-butyryl-4-aminomethyl benzoate (4-BAMB), behaved as one compound on TLC and GC. The mass spectrum of 4-BAMB was consistent with its structure.

Diazomethane was made in small quantities, fresh as required, from *p*-tosylsulphonylmethylnitrosamide [22], and was used as the ethereal solution. Other chemicals and solvents were of analytical grade.

Extract of *Helix pomatia* (β-glucuronidase plus arylsulphatase) was obtained from Boehringer (Mannheim, F.R.G.).

Apparatus

Glass TLC plates (20 × 20 cm) were coated with silica gel (0.25-mm thick) containing a fluorescent marker (Sigma type GF, size 10—40 μm; St. Louis, MO, U.S.A.). The mass spectrometer was a Vacuum Generators (U.K.) 70/70F double-focussing instrument, with a digital scan controller, linked-scan unit, mode switch, 8-channel M.I.D. unit and 2035 data system with magnet switching selected ion software. The instrument was operated in the electron impact mode at 70 eV and 4 kV main beam accelerating voltage.

Extraction and methylation

Urine was collected from subjects who had taken a dose of phenacetin (10 mg/kg in two gelatin capsules), and samples were kept at −20°C until analysed. The analysis was commenced on the day of collection, since the glucuronide conjugate of NHP is known to be unstable in aqueous solution [24].

Calibration curves were obtained by addition of known amounts of NHP (in methanol) to urine from an undosed subject.

A 2-ml aliquot of urine was placed in a 30-ml stoppered centrifuge tube, 200 μl of 1.10 *M* acetate buffer, pH 5.2 were added and the final solution was adjusted to pH 5.2, if necessary, with 5 *M* hydrochloric acid. To this were added 100 μl extract of *Helix pomatia* and 25 μl of methanol containing the internal standard. The internal standard was originally 25 μg of 4-BABA, but eventually 5 μg of DNHP were used when DNHP became available. The mixture was incubated overnight (17 h) at 37°C to hydrolyse conjugated NHP.

The tubes were then cooled in ice, the hydrolysate adjusted to pH 1.0 with 5 *M* hydrochloric acid (200 μl) and extracted with 15 ml methylene chloride by vortexing for 30 sec. The phases were separated by centrifugation (1200 *g*, 5 min), and freezing (dry ice—acetone), and the methylene chloride decanted into a 50-ml round bottomed flask. The extract was taken to dryness on a rotary evaporator (40°C), the residue redissolved in 2 ml methanol, and cooled in ice. Methylation was achieved with 2 ml ethereal diazomethane (1 h, on ice) and the reaction mixture again evaporated to dryness.

Thin-layer chromatography

The residue in the flask was redissolved in 200 μl methylene chloride and applied as a 5-cm strip to a silica gel TLC plate, which was developed twice with chloroform. A band which moved with reference NMP (R_F 0.26) was scraped off and eluted by vortexing for 30 sec with 10 ml methanol. After sedimenting the silica gel by centrifugation (1200 *g*, 5 min) the methanol was transferred by Pasteur pipette to a 50-ml round bottom flask, and the solvent removed with a rotary evaporator. The residue was redissolved in 200 μl methylene chloride and transferred to a conical-tipped tube ready for analysis.

Mass spectrometry

The daughter ions chosen for monitoring NMP and DNMP were *m/z* 135

and 139, respectively (see Results). The two metastable peaks were alternately brought to focus by a combination of linked magnetic field/electric sector (B/E) and accelerating voltage switching, through a modification of a previously described method [21].

At a resolution of 1000, the ion at *m/z* 214 in the mass spectrum of perfluorotributylamine was selected as the starting point of a theoretical B/E linked scan (i.e. the magnetic field which transmitted *m/z* 214 was linked to the electric sector voltage). B/E scans from *m/z* 209 and 213 could then be easily focussed by small accelerating voltage adjustments [25]. Accelerating voltages $V_1 = (209/214) \times V_0$ and $V_2 = (213/214) \times V_0$, where V_0 was the accelerating voltage at which the main beam was being transmitted, were entered into two channels of the M.I.D. unit, the output of which was routed directly to the accelerating voltage programmed power supply, bypassing the normal electric sector coupling. The appropriate constant parent ion scans were then focussed when the respective channel was selected.

Two genuine daughter ion channels were entered, at *m/z* 85.16 ($= 135^2/214$) (channel 2), and *m/z* 90.28 ($= 139^2/214$) (channel 4) into the magnetic selected ion software, along with two additional channels at *m/z* 82.66 ($= 133^2/214$) (channel 1) and *m/z* 87.70 ($= 137^2/214$) (channel 3), with the linked scan unit remaining on. These channels resulted in *m/z* 133, 135, 137 and 139 daughter ions being selected, and to be shown as such on the mass indicator of the 70/70.

A small sample of standard NMP and DNMP was loaded onto the direct insertion probe, and using a small sweep of the B/E scan over 1 mass unit each genuine daughter channel was accurately centred with the appropriate M.I.D. channel selected. The collector slit was then opened to give flat topped peaks.

The accelerating voltage was cycled to switch during daughter channels 1 and 3 so that the appropriate voltage was selected when genuine daughter channels 2 and 4 were selected. Dwell time was 1 sec for each accelerating voltage channel, and 500 msec, including reset time, for each daughter ion channel, with a full cycle resulting every 2 sec. For synchronization, the accelerating voltage cycle was started and the magnet switching was then initiated at the appropriate instant to result in V_1 being selected during channel 1 and V_2 during channel 3.

To avoid a very rapid distillation of NMP from the probe, a relatively cool (120°C) source temperature was used. This resulted in the distillation maximizing at about 20 sec after insertion of the probe, providing 20 sampling points.

A 1-μl aliquot of the sample was loaded on to the direct insertion probe and the results of the 209 → 135 and 213 → 139 decompositions were acquired in the manner described, being displayed as intensity versus time. The area of each was measured on the data system and the ratio calculated for determination of unknown concentrations.

RESULTS AND DISCUSSION

Thin-layer chromatography

Preliminary experiments showed that the extracted hydrolysate contained

too many interfering substances for NHP to be determined by direct insertion MS—MS, as we had previously found it to be too impure for GC analysis [8]. TLC was used to separate NHP from most of these interfering compounds.

Methylation of NHP gave a stable derivative for TLC. NHP itself tails badly on silica gel [8], possibly because of chelation to trace metals [4]. Hinson and Mitchell [7] found it necessary to add non-radioactive NHP to minimize adsorption losses of [^{3}H]NHP during their TLC assay.

Recovery from urine

Blank urine was spiked with NHP to examine the effect of pH on the extraction of NHP into methylene chloride. In this experiment 4-methylacetanilide was used to standardize the GC assay [8]. Recovery of NHP was better at pH 1 (95%) than at pH 3 or 5 (65%).

The methylation reaction mixture was analysed at various times up to 24 h to check on the completeness of NHP conversion to NMP. The reaction was 90% complete in 30 min, and no further NMP was formed after 1 h.

Optimal conditions for hydrolysis of conjugated NHP were found by treating urine from a subject dosed with phenacetin with different amounts of enzyme and various incubation times. It was found that 0.1 ml extract *Helix pomatia* per 2 ml urine gave the highest recovery of NHP, as well as most phenolic metabolites of phenacetin (to be reported elsewhere). An exception was 2-hydroxyphenacetin (2HP), whose recovery, and therefore hydrolysis, was maximal with 0.04 ml enzyme mixture. Incremental additions of enzyme (0.02 ml) resulted in a progressive decline in 2HP recovery, and a concomittant increase in NHP found. It seems likely that this reciprocal relationship is due to two competing reactions: the rearrangement of NHP-glucuronide to 2HP-glucuronide [24] and the enzymatic hydrolysis of the NHP-glucuronide. More enzyme, and therefore a faster hydrolysis, leaves less time for the rearrangement to occur. Lacking an NHP-glucuronide standard, we were unable to check on the extent of residual isomerization under conditions giving maximum recovery of NHP.

Using 0.1 ml enzyme, hydrolysis was found to be complete after incubation for 17 h (overnight).

Mean overall recovery was 63%, estimated from the NMP/DNMP ratio found when 5 μg NHP were added to urine and 5 μg DNHP added at the methylation step.

Mass spectrometry

Fig. 1 shows the normal 70-eV mass spectrum of NMP, with that of DNMP, in which all the major ions are shifted by 4 a.m.u. A full B/E linked scan from the molecular ion (*m/z* 209) of NMP, to find the direct daughter ions from the first field-free region, gave a spectrum with *m/z* 179 (2.5%), 178 (5%), 167 (100%), 166 (10%), and 135 (18%) as the only significant peaks. The ions at *m/z* 167 and 166 corresponded to the loss of ketene and an acetyl radical respectively, and were also found to occur in the normal and B/E spectra of the isomeric ring methoxylated compounds, 2- and 3-methoxyphenacetin [23]. Therefore these daughter ions were not suitable for monitoring NMP in the presence of isomeric phenolic metabolites of phenacetin. However, the

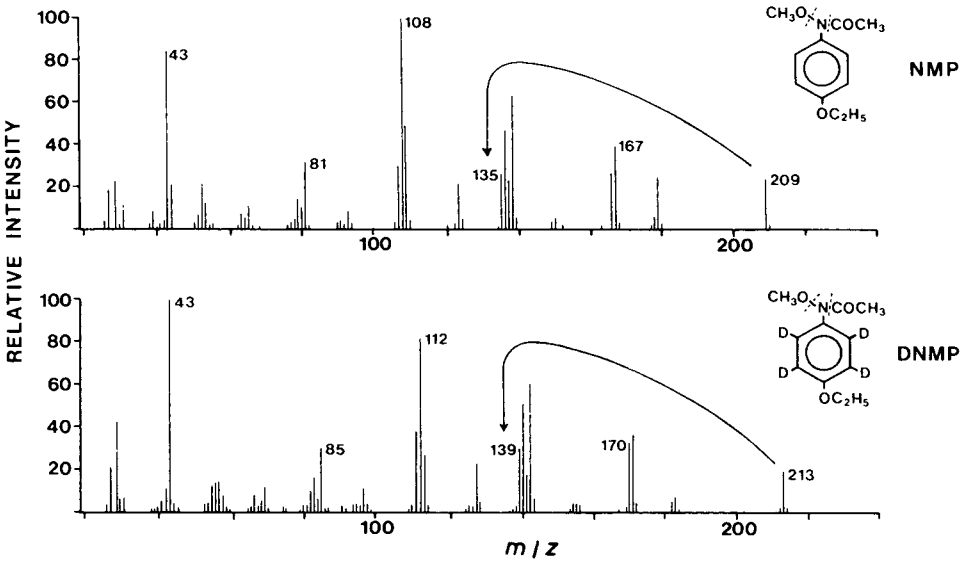

Fig. 1. Electron impact mass spectra of NMP and DNMP, with arrows showing the metastable reactions monitored for quantitative analysis. Broken lines show the sites of cleavage in these reactions.

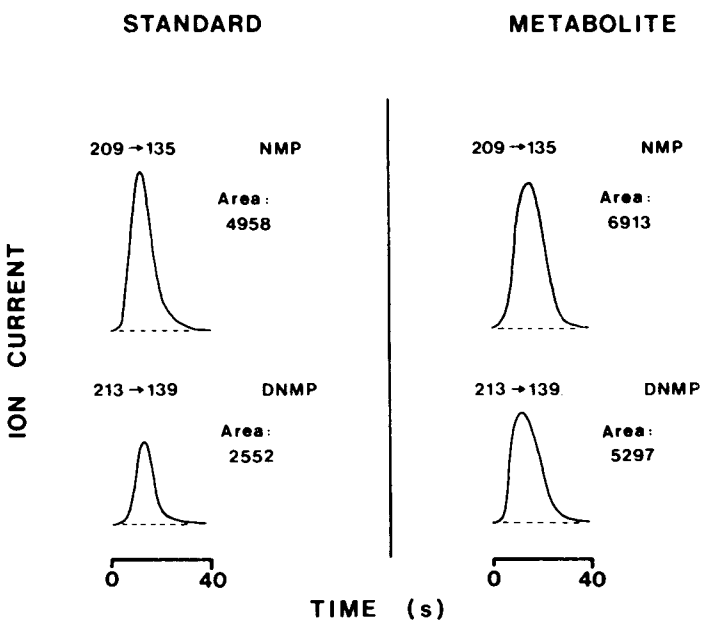

Fig. 2. Ion traces obtained during metastable peak monitoring of NMP and DNMP. The left side shows a calibration standard, in which 10 μg NHP was added to 2 ml blank urine. The right side shows analysis of urine following a dose of phenacetin; 6.7 μg NHP was found per 2 ml urine.

daughter ions at *m/z* 179, 178 and 135 were not found to be present in the normal or B/E spectra of the ring-substituted compounds, making them suitable candidates for a specific assay. Due to the relative size, and specificity of the loss, the ion at *m/z* 135 was chosen as being the most suitable. This ion was found from the high-resolution studies of the main beam *m/z* 135 ion to be C_8H_9NO, corresponding to the loss of the elements of the acetyl and methoxy groups by an undetermined mechanism. Thus the decompositions monitored were 209 → 135 for NMP, and 213 → 139 for DNMP, as indicated in Figs. 1 and 2.

An extract of hydrolysed urine from an undosed subject, without any preliminary purification, was monitored for these decompositions to determine the extent, if any, of interference from compounds other than NMP. This indicated there was some interference from other 209 → 135 decompositions, or additional reactions which could be focussed under the conditions employed [21]. Although the interference did not display the distillation profile of NMP from the direct insertion probe, it would nevertheless have made accurate quantification difficult. Hence a preliminary purification was necessary, and the TLC procedure was employed. An examination of the extract of a TLC scrape at the R_F of NMP from an undosed subject indicated virtually no detectable signal in either channel being monitored.

Calibration curve

Addition of NHP (1—20 μg) and DNHP (5 μg) to 2 ml blank urine gave a good linear relationship using either peak heights or peak areas (ratio NMP/DNMP versus amount NHP added). Peak areas gave a slope of 0.191, *y*-intercept 0.03, and correlation coefficient 0.9983. Peak heights gave a slope of 0.186, *y*-intercept 0.055, and correlation coefficient 0.9994. Repeated measurements on the same sample gave an instrumental coefficient of variation (C.V.) of 1.94% (n = 8). Replicate assays using eight lots of 2 ml urine from one urine collection sample from a subject who had taken phenacetin gave a C.V. of 9.2% (by peak area) and 7.2% (by peak height).

The selectivity of the assay depends on two separation steps: (1) TLC and (2) the selection of specific daughter ions produced from the molecular ions of NMP and DNMP in the first field-free region of the mass spectrometer. Thus NHP can be assayed in the presence of many other metabolites of phenacetin which are mostly formed in far greater amounts.

In early experiments, before DNHP was available, 4-BABA was used successfully as an internal standard. In this case the metastable reaction monitored was 221 → 151 (the molecular ion losing C_4H_6O). There was no interference from blank urine, and the calibration curve was linear (slope 0.0098, *y*-intercept 0.0018, correlation coefficient 0.9993). The only unsatisfactory aspect of 4-BAMB was that it distilled off the probe much more slowly than NMP, which considerably prolonged the MS analysis time. DNMP, having the same distillation profile as NMP, enabled much faster analyses, and is the ideal internal standard.

Formation of NHP from phenacetin in man

Fig. 3 shows the urinary excretion of NHP by a male subject following a

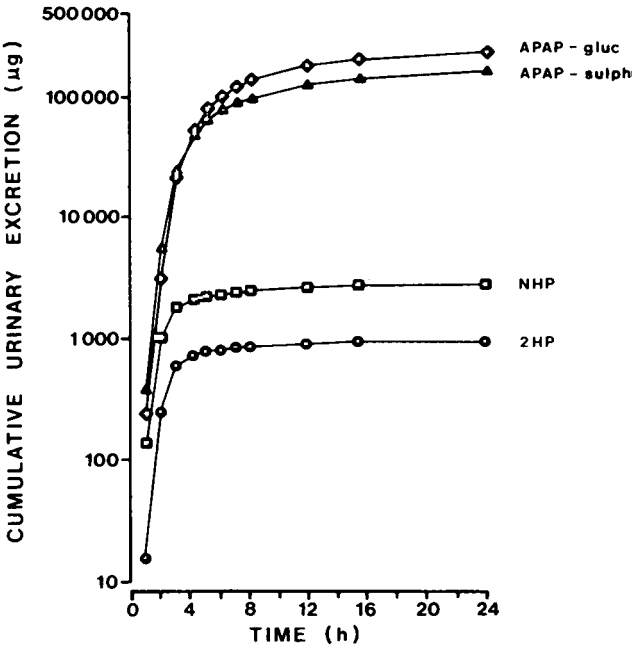

Fig. 3. Cumulative urinary excretion of NHP and some other phenacetin metabolites, following an oral dose of 10 mg/kg phenacetin in a male subject. APAP-gluc (♦) is paracetamol glucuronide and APAP-sulph (▲) is paracetamol sulphate. These two major metabolites of phenacetin are included for comparison with NHP (■) and 2HP (●), and their HPLC determination will be described in a separate communication.

dose of phenacetin (10 mg/kg, orally in two gelatin capsules). The presence of NHP in urine was separately confirmed by GC—MS, as previously described [8]. The total amount of NHP excreted in 24 h corresponded to 0.40% of the dose of phenacetin. This is comparable with the finding of 0.28% NHP after 900 mg phenacetin, in which NHP was determined by its ability to chelate copper and extract it into water [2]. However, in view of the complex metabolism of phenacetin, we believe that our structurally specific assay is preferable.

The assay is sensitive to less than 0.5 µg NHP per ml urine, which appears quite adequate for metabolic studies involving urine analysis.

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Note

High-performance liquid chromatographic determination of (*Z*)- and (*E*)-urocanic acid in human skin

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Urocanic acid [3-(1H-imidazol-4-yl)-2-propenoic acid] (UCA), a constituent of mammalian stratum corneum, is believed to act as a natural protective agent against sunburn [1—6]. Several high-performance liquid chromatographic (HPLC) procedures for the determination of UCA have been reported in recent years [7—12]. Especially the methods of Morrison et al. [11] and Caron et al. [12] seem to be very attractive at first glance for separation of both possible geometric isomers of UCA. In agreement with Caron et al. we found the method of Morrison et al. to be unreliable, because the described elution conditions caused rapid deterioration of the stationary phases. Caron et al. determined (*Z*)- and (*E*)-UCA in human plantar callus. In our opinion their determination methods are less attractive for the following reasons: (1) The use of a relatively high amount of biological material (50 mg) and the location (plantar callus) from which the sample was obtained. (2) Study of the effect of light on the *E* to *Z* isomerization of UCA is not possible

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under normal circumstances of daily routine using plantar callus. (3) Polar phases of the amino type show poor reproducibility and stability after prolonged use. (4) The mechanical homogenization of plantar callus, by means of perchloric acid, is a very tricky and time-consuming procedure and a significant decomposition of UCA may occur.

In our experiments, we used the reversed-phase mode, which is in principle capable of separating *Z* and *E* isomers [13].

A new HPLC procedure for the determination of (*Z*)- and (*E*)-UCA in 1—2 mg of the horny layer of human skin has been developed, which is sensitive enough to measure concentrations of UCA down to 150 ng/mg wet weight of human stratum corneum. This makes the method particularly attractive for paediatric work, especially in the diagnosis of histidinaemia, in which a decreased concentration of UCA in the stratum corneum has been reported [14—16].

A disturbance of the epidermal UCA content may be one of the etiological factors in hydroa vacciniformia, a very rare skin disease [17].

EXPERIMENTAL

All chemicals used were of the highest purity available. The mobile phase was filtered through a 0.45-μm filter and degassed by ultrasonication prior to use.

Procedure

With an ordinary fingernail clipper a small amount of stratum corneum (1—2 mg) was removed from the fingertips, weighed and transferred to a centrifuge tube. Then 100 μl of potassium hydroxide solution (1 mol/l) were added and the tube was vortexed briefly. After 10 min, the tube was centrifuged for 10 min at 6000 *g* and 4° C. Subsequently the supernatant was transferred to a 5-ml volumetric flask. This step was repeated. The residue was washed with 200 μl of water followed by 10 min centrifugation. The combined supernatants were acidified with 200 μl of orthophosphoric acid solution (2/3 mol/l) and made up to 5 ml with mobile phase; 50 μl were injected on the column.

Standard aqueous solutions were prepared using authentic UCA (anhydrous, stored desiccated in the dark at 4° C; Sigma, Munich, F.R.G.), under protection against light, in the range 20 μg per 0.5 ml to 0.31 μg per 0.5 ml and stored at —20° C. Samples were stable for at least two months.

Liquid chromatography

The analyses were performed using a liquid chromatograph consisting of a Beckman 112 solvent delivery module, a Beckman 420 controller, and a Beckman 340 organizer fitted with an Altex 210 inject system with 100-μl sample loop. Two columns, an Ultrasphere TM ODS, 25 cm × 4.6 mm I.D., 5 μm (Altex), and a MPLC®-microbore separation cartridge with an RP-18 3 cm × 2.1 mm, 5-μm guard column and an RP-18 22 cm × 2.1 mm, 5-μm main column (Kontron, Eching, F.R.G.), were tested. A Uvikon 720 LC spectrophotometer (Kontron) combined with a plotting integrator (C-R2AX, Shimadzu, Düsseldorf, F.R.G.) was used as detector; this was operated at 277

nm. The mobile phase was a mixture of potassium dihydrogen phosphate (0.02 mol/l) pH 3.7 with 1 g/l heptanesulphonic acid (sodium salt) and acetonitrile 93:7 (v/v). The flow-rate was 0.8 ml/min.

Mass spectrometry

Mass spectra were recorded on a double-focussing mass spectrometer (Varian Mat 311 A, Varian, Bremen, F.R.G.) in the electron impact mode with an electron energy of 70 eV.

RESULTS AND DISCUSSION

During the experiments the Ultrasphere ODS column showed slight tailing. Thus for quantitative analyses the MPLC-system was used. The accuracy of the method was tested with a standard sample containing 5 μg per 0.5 ml of water irradiated for 2 h with sunlight. The total amount of UCA was 5.02 ± 0.14 (S.D.) μg per 0.5 ml.

The coefficient of variation (C.V.) was 2.8% (n = 10, within-assay). The corresponding data for the between-assay were: 5.50 ± 0.19 μg per 0.5 ml, C.V. = 3.8%. Recovery was determined by adding 10, 20 and 30 μg of UCA to three preextracted stratum corneum samples of 1.2, 1.3 and 1.9 mg wet weight. A mean recovery of 96.0 ± 2.4%, C.V. = 2.5%, was found.

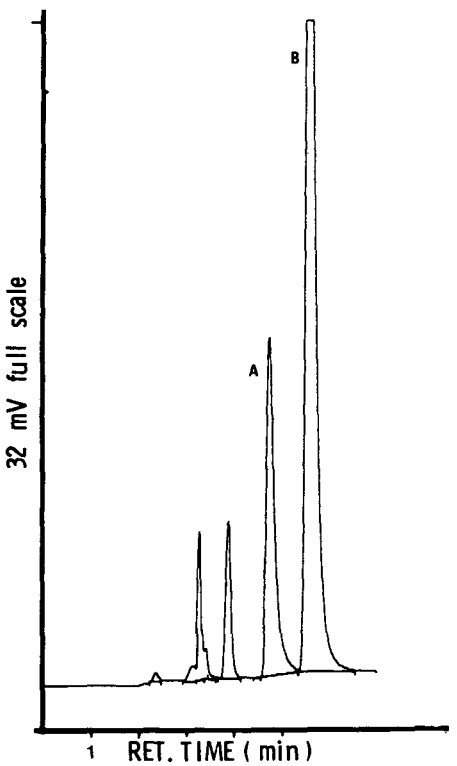

Fig. 1. Typical chromatogram of a stratum corneum extract of a 4-year-old girl. Peak A is (*Z*)-urocanic acid, peak B (*E*)-urocanic acid. For chromatographic conditions see experimental section.

The calibration was linear between 0.31 μg per 0.5 ml and 20 μg per 0.5 ml.

Solid authentic UCA and its aqueous solution protected from light had the *E* configuration. This could be proved by HPLC and infrared (IR) spectroscopy. Only one peak at 5.6 min appeared in the chromatogram. In the IR spectrum (solid material) a sharp absorption at 966 cm^{-1} is apparent, which is characteristic for the *E* configuration at a $\geq C=C\leq$ double bond. After 2 h exposure in sunlight in a plastic vessel, the 966 cm^{-1} band decreased, coinciding with a diminution of the peak at 5.6 min. A second peak, at 4.8 min, appeared. Control samples, protected from light with aluminium foil, did not show these alterations. After irradiation, the chromatogram resembled that in Fig. 1, which is a chromatogram of a normal human stratum corneum extract.

These results provided strong evidence for *E* to *Z* isomerization due to light energy. The effluents of peaks A and B were collected separately and evaporated in the dark in a vacuum desiccator over anhydrous calcium chloride. Mass spectra of the residues were then recorded. Both spectra showed the same fragmentation pattern and were identical with authentic UCA. Residues from a second run were redissolved in 50 μl of water and reinjected. In both residues only one appropriate peak could be found. Thus, no reisomerization occurred. The conclusion of all the experimental results is that peak A (4.8 min) is (*Z*)-UCA and peak B (5.6 min) is (*E*)-UCA.

Quantification of (*Z*)- and (*E*)-UCA was made as follows: a 5 μg per 0.5 ml sample of authentic UCA (pure *E* isomer) was injected and the area under the single peak (peak B) recorded. Subsequently, the sample was irradiated and re-injected. The area of the now arising peak A was compared to standard samples of pure (*E*)-UCA. This is possible because the difference between (*Z*)- and (*E*)-UCA in terms of spectrum shape and absorption intensity is negligible within the limits of analytical error.

The described method was tested in eight children between the ages of 1.5 and 8 years (mean 4.6 ± 2.3 years) with the informed consent of their parents. Samples were taken in winter. The results are shown in Table I.

The method presented offers the opportunity to determine both geometrical isomers of UCA in human skin in one step without lengthy solvent extraction

TABLE I

UROCANIC ACID CONTENT OF STRATUM CORNEUM IN EIGHT CHILDREN

Volunteer	Age (years)	Sex*	UCA content (μg/mg wet weight)			*Z/E* ratio
			(*Z*)-UCA	(*E*)-UCA	Total UCA	
1	7	f	1.22	4.71	5.93	1:4
2	8	m	—	6.51	6.51	—
3	1.5	m	0.81	5.25	6.06	1:6
4	6	f	0.70	4.51	5.21	1:6
5	4	f	0.49	1.90	2.39	1:4
6	4	m	0.23	5.36	5.59	1:23
7	4	f	0.26	3.43	3.69	1:13
8	2	m	—	3.46	3.46	—

*m = male; f = female.

or derivatization procedures. Furthermore, seasonal variations in UCA content as well as alterations in the isomer ratio can be delineated in a very convenient manner.

Another practical aspect of this method, besides the diagnosis of histidinaemia, would be the possibility of evaluating the efficacy of sun-protective agents.

UCA determinations with the described method are currently being used to elucidate the role of UCA in hydroa vacciniformia. The results will be published at a later date.

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Note

Determination of morphine in serum and cerebrospinal fluid by gas chromatography and selected ion monitoring after reversed-phase column extraction

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A wide range of methods for the determination of nanogram levels of morphine in biological fluids have been reported. Assays based on scintillation counting of radioactively labelled morphine and radioimmunoassay are capable of detecting picogram amounts of this drug, but are relatively non-specific [1—5].

Gas chromatographic procedures require extraction and formation of a volatile morphine derivative by silylation or alkylation. Quantitation is achieved by electron capture detection when a fluoroalkyl derivative is prepared [6—10]. Assessment of morphine after extraction by liquid chromatography with ultraviolet as well as electrochemical detection has been described [11—14]. Gas chromatography combined with mass spectrometry offers a highly specific and sensitive method and is a most suitable assay to obtain an unequivocal identification of morphine [15—23].

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Extraction procedures of morphine from biological specimens prior to analysis are mainly performed by liquid—liquid extraction. However, this isolation technique is rather time consuming. Todd et al. [13] described an extraction procedure on a Clin-Elut CE 1001 silica gel column. Reversed-phase column extraction offers the advantage of smaller elution volumes compared to normal-phase silica gel column extraction. Sep-Pak C_{18} cartridges were used for extraction by Svensson et al. [14].

In this paper we describe a rapid and efficient extraction procedure for the isolation of morphine from biological fluids. Morphine is extracted onto a reversed-phase Bond-Elut C_{18} column and the derivatized extract is analysed by selected ion monitoring, under chemical ionization conditions. This technique is being applied in a combined pharmacokinetic and clinical study; preliminary results illustrate the practical use of the method.

MATERIALS AND METHODS

Reagents

Morphine hydrochloride (pharmacopee grade) was obtained from Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands). Deuterated morphine was prepared by reaction of normorphine and deuterated methyl iodide ($C[^2H_3]I$) according to the procedure of Ebbinghausen et al. [16]. Methanol was high-performance liquid chromatography grade from Baker (Deventer, The Netherlands). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Oud Beijerland, The Netherlands) and β-D-glucuronidase was obtained from Sigma (Amsterdam, The Netherlands). All other chemicals were analytical grade. Baker octadecyl C_{18} columns were used for the extraction procedure.

Instrumentation

Gas chromatography was performed isothermally at 300°C on a 25 m × 0.32 mm I.D. fused-silica column with a chemically bonded stationary phase CP Sil 8 from Chrompack (Middelburg, The Netherlands). The carrier gas was helium, flow-regulated at 1.2 ml/min. A splitless type of injector was employed. Mass spectrometry was performed on a Finnigan 3200 F instrument in chemical ionization (CI) mode. Ammonia—methane (1:5) as reagent gas was directly introduced into the ion source. In selected ion monitoring at high sensitivity levels, a four-channel peak selection (PROMIM) was used. The temperature of the CI ion source was digitally controlled at 140°C ± 1°C. The temperature stated was measured at the elution time of morphine. The mass spectrometer was tuned for optimal sensitivity at the selected ions.

Sample preparation

Before use the columns were prewashed with 5.0 ml of methanol, 3.0 ml of distilled water and 1.0 ml of 0.05 mol/l borax buffer pH 9.0. Serum or liquor (100 μl to 1.0 ml) was mixed with 50 μl (= 50 ng) of internal standard and 1.0 ml of buffer pH 9.0, and transferred to the Bond-Elut C_{18} column. The column was washed twice with 500 μl of water and once with 100 μl of 80% methanol. Morphine was eluted with 0.5 ml of methanol and collected in a 1-ml reaction

vial. The solvent was evaporated under a stream of nitrogen and the residue was derivatized with 20 μl of BSTFA in pyridine (1:1) by heating at 85°C for 15 min; 2 μl of the mixture were injected into the gas chromatograph.

The total amount of morphine (i.e. free morphine base and morphine glucuronide) was estimated as follows: 0.5 ml of the serum sample was buffered at pH 5.0 and hydrolysed by 5 mg of β-D-glucuronidase at 37°C for 16 h. The mixture obtained was made alkaline with 4 *M* sodium hydroxide solution and adjusted to pH 9.0 with borax buffer. The precipitate was centrifuged and the supernatant was transferred to the extraction column and treated as described above.

RESULTS AND DISCUSSION

Previous reported procedures [17—23] for the determination of morphine in biological specimens using liquid—liquid extraction for the isolation of the drug have been examined. Compared to the present extraction procedure for serum and liquor these methods were less efficient and also time consuming.

To establish a standard curve we added known amounts of morphine and [N-C^2H_3]morphine to aliquots of morphine-free serum and liquor. The standard curve indicates that the measurement of morphine concentrations is linear over the range 5—200 ng/ml. The linear regression line of the data was calculated using the least-squares regression method and is expressed by the equation $y = 0.033 - 0.015x$ (y = peak ratio of morphine versus internal standard, x = morphine concentration in ng/ml) with a correlation coefficient of 0.996. Day-to-day precision of the morphine concentration of 50 ng/ml in serum ($n = 5$) was calculated, with a coefficient of variation of 5.4%.

The recovery of morphine at a concentration of 10 ng/ml from serum as well as from liquor was 90%.

Using the described procedure, morphine could not be detected when morphine glucuronide was added to serum at a concentration of 100 ng/ml. Svensson et al. [14] reported that morphine and its conjugate were eluted from a Sep-Pak C_{18} column. After hydrolysis with β-glucuronidase of the same concentration a 95% recovery of morphine was obtained.

Selected ion monitoring was applied to the analysis of morphine using a quadrupole mass spectrometer under CI conditions. Two masses were monitored at *m/e* 340 and *m/e* 343, respectively. They correspond to the prominent fragment ion $(M-89)^+$ [loss of $(CH_3)_3SiOH$] and the corresponding deuterated compound in the ammonia—methane CI spectrum of bis(trimethylsilyl)morphine and [N-C^2H_3] bis(trimethylsilyl)morphine (Fig. 1).

The procedure was applied to serum and liquor samples of two patients. One of them was undergoing surgery by standardized epidural anaesthesia, the other by intravenous administration of morphine. Morphine hydrochloride (1 mg/kg body weight) was injected intravenously and 0.1 mg/kg was injected through the epidural catheter. Eight liquor samples as well as serum samples were taken simultaneously within the time interval 0—240 min after injection.

In order to exclude any interference in the determination of morphine by drugs administered as premedication before surgery (e.g. methylatropine, diazepam and promethazine or/and their metabolites), a serum sample was

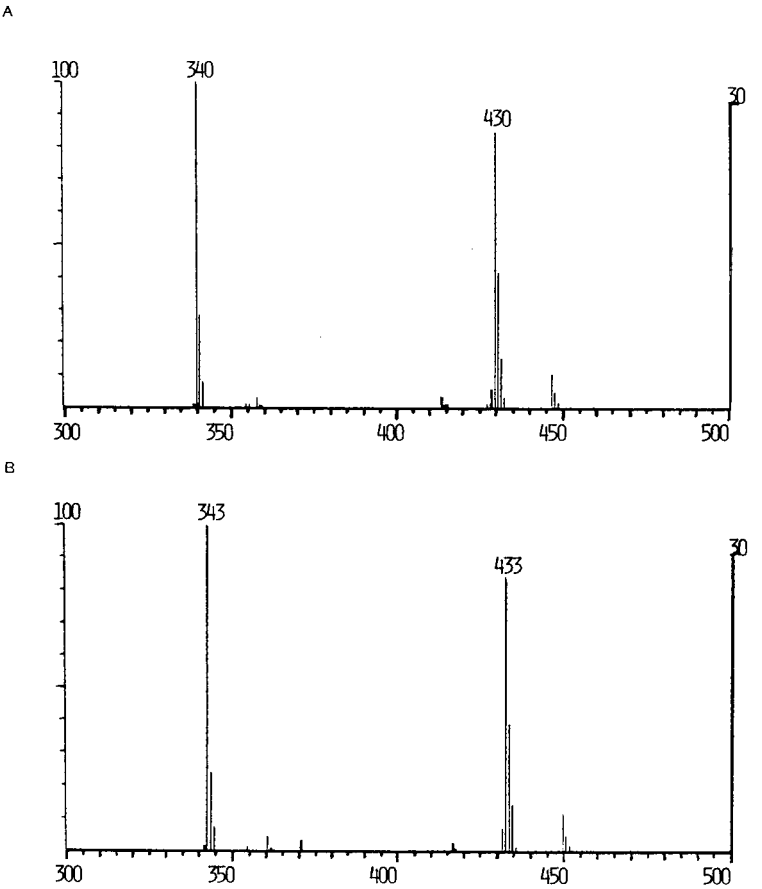

Fig. 1. Ammonia—methane chemical ionization spectra of bis(trimethylsilyl)morphine (A) and [N-$^2H_3$]bis(trimethylsilyl)morphine (B).

taken just before anaesthesia was initiated. No response was observed in these samples for the ion pair 340/343. These patients did not receive any other structurally related drugs like codeine, since morphine is a possible metabolite of codeine.

Fig. 2 shows the serum morphine concentration of the patient receiving the drug by intravenous route before and after hydrolysis. The major metabolite of morphine in man is the glucuronide conjugate. By examining the concentration of morphine before and after hydrolysis with β-glucuronidase, the decline is demonstrated between free and conjugated morphine in serum. The results indicate that the maximum ratio of morphine glucuronide to morphine in the serum of this patient was 10 to 1, which is in good agreement with the findings of Murphy and Hug [24]. From the free morphine concentrations, a terminal half-life of about 2 h was calculated, which is also in agreement with previous estimates [24].

Fig. 3 presents the morphine concentration in liquor and serum of a patient undergoing epidural anaesthesia, taken at regular time intervals. From this experiment it can be deducted that the decay of the morphine concentration in

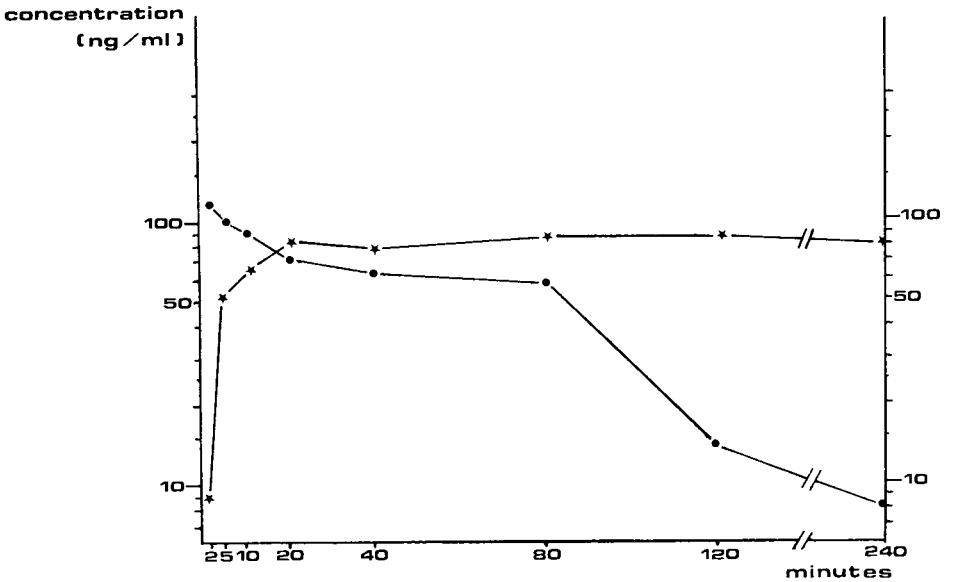

Fig. 2. Morphine concentration in serum before and after hydrolysis with β-glucuronidase. (•), free morphine (ng/ml); (⋆), morphine glucuronide (ng/ml).

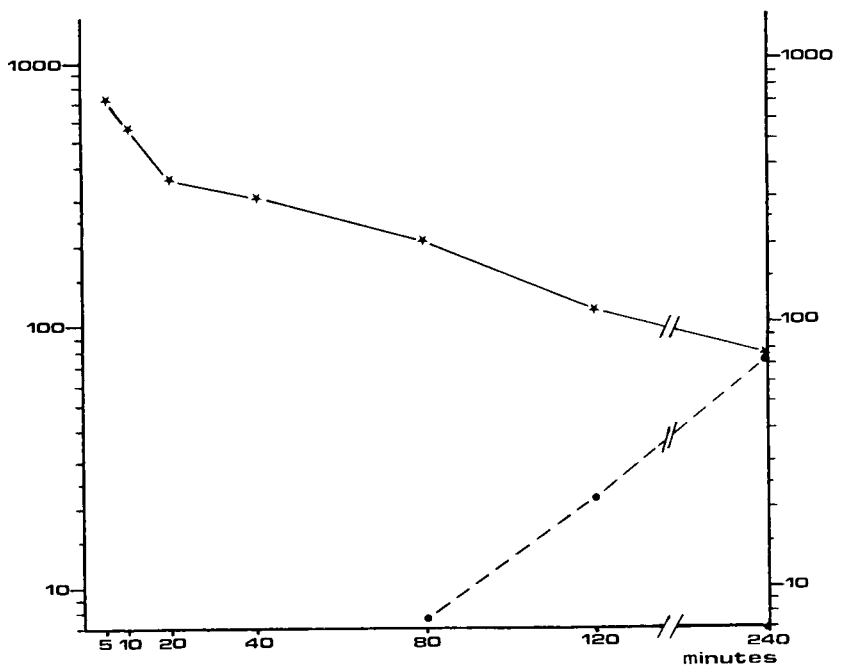

Fig. 3. Morphine concentrations (ng/ml) in liquor (⋆) and serum (•) after epidural administration of 0.1 mg of morphine per kg body weight.

liquor parallels the serum levels after intravenous administration. Furthermore, these data show that 1 h after epidural administration morphine can be detected in the serum.

In summary, it can be concluded that the described procedure offers a reliable analytical method for the determination of morphine in biological fluids, and had proved to be a suitable procedure for studying the pharmacokinetics and metabolism of the drug.

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Note

Determination of plasma phenytoin by capillary gas chromatography with nitrogen—phosphorus detection and with selective ion monitoring

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Phenytoin (5,5-diphenylhydantoin, DPH) is an important drug used mostly for the control of grand-mal epilepsy. It has the disadvantage of a narrow therapeutic range and thus it is important for individual therapy to monitor plasma concentrations. The half-life of the drug in plasma is variable being influenced by a number of factors such as dose, degree of tissue saturation and duration of administration [1]. Pregnancy also may affect the pharmacokinetics of the drug [2, 3] but in a way that is not completely understood. As part of a study of DPH therapy in pregnancy we have developed a reliable method of assay based on the use of capillary gas chromatography (GC) with nitrogen—phosphorus detection (NPD) for determining DPH in patients plasma, which is accurate down to concentration levels of 0.5 μg/ml. The pharmacokinetics of the drug can be investigated in the steady state by using the mass spectrometer as a detection system [4]. In this study a single oral dose of isotopically labelled [$^2H_5$]DPH was given to chronically dosed pregnant epileptic women and the unlabelled and labelled drug were measured by capillary GC with selective ion monitoring (SIM).

EXPERIMENTAL

Standards and reagents

DPH, 5-(*p*-methylphenyl)-5-phenylhydantoin (PTH) and [$^2H_6$]benzene (99.5 atom% deuterium) were obtained from Aldrich Chemical (Gillingham, U.K.). 5-[$^2H_5$]Phenyl-5-phenylhydantoin ([$^2H_5$]DPH) was prepared by a method described [5] for the unlabelled compound from the corresponding labelled benzophenone. The reaction under Friedel-Craft's conditions in the

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presence of aluminium chloride of benzoyl chloride with [$^2H_6$]benzene gave [$^2H_5$]benzophenone. Stock standard solutions of the reference compounds prepared in ethyl acetate at a concentration of 1 mg/ml were stored at 0° C. These solutions were diluted in ethyl acetate (1:20) before use. Solvents (ethyl acetate, methylene chloride, hexane) were BDH (Poole, U.K.) AnalaR grade and were used without further purification. Methyl iodide (laboratory reagent grade) was also obtained from BDH. Tetrabutylammonium hydrogen sulphate (TBAH, Aldrich Chemical) was prepared as a 0.5 *M* solution in 1.0 *M* sodium hydroxide.

Gas chromatography

The instrument used for the GC determinations was a Carlo Erba Fractovap 4160 equipped with an electrically heated alkali-bead nitrogen—phosphorus detector. Separations were made on a 20 m × 0.3 mm OV-1 (cross-linked) glass capillary column prepared according to the procedure of Grob et al. [6]. The column was maintained at 100° C for 3 min, then programmed at 30° C/min to 320° C. Helium was used as carrier gas at a linear flow-rate of 2 ml/min measured at 100° C. Samples were injected using a split injection system (10:1 split ratio). The nitrogen—phosphorus detector was operated at an air flow-rate of 120 ml/min and a hydrogen flow-rate of 4 ml/min. Quantitation was by determining peak heights computed with an electronic integrator (Hewlett-Packard, Model 3390A).

Gas chromatography—mass spectrometry

Analysis by gas chromatography—mass spectrometry (GC—MS) was carried out on a 70-70 VG Micromass double-focusing mass spectrometer interfaced to a Pye Unicam Series 204 gas chromatograph. The column used was a 25 m × 0.3 mm SE52 (cross-linked) fused-silica capillary. The outlet end of the column was located up to the entrance of the ion source. Samples were introduced into the capillary using an on-column injector or a split injection system (10:1 split ratio). Helium was used as carrier gas at a flow-rate of 2 ml/min. The column was maintained at 100° C for 30 sec followed by a 16° C/min programme to 250° C. The temperatures of the injection port, GC—MS interface and ion source were 250, 300 and 220° C, respectively. The mass spectrometer was operated in the electron impact mode with an ionisation potential of 70 eV and a trap current of 200 μA. For SIM the ions at *m/z* 280, 285 and 294 were measured (50 msec per mass channel) using as lock mass the ion *m/z* 295 from a constant septum bleed of heptacosafluorotributylamine. The ion intensity was recorded using a Rikadenki Series DBE-6 Multi-Pen recorder. The resolution of the mass spectrometer (10% valley) was 650.

Extraction and derivatization procedure

All operations were carried out in Eppendorf disposable microcentrifuge tubes. A 200-μl aliquot of plasma was spiked with 1 μg of the internal standard PTH and 500 μl hexane were added. After vortex mixing and centrifugation the hexane layer was removed and discarded. The plasma was then extracted twice by vortex mixing and centrifugation with 500-μl aliquots of ethyl acetate. The solvent extracts were combined and blown to dryness under

nitrogen. The sample was then derivatized by extractive methylation according to the procedure of Hoppel et al. [7]. To the dried extract were added 100 μl of 1.0 *M* sodium hydroxide, 50 μl TBAH solution, 500 μl methylene chloride and 25 μl methyl iodide. The mixture was vortexed for several seconds and the tubes then placed on a rotary mixer for 45 min. After centrifugation the upper aqueous layer was removed and the remaining solvent layer washed with 500 μl water. The solvent extract was transferred to a small glass tube, then taken to dryness and redissolved in 100 μl ethyl acetate for analysis by GC—NPD and GC—MS.

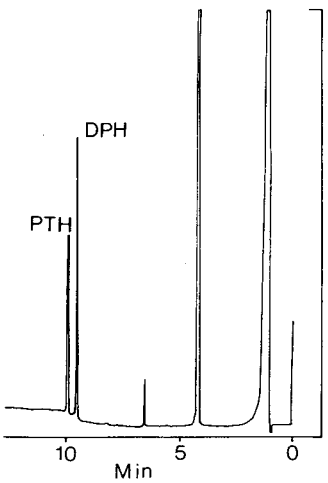
Quantitation of the drug in the plasma samples was made by reference to a standard calibration curve constructed with each batch of samples analysed. The standard curve for the GC—NPD determinations was obtained from the analysis of 200-μl aliquots of drug-free plasma to which had been added 1 μg PTH and various amounts of DPH (500 ng to 2 μg). The ratio of the peak heights (DPH/PTH) was plotted against the concentration of DPH in the plasma. The standard curves for the GC—MS analysis with SIM were constructed similarly but with PTH (2 μg per sample) and [$^2H_0$]DPH (0—4 μg) and [$^2H_5$]DPH (0—0.2 μg). The ratios of the peak heights of the molecular ions of [$^2H_0$]DPH (*m/z* 280) or of [$^2H_5$]DPH (*m/z* 285) to the molecular ion of PTH (*m/z* 294) were plotted against the concentration of DPH in the plasma. The standard curves for both the GC and GC—MS determinations typically had regression coefficients > 0.99.

RESULTS AND DISCUSSION

High-resolution capillary GC, usually with a selective GC detection system or combined with MS, is now an established method for quantifying drugs and their metabolites in biological samples [8]. We have previously demonstrated the advantage of capillary GC with NPD for determining the concentration of psychotropic drugs in plasma at the therapeutic level [9, 10] and have now successfully applied this technique to the measurement of DPH in plasma.

Extractive alkylation has been used previously in the GC and GC—MS analyses of a number of drugs including DPH [4, 7, 11, 12]. The permethylated derivatives of both DPH and the internal standard PTH were chemically very stable and had good GC properties. Fig. 1 shows a typical GC—NPD tracing from the plasma of a patient receiving a therapeutic dose of the drug. No interference from endogenous compounds was found in the analysis of over 100 samples from six women taken at various times during their pregnancies. Fig. 2 shows a SIM trace from a chronically dosed patient who had been given a single dose of [$^2H_5$]DPH.

The recovery of the drug from plasma by ethyl acetate extraction was determined by spiking two sets of drug-free plasma with DPH (1, 4, 8, 12 or 16 μg/ml). The PTH internal standard was added to one set of plasma before ethyl acetate extraction and to the ethyl acetate extracts from the other set. The mean recovery of DPH as measured by GC—NPD over the concentration range 1—16 μg/ml was 96.6 ± 3.01% S.D. The accuracy and precision of the GC—NPD method was determined from replicate recovery experiments of authentic DPH added to drug-free plasma at concentration levels of 1, 4, 8,



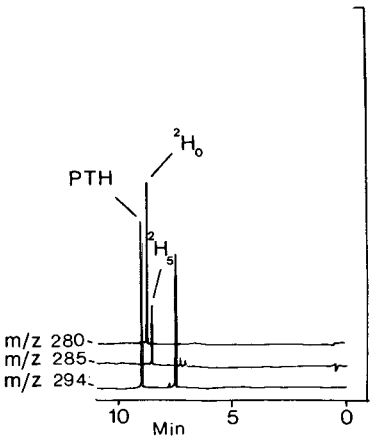


Fig. 1. GC—NPD trace of an extract from a plasma sample calculated to contain 3.6 μg of DPH per ml plasma. For conditions see text.

Fig. 2. SIM trace (*m/z* 280, *m/z* 285) of an extract of plasma from a pregnant epileptic woman dosed with [$^2H_0$]DPH and [$^2H_5$]DPH. For conditions see text.

TABLE I

PRECISION AND ACCURACY OF THE METHOD FOR DPH

DPH added (μg/ml)	DPH found mean ± S.D. (μg/ml, n = 6)	Percent recovery (mean ± S.D.)
1	1.07 ± 0.03	107.0 ± 2.65
4	4.01 ± 0.17	100.25 ± 4.19
8	7.75 ± 0.13	96.81 ± 1.59
12	12.17 ± 0.18	101.44 ± 1.49
16	16.32 ± 0.33	101.99 ± 2.07

12 and 16 μg/ml. The calculated recoveries from six replicate analyses at each of these levels are presented in Table I.

A number of plasma samples from patients receiving both the unlabelled and labelled drug were assayed by GC—NPD and by GC—MS. The sum of the concentrations of [$^2H_0$]DPH and [$^2H_5$]DPH determined by GC—MS was plotted against the total DPH concentration measured by GC—NPD. Taking the latter results as the standard the regression line of the GC—MS results on the GC—NPD results was calculated. The regression line (see Fig. 3) was $y = 1.042x - 0.087$. The slope of the line is 1.042 ± 0.037 which is not significantly different from 1.00 and the intercept is −0.087 ± 0.092 which is not significantly different from zero, x^2 = 96.8%.

Although in this work PTH has been used as the GC—MS internal standard in order to allow the simultaneous use of the MS and NPD detection systems, the GC—MS approach has also been satisfactorily applied using the analogue [$^2H_{10}$]DPH as an internal standard.

In conclusion the GC method described in this paper can be performed on

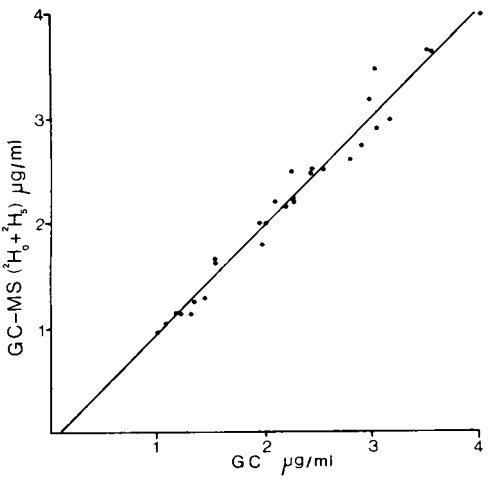


Fig. 3. The sum of [$^2H_0$]DPH and [$^2H_5$]DPH concentrations in plasma determined by GC—MS plotted against total DPH concentrations determined by GC—NPD.

small plasma volumes, and is both reliable and accurate down to the 0.5 µg/ml DPH level. This assay is suitable for routine clinical application and derivatized samples can if required be stored at 0° C for several months. The use of SIM has allowed pharmacokinetic studies to be made of a pulse dose of [$^2H_5$]DPH administered to patients who are taking a steady-state dose of the drug. The clinical implications of the pharmacokinetic studies carried out on pregnant epileptic women will be reported shortly.

ACKNOWLEDGEMENTS

The administration of labelled DPH to pregnant patients was approved by the ethical committee of Northwick Park Hospital, Harrow, Middlesex, U.K. Our thanks are due to Drs F.E. Hytten and M.J. Landon, Division of Perinatal Medicine, Northwick Park Hospital, for organising the clinical part of this work.

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Note

Effect of sodium dodecyl sulphate on the extraction of ubiquinone-10 in the determination of plasma samples

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(First received January 16th, 1984; revised manuscript received April 24th, 1984)

The determination of plasma ubiquinone-10 (UQ-10, the number ten indicating the number of isoprenoid side-chains) has been reported in connection with human bioavailability studies of UQ-10 [1, 2]. The most commonly used method is based on the extraction of UQ-10 from the plasma sample with *n*-hexane followed by analysis of the extract on a reversed-phase high-performance liquid chromatographic (HPLC) column. In the course of our bioavailability studies, we encountered difficulty with low sensitivity and poor reproducibility of the determination, which resulted from the low recovery of UQ-10 from the sample. We found that the low recovery was associated with the low temperature of our laboratory during the winter season.

This paper reports the finding that the addition of sodium dodecyl sulphate (SDS) to the plasma sample before extraction with the hexane significantly improves the recovery at low ambient temperatures.

EXPERIMENTAL

Reagents and apparatus

UQ-10 was received as a gift from Nisshin Chemicals (Tokyo, Japan). Other reagents and solvents were of the best commercially available grades. HPLC

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was carried out on a Shimadzu liquid chromatograph, Model 3A. UQ-10 was detected at 275 nm with an ultraviolet (UV) detector. Separation was obtained on a Zorbax ODS column (15 × 0.46 cm; particle size 5—6 μm) with a mobile phase consisting of 96% ethanol in water. The flow-rate was 1.0 ml/min at 54°C.

Determination

To a human plasma sample (1.0 ml) was added a solution (1.0 ml) of 200 m*M* SDS, and the mixture was shaken vigorously to obtain an emulsion. After the addition of methanol (3.0 ml) UQ-10 was extracted with *n*-hexane (5.0 ml).

TABLE I

EFFECT OF TEMPERATURE ON EXTRACTION OF PLASMA UQ-10 WITH HEXANE

Temperature (°C)	Additive	UQ-10 added (μg/ml plasma)	UQ-10 found (μg/ml plasma)			Recovery (%)		C.V. (%)
			Value		Mean found	Value	Mean ± S.D.	
7	None	0.0	0.13	0.15	0.14	—		
		0.5	0.18	0.18	0.18	8.0		
		1.0	0.25	0.33	0.29	15.0	12.3 ± 3.10	25.20
		1.5	0.33	0.36	0.35	14.0		
		2.0	0.39	0.36	0.38	12.0		
	SDS	0.0	1.09	1.06	1.08	—		
		0.5	1.53	1.58	1.56	96.0		
		1.0	2.09	2.09	2.09	101.0	100.9 ± 4.09	4.05
		1.5	2.59	2.59	2.59	100.7		
		2.0	3.17	3.22	3.20	106.0		
17	None	0.0	0.96	0.98	0.97	—		
		0.5	1.41	1.39	1.40	86.0		
		1.0	1.76	1.85	1.81	84.0	86.4 ± 2.06	2.38
		1.5	2.23	2.30	2.27	86.7		
		2.0	2.81	2.69	2.75	89.0		
	SDS	0.0	1.03	1.03	1.03	—		
		0.5	1.50	1.52	1.51	96.0		
		1.0	1.94	1.99	1.97	94.0	95.2 ± 0.99	1.04
		1.5	2.44	2.45	2.45	94.7		
		2.0	2.95	2.95	2.95	96.0		
30	None	0.0	1.02	1.07	1.04	—		
		0.5	1.48	1.56	1.52	96.0		
		1.0	1.96	1.99	1.98	93.5	94.8 ± 1.26	1.33
		1.5	2.46	2.44	2.45	94.0		
		2.0	2.94	2.97	2.96	95.8		
	SDS	0.0	1.06	1.04	1.05	—		
		0.5	1.53	1.51	1.52	94.0		
		1.0	2.03	2.00	2.02	97.0	95.6 ± 1.25	1.31
		1.5	2.49	2.48	2.49	96.0		
		2.0	2.99	2.93	2.96	95.5		

The hexane phase (4.0 ml) was removed and evaporated. The resulting residue was dissolved in the HPLC mobile phase (1.0 ml) at 70°C, and was analysed on the HPLC column.

The concentration of UQ-10 in plasma was obtained directly from a calibration curve obtained by analyses of control plasma samples (1.0 ml) to which known quantities (0.5—2.0 μg) of UQ-10 in ethanol (20 μl) were added. The curve was prepared from peak heights corresponding to UQ-10, for which the retention time was 14 min.

RESULTS AND DISCUSSION

SDS used as an additive was included in the hexane extract; however, it did not interfere with the separation of UQ-10. Over 400 samples in our bioavailability studies were analysed on a column without deterioration of the resolution.

Samples were analysed in the presence and absence of SDS at three different temperatures. At 30°C SDS had almost no effect on the recovery of UQ-10 and coefficient of variation (C.V.). However, at 7°C and 17°C, the additive improved both the recovery and C.V., especially at 7°C (Table I). These findings indicate the necessity of using SDS when conducting this analysis at temperatures below 17°C.

The recovery of UQ-10, which was added to a control plasma in a known amount, was studied at various concentrations of SDS at 7°C (Fig. 1). Although the recovery was 47% in the absence of SDS, no recovery was obtained in 8 and 43 m*M* SDS. The presence of at least 50 m*M* SDS resulted in almost complete recovery. Therefore the determination of UQ-10 by this method included SDS at the concentration of 100 m*M*.

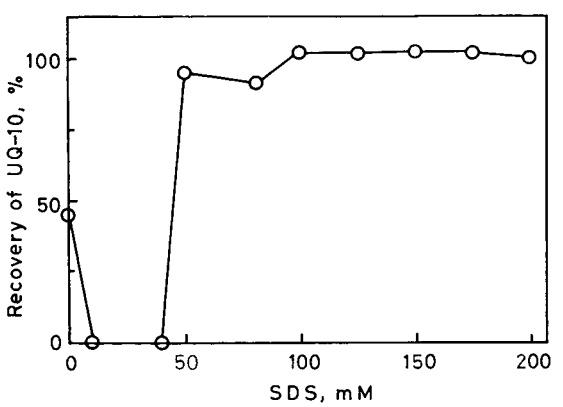

Fig. 1. Effect of SDS on recovery of UQ-10. UQ-10 (2.0 μmol) was added to control plasma (1.0 ml). SDS solutions (1.0 ml) were added to obtain plasma samples with varying concentrations of SDS. The analysis of UQ-10 was carried out at 7°C.

Although UQ-10 is freely soluble in *n*-hexane, it is not extracted from plasma at low temperatures by this solvent. The present work revealed that, when SDS was added to plasma, UQ-10 was extractable. It may be that this compound forms complexes with weakly binding sites on one or more plasma

proteins but that these complexes dissociate at higher temperatures. SDS would dissociate the complexes even at low temperatures by unfolding the proteins and disrupting the binding sites.

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Note

Direct determination of valproate in minute whole blood samples

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(First received December 12th, 1983; revised manuscript received April 10th, 1984)

Monitoring of valproate in blood is of importance in the treatment of epilepsy. Chromatographic procedures are most commonly used for analysis of this drug. Many methods include solvent extraction followed by gas chromatography (GC) without derivatization. The shortcomings of these methods are the use of relatively large sample volumes, the formation of emulsion causing loss of analyte, low sensitivity because of the volatility of valproic acid thus precluding solvent evaporation, use of unsuitable chromatographic columns for the accurate quantification of valproic acid. To overcome these last two drawbacks, some authors [1—4] proposed the conversion of valproic acid to its esters. Recently, a procedure has been described employing solvent extraction, esterification and quantification by GC with a nitrogen detector to analyse valproate in plasma [5]. More recently, another method couples a non-extractive preparative procedure with liquid chromatography of valproic acid as its phenacyl ester [6]. This approach is simple and original, but appears to be insufficiently accurate and precise for serum valproate levels lower than 50 mg/l.

Graphitized carbon black (Carbopack) is very effective both as packing material in GC for the separation of acidic compounds [7—10] and as an adsorbing material for the extraction of very polar compounds from biological liquids [8—10]. In this paper, we describe its use for isolating valproate directly from blood and quantifying it by GC. This method requires only 10 μl of blood, is simple and accurate even at the lowest therapeutic level.

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MATERIALS AND METHODS

Reagents

The following analytical grade reagents (Fluka, Buchs, Switzerland) were used: heptanoic acid, 1,3,5-tricarboxybenzene (trimesic acid), polyethylene glycol (PEG) 20 M. Stock solutions of sodium valproate (Labaz, Maassluis, The Netherlands) were prepared by dissolving the salt in water to give concentrations of 6.4, 2.4, 0.8, 0.4, and 0.2 mmol/l. Blood standards were prepared by adding 10 μl of the stock solutions to 100 μl of drug-free blood. The working internal standard was prepared by diluting heptanoic acid with 0.01 mol/l hydrochloric acid to give a concentration of 0.10 μg/ml. A second internal standard solution was prepared by dissolving octanoic acid in methanol to give a concentration of 30 μg/ml. Carbopack B and C (Supelco, Bellefonte, PA, U.S.A.) were 80—100 mesh.

Procedure

A 10-μl freshly collected blood sample is placed in a glass tube containing 1 ml of 0.01 mol/l hydrochloric acid and 0.1 μg/ml heptanoic acid. The mixture is well agitated and applied to a glass column filled with 0.25 g of Carbopack B. The bed (3 × 0.6 cm) of Carbopack B is prepared by suspending the material in water and introducing the suspension into a 15 × 0.6 cm glass column with a small flock of glass wool in the bottom. After the sample is passed, the column is washed with 10 ml of 0.05 mol/l hydrochloric acid followed by 1 ml of water—methanol (50:50, v/v) and 0.01 mol/l hydrochloric acid. Valproic acid is eluted by applying to the top of the column 2 ml of methanol, discarding the first 0.5 ml of the effluent, which is the residue of the previous washing phase, and subsequently collecting 1.5 ml. The collected fraction is made alkaline with 20 μl of methanol, 1.5 mol/l potassium hydroxide and evaporated under nitrogen at 50° C. The residue is dissolved with 40 μl of 0.5 mol/l oxalic acid and 1.6 μl are injected into the gas chromatograph.

GC was performed on a Carlo Erba (Milan, Italy) Model 4200 gas chromatograph, equipped with a flame ionization detector. The GC column adopted for quantification was glass, 1.2 m × 2 mm I.D. packed with Carbopack C coated with 0.2% trimesic acid and 0.4% PEG 20 M, which are added to Carbopack C from methanol—methylene chloride (50:50, v/v). Other details concerning the preparation of the packing material and the column packing procedure were described previously [11]. The GC column was conditioned overnight at 230° C with nitrogen. For quantification, the column was operated at 160° C with a dead time of 9 sec. The injector and detector temperatures were both 170° C.

To ensure that the column packing was totally conditioned to water, 1.6 μl of 0.5 mol/l oxalic acid were injected four times into the GC column. This operation was repeated whenever the column had been left unused for some hours.

RESULTS AND DISCUSSION

Analytical recovery and precison of the method were determined by analysing blood samples spiked with various known amounts of valproic acid. Absolute recovery was assessed by adding 10 μl of the octanoic acid solution to the collected fraction. Analytical and absolute recoveries were calculated by measuring the peak height of valproate relative, respectively, to those of heptanoic acid and octanoic acid and comparing them to those of a reference standard. Results are given in Table I. Relative and absolute recoveries in the concentration range considered average 99.2% and 98.5%, respectively. The fact that absolute recovery is not dependent upon the valproate concentration demonstrates that, under the experimental conditions selected, neither chemisorption nor saturation phenomena are present when the sample is passed through the Carbopack B column. Day-to-day coefficient of variation ranged from 5.6% at 19.5 μmol/l to 1.8% at 624 μmol/l. The limit of sensitivity of our method was about 8 μmol/l of blood. At this concentration, a well defined chromatographic peak for valproate could still be obtained and the coefficient of variation of the measurement was 7.2%. Fig. 1 shows typical chromatograms.

TABLE I

RESULTS OF SIX REPLICATE ANALYSES OF SODIUM VALPROATE IN SPIKED BLOOD SPECIMENS

Added (μmol/l)	Found (μmol/l, mean ± S.D.)	C.V. (%)	Analytical recovery (%)	Absolute recovery (%)
19.5	19.2 ± 1.0	5.3	98.4	98.3
39.0	38.4 ± 1.5	3.8	99.5	98.5
78.0	76.9 ± 1.8	2.4	99.2	98.6
254	249 ± 4	1.8	99.2	98.2
624	616 ± 11	1.8	99.0	98.7

Under the GC conditions selected, lactic acid among the endogenous compounds and ethosuximide among the exogenous ones were taken into consideration as possible interfering substances in the valproate determination. Although lactic acid is eluted by the GC column with a broadened peak whose retention time is close to that of valproic acid, it has been reported [9] that lactic acid is scarcely retained by the extraction column and a few millilitres of acidic water are sufficient to eliminate any trace of the hydroxy acid considered. A blood sample supplemented with ethosuximide at level of 100 μg/ml was carried through the procedure described. It was found that the drug was almost completely lost during the washing step with the water—methanol mixture and the last vestiges of ethosuximide produced a GC peak with a retention time of 10 min.

A stability test was performed by adding a blood sample spiked with valproic acid to 0.01 mol/l hydrochloric acid and assaying aliquots in duplicate on the day of collection and after 1, 3, 5, and 10 days' storage at 4°C. No significant deterioration of the sample occurred during the storage (Table II).

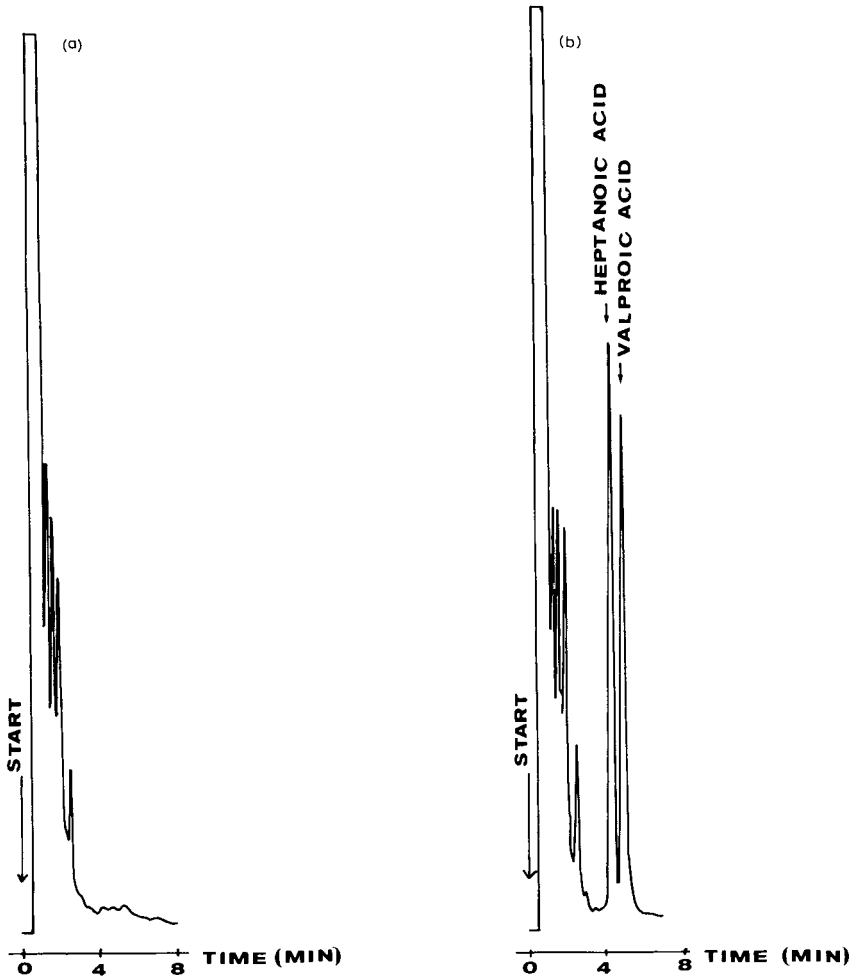

Fig. 1. Gas chromatograms obtained from blood specimens: (a) blank blood; (b) blood spiked with valproate (80 μmol/l) and heptanoic acid.

TABLE II

EFFECT OF STORAGE AT 4°C ON BLOOD VALPROATE CONCENTRATION

$n = 4$.

Days of storage	Concentration (μmol/l, mean ± S.D.)
0	76.8 ± 1.5
1	77.0 ± 1.9
3	77.9 ± 1.5
5	76.2 ± 1.7
10	75.3 ± 1.8

For rapid, accurate and simple valproate determination, analytical optimization studies were performed by changing sequentially the analytical conditions of the sample preparation procedure. The initial pH of the mixture of

blood and acidified water was increased by either increasing the blood volume or decreasing the hydrochloric acid concentration. Concurrently, a steady decrease in the absolute recovery of valproate was observed from 98.6% at pH 2.5 to 84.4% at pH 4.5. This loss can be explained on the basis that valproic acid is increasingly ionized as the pH increases and that some chemical heterogeneities present on the Carbopack surface are promptly rearranged in the presence of water to form salts able to exchange anions [12]. To avoid this undesirable effect, we found it useful to mix 1 ml of 0.01 mol/l hydrochloric acid with 10 μl of blood sample, the resulting pH being 2.3—2.5. When larger volumes of blood were analysed, we took care to increase the volume of 0.01 mol/l hydrochloric acid proportionally, letting other parameters of the analytical procedure remain unmodified.

The flow-rates at which the sample volume and the other liquids percolated through the Carbopack B bed were varied over the range 0.5—5 ml/min by varying the particle size of the Carbopack. No significant variation in the absolute recovery of valproate was noted.

A water bath temperature of 50°C was found to be the best compromise to avoid loss of sample and to shorten the evaporation time, which was about 15 min.

The reusability of the Carbopack B column for valproate determination in blood was studied by repeated extractions on the same column. After each extraction the column was regenerated with 5 ml of methanol and 5 ml of water, taking care to avoid formation of bubbles. After four extractions, the absolute recovery did not change in a significant way, while a 9.3% decrease of absolute recovery was observed after five extractions, even though the analytical recovery remained unaltered.

CONCLUSION

The procedure described here meets the important requirement of using very small blood samples; it is rapid, total analysis time being of about 30 min, as well as highly accurate and sensitive. The stability test has shown the possibility of day-to-day home monitoring of valproate in blood for patients affected by epilepsy.

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Note

High-performance liquid chromatographic determination of nitrazepam and its metabolites in human urine

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(First received January 4th, 1984; revised manuscript received April 4th, 1984)

Nitrazepam, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, is a benzodiazepine analogue which has been used extensively as a hypnotic. Although this drug is known for its low toxicity and high safety margin, it has recently been used for purposes of suicide and homicide. In addition, it is known that the side-effects of this drug will influence psychomotor abilities related to driving [1].

Many reports have been published on the determination of nitrazepam in biological fluids, gas chromatography (GC) being used extensively [2—5]. The GC methods require somewhat lengthy clean-up procedures, and in some cases derivatization or hydrolysis to the more volatile benzophenone [4]. Several methods were reported on the analysis of nitrazepam alone by high-performance liquid chromatography (HPLC) [6—9], while few reports have been published on the analysis of nitrazepam together with its metabolites in biological fluids. Although the analysis of nitrazepam and its metabolites by HPLC on an anion-exchange packing was reported [10], this method failed to yield reliable results, due to broad, tailing peaks.

In this paper, the simultaneous determination of nitrazepam and metabolites by HPLC on Nucleosil $5C_{18}$ packing gave sharp peaks and good separation.

EXPERIMENTAL

Reagents

Nitrazepam (Benzalin; Shionogi Pharmaceutical, Japan) was recrystallized from ethanol. 7-Amino-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (7-aminonitrazepam) and 7-acetamido-1,3-dihydro-5-phenyl-2H-1,4-benzo-

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diazepin-2-one (7-acetamidonitrazepam) were supplied by courtesy of Professor H. Sawada, Gifu College of Pharmacy. 1-Methyl-7-nitro-5-phenyl-1H-1,4-benzodiazepin-2-(3H)-one (nimetazepam, internal standard) was kindly provided by Sumitomo Kagaku Kogyo, Japan. Nucleosil $5C_{18}$ (Macherey-Nagel, 5 μm) was used as column packing.

Apparatus and chromatographic conditions

The liquid chromatograph was a Shimadzu LC-3A with a spectrophotometric detector SPD-1 connected to a Chromatopac C-R1A recorder. The column was a steel column (150 mm × 4.0 mm I.D.) packed with Nucleosil $5C_{18}$ (particle size 5 μm) using a slurry packing technique. A guard column was used for protecting the analytical column from interfering materials. The chromatographic conditions were as follows: mobile phase, methanol—water (35:65) (adjusted to pH 4.0 with phosphoric acid); flow-rate, 1.3 ml/min; pressure, 120 kg/cm^2; column temperature, 50°C; detector, ultraviolet (UV) at 254 nm.

Extraction procedures

Urine samples were adjusted to pH 10 by the addition of 0.28% ammonia solution before extraction. Columns for extraction of nitrazepam and its metabolites from human urine were prepared from a 30-ml glass syringe filled with 5.0 g of Extrelut® (E. Merck, Darmstadt, F.R.G.), which is similar to

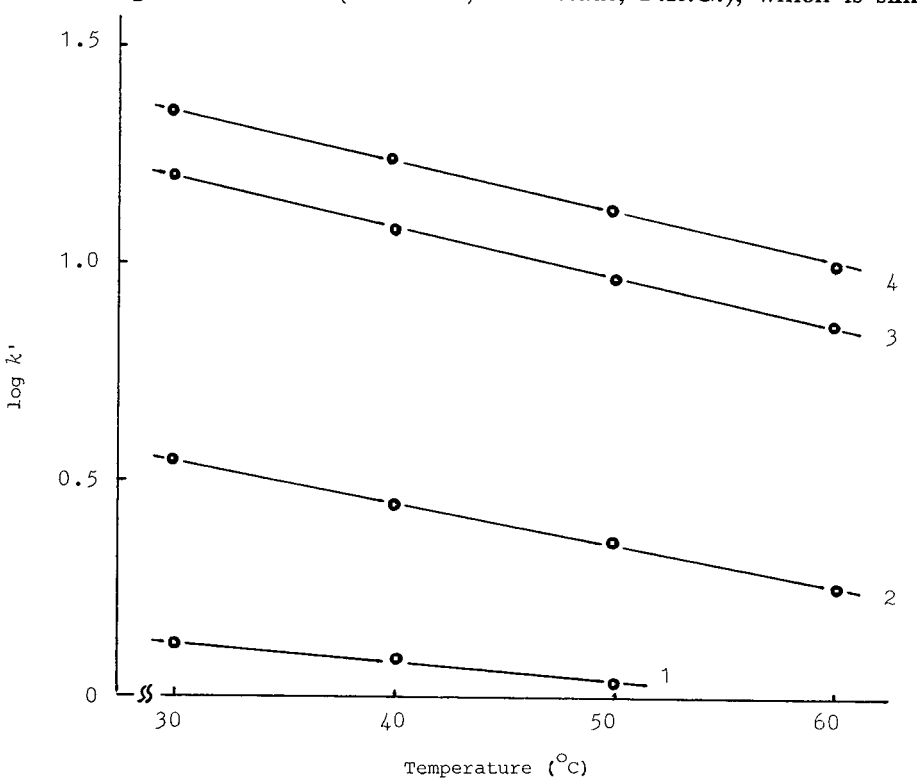

Fig. 1. Relationship between the capacity factor, k', and the temperature at pH 4.0. 1 = 7-Aminonitrazepam; 2 = 7-acetamidonitrazepam; 3 = nitrazepam, 4 = nimetazepam (internal standard).

Bond-Elut® [11]. A 10-ml aliquot of a urine sample was applied onto the column and, after soaking for 15 min, the column was eluted with chloroform. The eluate was evaporated to dryness using a rotary evaporator.

RESULTS AND DISCUSSION

Chromatographic conditions

Liquid chromatography of nitrazepam has been performed on columns packed with Corasil II [6], Carbowax 400 on Corasil [6], C_{18} (octadecyltrichlorosilane) on Corasil [6], LiChrosorb Si 100 [7], Spherisorb S-W10 [8], porous silica gel [9], and Zipax SAX [10] using a variety of solvents. In this study, various chromatographic conditions have been evaluated, varying column packings, the composition and the pH of the mobile phase, and the column temperature. Liquid chromatography of nitrazepam and its metabolites was performed on columns packed with Nucleosil 5CN, Nucleosil $5NH_2$, Nucleosil $5C_{18}$ and Zipax SAX. Nitrazepam was well separated from its metabolites and from the internal standard by using the Nucleosil $5C_{18}$ column and methanol—water (35:65) (pH 4.0) at a flow-rate of 1.3 ml/min (pressure 120 kg/cm^2). The column temperature ws thermostated at 50°C. The logarithm of the capacity factor (k') between nitrazepam, 7-aminonitrazepam, 7-acet-

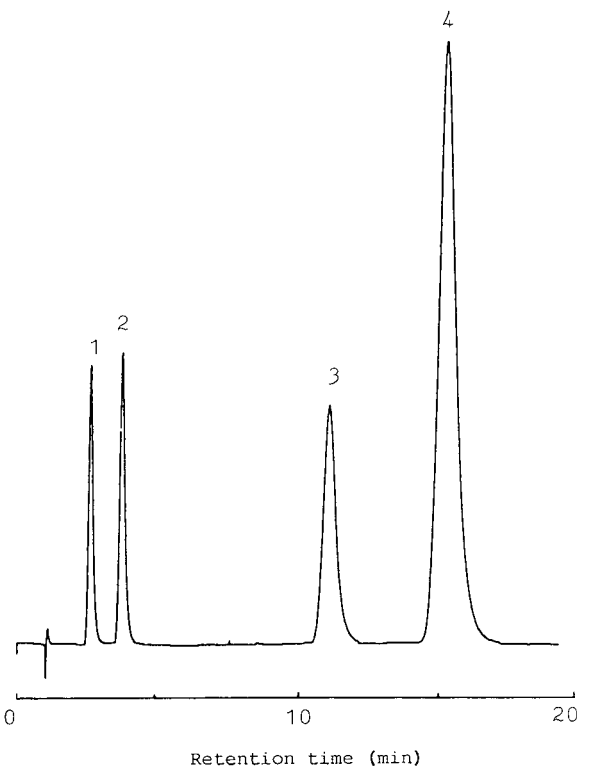

Fig. 2. Typical chromatogram of nitrazepam and its metabolites. 1 = 7-Aminonitrazepam; 2 = 7-acetamidonitrazepam; 3 = nitrazepam; 4 = nimetazepam (internal standard). Column, Nucleosil $5C_{18}$; mobile phase, methanol—water (35:65) (pH 4.0); column temperature, 50°C; flow-rate, 1.3 ml/min (pressure, 120 kg/cm^2); detector, UV photometer (254 nm).

amidonitrazepam and nimetazepam plotted against the temperature at pH 4.0 gave a straight line (Fig. 1).

Analysis of a standard mixture

A typical chromatogram of a methanolic mixture of 7-aminonitrazepam (100 μg/ml), 7-acetamidonitrazepam (100 μg/ml), nitrazepam (160 μg/ml) and nimetazepam (the internal standard, 800 μg/ml) is shown in Fig. 2.

Calibration curve

Standard solutions of nitrazepam, 7-aminonitrazepam and 7-acetamidonitrazepam were dissolved in methanol at concentrations of 40–200 μg/ml for nitrazepam, and 25—125 μg/ml for 7-aminonitrazepam and 7-acetamidonitrazepam. Nimetazepam (the internal standard) was dissolved in methanol at a concentration of 800 μg/ml. Calibration curves were constructed by plotting the ratio of peak area of each compound to that of the internal standard. Regression equations were as follows: $Y = 0.1421X - 0.0100$ for nitrazepam, $Y = 0.2282X - 0.0015$ for 7-aminonitrazepam, and $Y = 0.1857X - 0.0005$ for 7-acetamidonitrazepam.

Extraction and recoveries

The recovery of nitrazepam (2.4 μg/ml), 7-aminonitrazepam (1.5 μg/ml) and 7-acetamidonitrazepam (1.5 μg/ml) added to normal human urine was evaluated after elution with various organic solvents after adjusting the samples to pH 10 with 0.28% ammonium hydroxide and pH 12 with 1 *M* sodium hydroxide. For 10 ml of urine 30 ml of solvent were used, in which 100% of the extractable substances was eluted. The dried residue of the eluate was unsuitable for analysis by HPLC using ethyl acetate and dichloromethane—isopropanol mixture; the solvent for optimum elution was chloroform (Table I).

TABLE I

RECOVERIES OF 7-AMINONITRAZEPAM, 7-ACETAMIDONITRAZEPAM AND NITRAZEPAM ADDED TO NORMAL HUMAN URINE AT pH 10 AND pH 12

$n = 5$.

Solvent	pH	7-Amino-nitrazepam (%)	7-Acetamido-nitrazepam (%)	Nitrazepam (%)
Ether	10	88.39 ± 4.11	62.18 ± 3.54	97.24 ± 3.74
	12	81.45 ± 3.09	56.44 ± 1.47	86.06 ± 4.65
Ethyl acetate	10	104.92 ± 3.03	105.12 ± 4.23	96.97 ± 1.80
	12	86.20 ± 2.61	99.06 ± 2.28	88.64 ± 2.30
Dichloromethane—isopropanol (85:15)	10	93.10 ± 1.51	98.64 ± 1.58	96.92 ± 1.20
	12	98.00 ± 4.23	99.56 ± 5.79	88.05 ± 4.89
Chloroform	10	99.39 ± 1.78	95.90 ± 2.02	99.93 ± 3.18
	12	83.63 ± 3.72	99.52 ± 3.78	98.95 ± 3.73
Hexane	10	—	—	—
	12	—	—	—

Quantitation of the sample

Urine from a person suspected of having ingested nitrazepam was analysed. After evaporation of the extracted urine, the residue was dissolved in 0.2 ml of the internal standard solution. Usually, 5-μl aliquots were injected into the liquid chromatograph. The concentration of the drug and its metabolites in the sample was determined from the calibration curves. The chromatogram and the

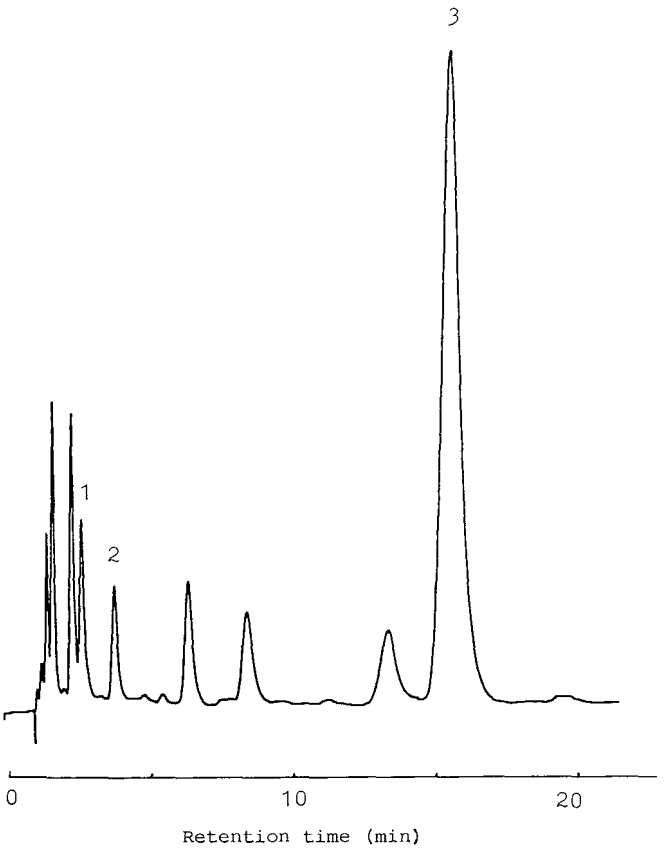

Fig. 3. Chromatogram of the extract of human urine from a person suspected of ingesting nitrazepam. 1 = 7-Aminonitrazepam; 2 = 7-acetamidonitrazepam; 3 = nimetazepam (internal standard). Chromatographic conditions are the same as in Fig. 2.

TABLE II

CONTENTS OF 7-AMINONITRAZEPAM AND 7-ACETAMIDONITRAZEPAM IN THE SAMPLE

Sample No.	7-Aminonitrazepam (μg/ml)	7-Acetamidonitrazepam (μg/ml)
1	38.34	30.37
2	37.20	30.48
3	35.71	31.13
Mean	37.08	30.66

concentrations of 7-amino nitrazepam and 7-acetamido nitrazepam detected in the sample are shown in Fig. 3 and Table II, respectively. Nitrazepam was not detected in this sample.

ACKNOWLEDGEMENTS

The author is much obliged to Dr. H. Sawada for supplying 7-amino-nitrazepam and 7-acetamidonitrazepam, and also indebted to the Nagano Prefectural Foundation for Scientific and Technical Advancement, Japan, which supported this study in part by a grant.

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Note

High-performance liquid chromatographic determination of nifedipine in plasma

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(First received January 12th, 1984; revised manuscript received March 23rd, 1984)

Nifedipine [dimethyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylate] is one of the most useful drugs known as a calcium antagonist, and has been widely used clinically. Thus the measurement of nifedipine in plasma is required for pharmacokinetic studies and to examine relationship between plasma levels and clinical effects.

Several methods for the assay of plasma levels of nifedipine have been described, including a fluorescence method [1], gas chromatography [2—4], gas chromatography—mass spectrometry [5], and high-performance liquid chromatography (HPLC) [6, 7], but many of these methods are inappropriate for clinical use because they have a low sensitivity, are time-consuming, need a large amount of plasma, and require expensive equipment not usually available in a clinical laboratory. A recent paper [8] describes a rapid assay method using HPLC, but the reproducibility of this method at low concentrations (10 ng/ml in plasma) is relatively inferior. Here we describe a simple and reproducible HPLC method using a reversed-phase column and an ultraviolet (UV) detector.

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EXPERIMENTAL

Materials

Nifedipine was kindly supplied by Kanebo (Tokyo, Japan). Butamben (*n*-butyl-*p*-aminobenzoate) was purchased from Kishida Chemicals (Osaka, Japan). All chemicals were of reagent grade and used without further purification.

Procedures

Methanol (100 μl) and acetonitrile (2 ml) were added to 0.5 ml of plasma in a brown test tube, and agitated with a Vortex mixer. After centrifugation at 1500 *g* for 5 min, 2 ml of the supernatant were transferred into a brown test tube containing 1 ml of distilled water, and then 4.5 ml of acetone–chloroform mixture (1:1, v/v) were added. The mixture was shaken for 10 min and then centrifuged at 1500 *g* for 5 min. After aspirating the aqueous layer, 5 ml of the organic layer were transferred into a brown test tube, and reduced to dryness in a centrifugal evaporator (Model RD-21, Yamato Scientific, Tokyo, Japan) at 45°C for 30 min. The residue was dissolved in 100 μl of the mobile phase containing butamben as internal standard (2 μg/ml), and 20 or 30 μl of the solution were injected into the HPLC system.

Chromatographic conditions

A liquid chromatograph (Hitachi 635 A) equipped with a high-pressure sampling valve (638-0801, 1–150 μl) and multiwavelength UV detector (Hitachi 638-41) was used. For the stationary phase, a reversed-phase column (Zorbax ODS, 4–6 μm 25 cm × 4.6 mm I.D.; Du Pont de Nemour, Wilmington, DE, U.S.A.) was used; the column was warmed at 55°C using a constant-temperature water bath circulator. The mobile phase consisted of 0.01 *M* disodium hydrogen phosphate buffer–methanol (45:55). Before mixing, the buffer was brought to pH 6.1 with 50% phosphoric acid. The flow-rate was 0.8 ml/min and the pressure was approximately 70 kg/cm^2. The wavelength was 280 nm at 0.0025 a.u.f.s.

Calibration graph

Standard solutions containing 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml nifedipine in methanol were prepared under very subdued light. Instead of 100 μl of methanol, 100 μl of each standard solution were added to 0.5 ml of drug-free plasma and the samples were processed as described above. The ratios of the peak height of nifedipine to that of butamben (internal standard) were used to construct a calibration graph. Stock solutions of both nifedipine and internal standard (1 mg/ml) in methanol were stored in complete darkness; these solutions were freshly prepared every month.

Monitoring of plasma concentrations

An experiment was performed with two healthy subjects aged 27 and 29 years, weighing 60 and 53 kg, respectively. First, basal blood samples were taken after overnight fasting. Then 10 mg of nifedipine (Adalat; Bayer Yakuhin, Osaka, Japan) were administered orally with 100 ml of tap water. Blood samples were drawn through an indwelling venous catheter at 0.33,

0.5, 0.75, 1, 1.5, 2, 3, and 4 h. Food and beverages were restricted for 4 h after administration.

RESULTS AND DISCUSSION

Sample preparation

Nifedipine can be extracted at neutral and alkaline pH by several organic solvents such as heptane, diethyl ether, chloroform, and ethyl acetate. In preliminary experiments, however, endogenous biological interfering substances were also extracted by these organic solvents, and showed a peak immediately after the peak of the internal standard. However, it was found that these interfering peaks became negligible when a mixture of acetone–chloroform (1:1, v/v) was used for the extraction.

Selectivity

Fig. 1 shows chromatograms of blank plasma, plasma sample spiked with 40 ng/ml nifedipine, and plasma sample at 3.0 h after the administration of nifedipine to the patient (Adalat, 10 mg). Nifedipine and internal standard were well separated from endogenous substances. In this procedure a peak due to metabolites of nifedipine was not observed near the peak of nifedipine.

The calibration curve of peak height ratio was linear with a correlation coefficient of 0.9998. The coefficient of variation at 10 ng per 0.5 ml of plasma was 3.02% (n = 7).

The relative recovery of nifedipine from plasma containing 40 ng/ml was estimated by comparing it with the recovery from an aqueous sample (distilled water) and was found to be 99.1 ± 3.2% (mean ± S.D., n = 7). Plasma was spiked with nifedipine by the same procedure as described for the calibration graph. The limit of sensitivity for quantitation was 5 ng/ml plasma.

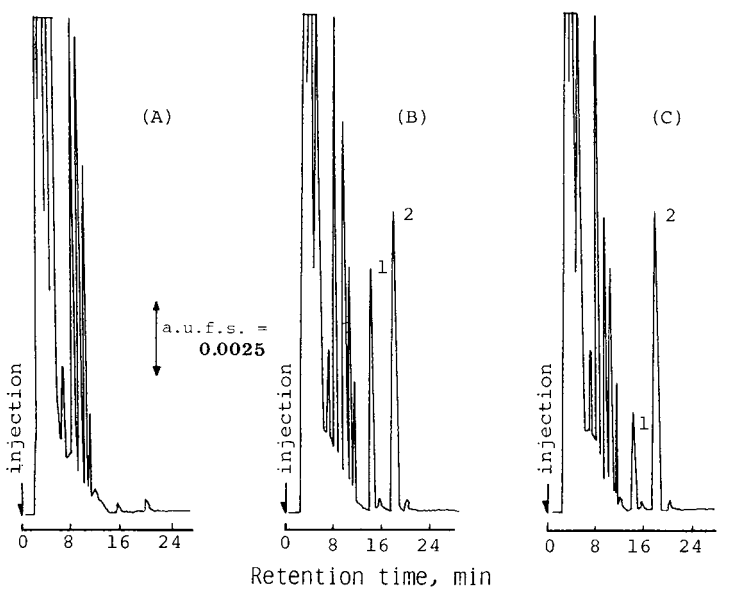

Fig. 1. High-performance liquid chromatograms of blank plasma (A), plasma sample spiked with 40 ng/ml nifedipine (B), and plasma sample after the administration of nifedipine to a patient (C). Peaks: 1 = nifedipine, 2 = internal standard.

No interfering peaks were detected for plasma spiked with propranolol, trichloromethiazide, or L-α-methyldopa in accepted therapeutic concentrations.

Plasma concentration profile

Plasma concentrations of nifedipine were monitored using the newly developed assay method. The plasma concentration profiles are shown in Fig. 2. The absorption rates and the elimination profiles were significantly different between the two subjects. In our recent studies investigating the relationship between the plasma levels of nifedipine and the lowering of the blood pressure in hypertensive patients, substantial variations in the rate of appearance in plasma were seen (unpublished data). Also, substantial variations in plasma levels of nifedipine [8] and its analogue [9] among the subjects were reported.

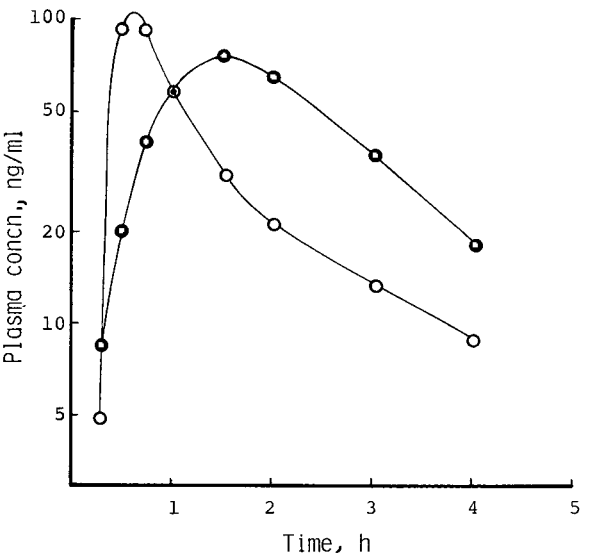

Fig. 2. Plasma concentration profiles of nifedipine after oral administration of 10 mg of nifedipine to each of two volunteers.

It is possible to determine low plasma concentrations of nifedipine rapidly, reproducibly, and sensitively by the method described in this report. Our results suggest that the method is useful for both therapeutic drug monitoring and pharmacokinetic studies.

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JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

21st INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY, OSLO, NORWAY, JUNE 3–6, 1985

The above symposium will be held at the Hotel Scandinavia in Oslo, Norway. The scope of the meeting will cover papers, poster sessions and informal discussion groups by outstanding researchers from throughout the world in all fields of chromatography. In particular, new developments in gas, liquid, supercritical fluid and thin-layer chromatography will be included. There will also be a commercial exhibition of the latest instrumentation and books. Participation in the symposium will be on the basis of invited papers as well as unsolicited contributions. Authors desiring to present papers or posters must submit 200-word abstracts by December 1st, 1984. Complete manuscripts of accepted authors will be due on June 3rd, 1985 at the meeting in Oslo. All correspondence pertaining to the symposium and exhibition space should be directed to: Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.

9th INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY, EDINBURGH, U.K., JULY 1–5, 1985

The Ninth International Symposium on Column Liquid Chromatography (HPLC '85) will be held in the George Square complex of the University of Edinburgh, Scotland, from 1–5 July 1985. The Symposium will cover all aspects of modern liquid chromatography, including microbore to preparative systems, computer-aided developments, novel detection systems, electrophoretic chromatography along with the latest developments in fundamental theory and applications in biomedical, environmental and industrial analysis. The Symposium will include presentations by invited speakers of international distinction.

The Ninth Symposium like its predecessors will be organised by a permanent international committee and a local UK commitee, the latter chaired by Professor J.H. Knox, F.R.S. It will be supported by major Chemical and Chromatographic Societies from throughout the world.

Authors wishing to present papers at the Symposium should send definitive abstracts to the 9th ISCLC Secretariat *no later than 15 November 1984,* stating their preference for oral or poster presentation. Publication, subject to refereeing, will be in the *Journal of Chromatography.*

A technical exhibition will form an integral part of the Symposium. Exhibitors wishing to display equipment or accessories for liquid chromatography should contact the 9th ISCLC Secretariat as soon as possible as prime exhibition space is limited.

A full social programme will be offered as part of the Symposium, including receptions, a banquet and tours to places of cultural and historical interest in and around Edinburgh.

The official carrier for the Symposium is British Airways.

All correspondence and requests for further information should be directed to: 9th ISCLC Secretariat, CEP Consultants Ltd, 26 Albany Street, Edinburgh EH1 3QH, Scotland, U.K.; Tel: 031-557 2478.

16th INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY, PARIS, FRANCE, SEPTEMBER 21–26, 1986

The G.A.M.S., in association with the Arbeitskreis Chromatographie der Fachgruppe Analytische Chemie der Gesellschaft Deutscher Chemiker (F.R.G.) and the Chromatographic Society (U.K.), organizes the 16th International Symposium on Chromatography.

This meeting will be held at the Conservatoire National des Arts et Métiers, Paris, France.

The scientific programme will include plenary lectures from invited speakers, contributed papers, poster and discussion sessions.

The scope of this symposium will cover all aspects of chromatography and related techniques, with special emphasis on the latest development of this method in the field of theory, instrumentation and applications.

In addition to the scientific programme, an exhibition of instruments and a social programme will also be arranged.

For further information contact: G.A.M.S., 88, Boulevard Malesherbes, F-75008 Paris, France. Tel. (1) 563.93.04.

CALENDAR OF FORTHCOMING EVENTS

Oct. 1–5, 1984 Nürnberg, F.R.G.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), F.R.G. (Further details published in Vol. 281.)
Oct. 22–23, 1984 Tarrytown, NY, U.S.A.	**3rd International Symposium on Capillary Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, TX 77004, U.S.A. (Further details published in Vol. 308.)
Oct. 24–26, 1984 Montreux, Switzerland	**3rd Workshop on LC–MS and MS–MS** Contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 276, No. 1.)
Nov. 13–16, 1984 New York, NY, U.S.A.	**23rd Annual Eastern Analytical Symposium** Contact: Dr. S. David Klein, EAS Publicity, Merck & Co., Inc., P.O. Box 2000/R80L-106, Rahway, NJ 07065, U.S.A. Tel.: (201) 846-1582.
Nov. 19–24, 1984 Barcelona, Spain	**EXPOQUIMIA 84 – Salón Internacional de la Quimica** Contact: EXPOQUIMIA, Feria de Barcelona, Barcelona 4, Spain.
Nov. 19–20, 1984 Barcelona, Spain	**2nd Workshop on the Chemistry and Analysis of Hydrocarbons in the Environment** Contact: Expoquimia, Avda. Reina Ma. Cristina, Barcelona-4, Spain. Tel.: (93) 223 31 01. Telex 53117 FOIMB-E.

Nov. 22–24, 1984 Barcelona, Spain	**14th Annual Symposium on Analytical Chemistry of Pollutants** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E.
Nov. 22–24, 1984 Barcelona, Spain	**3rd International Congress on Analytical Techniques in Environmental Chemistry** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel: 223.31.01: Telex: 50458 FOIMB-E. (Further details published in Vol. 285, No. 1.)
Dec. 10–12, 1984 Baltimore, MD, U.S.A.	**4th International Symposium on HPLC of Proteins, Peptides, and Polynucleotides** Contact: Shirley E. Schlessinger, Symposium Manager, Fourth International Symposium on HPLC of Proteins, Peptides, and Polynucleotides, 400 East Randolph, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011. (Further details published in Vol. 285, No. 1.)
Jan. 28–30, 1985 Kyoto, Japan	**International Symposium on High Performance Liquid Chromatography** Contact: Prof. H. Hatano, Symposium Chairman, Dept. of Chemistry, Faculty of Science, Kyoto University, Kyoto 606, Japan. Tel.: (075) 721-9100; telex: 05422693 LIBKU J.
Feb. 25–March 1, 1985 New Orleans, LA, U.S.A.	**36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A.
April 28–May 3, 1985 Miami Beach, FL, U.S.A.	**189th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
May 14–16, 1985 Riva del Garda, Italy	**6th International Symposium on Capillary Chromatography** Contact: Dr. P. Sandra, Laboratory of Organic Chemistry, University of Ghent, Krijgslaan 281 (S.4), B-9000 Ghent, Belgium. (Further details published in Vol. 298, No. 1.)
June 3–6, 1985 Oslo, Norway	**21st International Symposium on Advances in Chromatography** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623. Telex.: 762878
July 1–5, 1985 Edinburgh, U.K.	**HPLC '85. 9th International Symposium on Column Liquid Chromatography** Contact: 9th ISCLC Secretariat, CEP Consultants Ltd., 26 Albany Street, Edinburgh EH1 3QH, Scotland, U.K. Tel.: (031) 557 2478
Aug. 11–16, 1985 Espoo, Finland	**XIVth International Conference on Medical and Biological Engineering and VIIth International Conference on Medical Physics** Contact: Hannu Seitsone, Secretary General, P.O. Box 105, 00251 Helsinki, Finland. Tel.: 358-0-4713070.
Sept. 1–6, 1985 Prague, Czechoslovakia	**6th International Symposium on Bioaffinity Chromatography and Related Techniques** Contact: Dr. J. Turková, Institute of Organic Chemistry and Biochemistry ČSAV, Flemingovo No. 2, CS-166 10, Prague 6, Czechoslovakia. Tel.: (422) 324541, int. 080. (Futher details published in Vol. 308.)

Sept. 5–8, 1985 Birmingham, U.K.	**Flow Analysis III – An International Conference on Flow Analysis** Contact: Flow Analysis III, Dr. A.M.G. Macdonald, Department of Chemistry, The University, P.O. Box 363, Birmingham B15 2TT, U.K. (Further details published in Vol. 288, No. 2.)
Sept. 8–13, 1985 Chicago, IL, U.S.A.	**190th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
Sept. 9–13, 1985 Manchester, U.K.	**30th International Congress of Pure and Applied Chemistry** Contact: The Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K.
Nov. 11–16, 1985 Yalta, U.S.S.R.	**5th Danube Symposium on Chromatography** Contact: Dr. L.N. Kolomiets, The Scientific Council of Chromatography, Academy of Sciences of the U.S.S.R., Institute of Physical Chemistry, Lenin-Prospect 31, Moscow 117312, U.S.S.R. (Further details published in Vol. 281.)
May 18–23, 1986 San Francisco, CA, U.S.A.	**New Frontiers in HPLC. 10th International Symposium on Column Liquid Chromatography** Contact: Ms. Shirley Schlessinger, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A.
Sept. 21–26, 1986 Paris, France	**16th International Symposium on Chromatography** Contact: G.A.M.S., 88, Boulevard Malesherbes, F-75008 Paris, France. Tel.: (1) 563 93 04.

NEW BOOKS

Gas chromatography/mass spectrometry applications in microbiology, edited by G. Odham, L. Larsson and P.-A. Mårdh, Plenum, New York, 1984, 436 pp., price US$ 59.50 (U.S.A. and Canada), US$ 71.40 (rest of world), ISBN 0-306-41314-0.

Clinical liquid chromatography, Vol. I, Analysis of exogenous compounds, and **Vol. II, Analysis of endogenous compounds,** edited by P.M. Kabra and L.J. Marton, CRC Press, Boca Raton, FL, 1984, XIII + 212 pp. (Vol. I) and XIII + 226 pp. (Vol. II), prices: Vol. I: US$ 63.00 (U.S.A.), US$ 72.00 (other countries), Vol. II: US$ 72.00 (U.S.A.), US$ 83.00 (other countries), ISBN: Vol. I: 0-8493-6637-2, Vol. II: 0-8493-6638-0.

Topics in forensic and analytical toxicology (*Proc. Annual European Meeting of the International Association of Forensic Toxicologists, Munich, August 21–25, 1983; Analytical Chemistry Symposia Series,* Vol. 20), edited by R.A.A. Maes, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 214 pp., price Dfl. 150.00, US$ 57.75, ISBN 0-444-42313-3.

Hazardous metals in human toxicology (*Techniques and Instrumentation in Analytical Chemistry, Vol. 4, Evaluation of Analytical Methods in Biological Systems*, Part B) edited by A. Vercruysse, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 338 pp., price Dfl. 190.00, US$ 73.00, ISBN 0-444-42207-2.

Clinically important adverse drug interactions, Vol. 2, Nervous system, endocine system and infusion therapy, edited by J.C. Petrie, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, 384 pp., price Dfl. 250.00, US$ 96.25, ISBN 0-444-80529-x.

Biosynthetic products for cancer therapy, Vol. 4, by G.R. Pettit, G.M. Cragg and C.L. Herald, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XII + 430 pp., price US$ 13.50 (U.S.A. and Canada), Dfl. 295.00 (rest of world), ISBN 0-444-42348-6.

PUBLICATION SCHEDULE FOR 1984

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1983	J	F	M	A	M	J	J	A	S	O	N
Journal of Chromatography	282	283 284/1	284/2 285/1	285/2 285/3 286 287/1	287/2 288/1 288/2 289	290 291 292/1	292/2 293 294	295/1 295/2 296	297 298/1 298/2 298/3	299/1 299/2 301/1	301/2 302 303/1	303/2
Chromatographic Reviews		300/1					300/2					300/3
Bibliography Section		304/1	304/2			304/3					304/4	304/5
Biomedical Applications		305/1	305/2	306	307/1	307/2	308	309/1	309/2	310/1	310/2	311/1 311/2

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 295, No. 2, pp. 555–558. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

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Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

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