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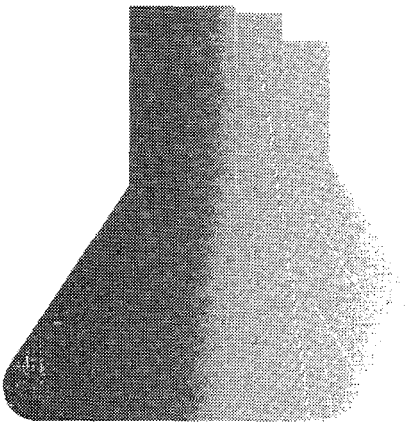
ANALYTICAL METHODS AND PROBLEMS IN BIOTECHNOLOGY

AN INTERNATIONAL SYMPOSIUM



17–19 April, 1984, Leeuwenhorst Congress Center, Noordwijkerhout, The Netherlands

Organised by:
Royal Netherlands Chemical Society (KNCV), Section for Analytical Chemistry
Netherlands Biotechnology Society (NBV) under the sponsorship of the Federation of European Chemical Societies

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Modern Liquid Chromatography of Macromolecules

by **B.G. BELENKII** and **L.Z. VILENCHIK,** Institute of Macromolecular Compounds, Academy of Sciences of the USSR, Leningrad, USSR

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Researchers, technologists and students in the fields of physics, chemistry, biology and medicine will all find this book of interest. It considers the chromatography of high-molecular-weight compounds and its main theoretical and methodological features.

Recommendations are made for preparing high-performance chromatographic systems and for selecting the optimum conditions for their operation. Particular attention is paid to the problems of interpreting chromatographic data in order to obtain various molecular-weight and structural characteristics of the macromolecules investigated: AMW, MWD, indices of polymer branching, the compositional homogeneity of copolymers, the functionality of oligomers, etc. The authors also provide examples of various combinations of chromatographic and other methods that can be used to analyse complex polymer systems.

Written mainly for the reader who has some practical experience in chromatography, the book also contains much useful information for the beginner.

CONTENTS: Chapter I. General Theory of Chromatography. II. Main Features of the Chromatography of Macromolecules. III. Interpretation of Data on the Analysis of Polymers by Gel Chromatography. IV. Methodological Problems of Gel-Permeation Chromatography (GPC). V. Use of Gel-Permeation Chromatography for the Analysis of Macromolecules, Investigation of their Interaction and the Characterization of the Porous Structure of Sorbents. VI. High Performance Gel-Filtration Chromatography (HPGTC). VII. Thin-Layer Chromatography of Polymers. VIII. One-Phase Chromatography: Field Fractionation and Hydrodynamic Chromatography.

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LIPOXYGENASE PRODUCTS

A NOVEL GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ASSAY FOR MONOHYDROXY FATTY ACIDS

P.M. WOOLLARD* and A.I. MALLET

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(First received August 1st, 1983; revised manuscript received October 21st, 1983)

SUMMARY

We present a novel mass spectrometric method for the profiling of monohydroxy fatty acids and illustrate its use for the analysis of lesional skin of patients with psoriasis.

By combination of vapour phase hydrogenation and gas chromatography—mass spectrometry with selected ion monitoring the position of the substituted hydroxyl group of each fatty acid was determined in their methyl ester trimethylsilyl ether derivatives. Reduction was instantaneous and quantitative allowing detection of less than 100 picogram of each compound. Biological extracts, derivatised as methyl ester *tert.*-butyldimethylsilyl ethers, were purified by reversed-phase high-performance liquid chromatography. Separation was dependent on carbon chain length and degree of unsaturation, but not on the position of the silyl ether group. Subsequent conversion of the *tert.*-butyldimethylsilyl ethers to trimethylsilyl ethers facilitated detection of each of the positional hydroxyl isomers as described. Distinction between double bond isomers was possible when they were separated on gas chromatography prior to reduction.

INTRODUCTION

Unsaturated fatty acids are metabolised by lipoxygenase enzymes to their respective hydroperoxide products. Subsequent enzymic or non-enzymic events lead either to the formation of further oxidative products or to their reduction to monohydroxylated fatty acids.

Both mammalian and plant enzymes exhibit varying degrees of specificity for a wide range of fatty acid substrates and the pattern of monohydroxylated metabolites produced tends to be characteristic of the tissue in which it is found. Whereas the major product of lipoxygenase action in human platelets is 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) [1], human eosinophils

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produce mainly 15-HETE [2], while human neutrophils produce 12-, 9-, 8- and 5-HETEs [3]. The availability of more than one substrate may also lead to the formation of a variety of metabolites. Rabbit polymorphonuclear leukocytes convert arachidonic acid primarily into 5-HETE, while 8-hydroxy-9,11,14-eicosatrienoic acid is the major product when dihomo-gamma-linolenic acid is utilised [4].

Changes in lipoxygenase activity have been demonstrated in certain human inflammatory diseases. In particular, patients with the common skin disorder, psoriasis, show significantly increased concentrations of certain monohydroxy fatty acids in their lesional as opposed to uninvolved skin [5, 6]. Since we have found that human skin produces several monohydroxylated products derived from at least three precursor fatty acids [6], we were interested in profiling metabolites from these and other possible precursors in patients with psoriasis and in unaffected individuals. The significance of these hydroxy fatty acids is unclear, but several metabolites derived from arachidonic acid have been shown to be chemotactic and chemokinetic for human neutrophils [7], the infiltration of which has been implicated in the pathogenesis of the disease [8]. As a large number of precursor fatty acids have been shown to be present in human skin [9] stimulation of lipoxygenases in normal or pathological conditions may result in the formation of a wide range of hydroxylated products.

For the screening of monohydroxy fatty acids in lesional psoriatic skin, published methods lack the required specificity: we therefore describe a novel gas chromatography—mass spectrometric (GC—MS) assay with improved selectivity and sensitivity capable of measuring a wide range of monohydroxy fatty acids.

EXPERIMENTAL

Materials

All unsaturated fatty acids and N,O-bis-trimethylsilyl trifluoroacetamide (BSTFA) were purchased from Sigma London (Poole, U.K.). 12-Hydroxystearic acid and ricinoleic acid were supplied by Applied Science Labs. (Chester, U.K.). All other hydroxy fatty acids were synthesised by a photooxidative method previously described [6] and outlined below.

[1-^{14}C] Arachidonic acid was supplied by Amersham International (Amersham, U.K.) and was used for the synthesis of radioactive hydroxy fatty acids [6]. The *tert.*-butyldimethylchlorosilane, imidazole, tetrabutylammonium fluoride, dimethylformamide, tetrahydrofuran and *tert.*-butylmethyl ether (Fluorochem) were purchased from Fluka (Glossop, U.K.). Col Treet^{TM} (Regis) was supplied by Phase Separations (Clwyd, U.K.).

The catalysts, palladium(II) chloride and rhodium(III) chloride trihydrate were obtained from Aldrich (Gillingham, U.K.) and the platinum(IV) oxide was from Fluka. The support phases on which the catalysts were coated were Diatomite CQ (100—120 mesh) obtained from Pye Unicam (Cambridge, U.K.) and Chromosorb G HP (100—120 mesh) supplied by Phase Separations.

Methanol and *n*-heptane, which were redistilled from the Analar-grade reagents and the 2,4,4-trimethylpentane were obtained from BDH (Poole, U.K.). All other solvents (HPLC grade) and Fisofluor I scintillation fluid were from Fisons (Loughborough, U.K.).

Preparation of monohydroxy fatty acids

Monohydroxy fatty acids were prepared by photolysis of their respective unsaturated fatty acids using a modification of the method of Porter et al. [10] as described previously [6]. Briefly, each fatty acid in solution in methanol containing 0.01% methylene blue (w/v) was irradiated with three 500-W Phillips PF 318 P2/3 bulbs, the reaction being cooled (less than 10°C) using a circulating water jacket. The hydroperoxides so formed were reduced with sodium borohydride and the products were subjected to preliminary purification on Lipidex 5000^{TM} (100 ml bed volume; 20 cm column length) (Packard, Reading, U.K.) reversed-phase gel partition chromatography using methanol—water—1,2-dichloroethane—acetic acid (750:150:150:1), from which the monohydroxy fatty acid fraction was collected. Isolation of the positional isomers was achieved by using a semipreparative Spherisorb S5W straight-phase high-performance liquid chromatography (HPLC) column (25 cm × 8 mm) eluted with hexane—propan-2-ol—methanol—acetic acid (975:22:26:1). Where individual components co-eluted, separation was attained, where possible, by adjusting the solvent system. 14-HETE, which is not separated from 15-HETE using the above solvent system, was purified by using the same solvents in the proportion 1070:5:15:1. Those compounds containing a conjugated diene were detected by UV absorbance at 235 nm; 14-HETE was detected at 205 nm.

Extraction

Samples of psoriatic scale (25 mg) obtained by gentle abrasion of skin lesions were vortexed with a mixture of sodium acetate buffer (0.1 *M*, pH 3.5, 3 ml) and ethyl acetate (3 ml). After centrifugation the organic phase of each sample was removed and the process was repeated with an equal volume of ethyl acetate. The pooled organic phase was evaporated under nitrogen. Non-polar lipids were then removed by partition of the residue between methanol (3 ml) and *n*-heptane (4 ml), the heptane layer being discarded. The methanol was evaporated prior to derivatisation. (See Fig. 1, step 1.)

Methylation

Methyl esters were prepared by reacting the methanolic residues twice (Fig. 1, step 1) with methanolic—ethereal (1:9) diazomethane (100 μl). (See Fig. 1, step 2.)

tert.-Butyldimethylsilyl ether formation

tert.-Butyldimethylsilyl (tBDMS) ether derivatives were prepared using a solution of *tert.*-butyldimethylchlorosilane (1 *M*) and imidazole (0.5 *M*) in dimethylformamide (100 μl), a modification of the reagent described by Corey and Venkateswarlu [11]. The reaction was carried out at 40°C for 30 min when it was terminated by addition of water (100 μl). Fatty acid methyl esters and methyl ester, tBDMS ether derivatives were extracted twice into hexane (2 × 1 ml). The pooled hexane phase (2 ml) was then washed with an equal volume of water to remove traces of imidazole prior to evaporation of the solvent and HPLC of the derivatives. (See Fig. 1, step 2.)

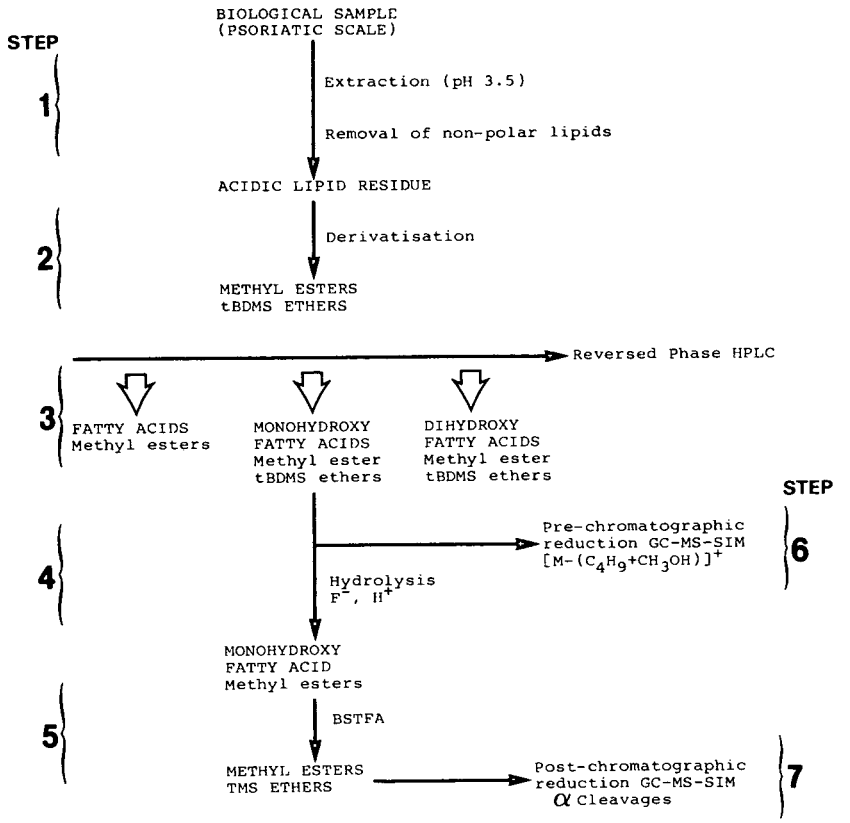

Fig. 1. Flow diagram of assay procedure.

Reversed-phase high-performance liquid chromatography

This was carried out on a Spherisorb analytical S5 ODS column eluted with methanol—water (925:75) at a flow-rate of 1 ml/min. Effluent was collected at the relevant times (see Results) and the solvent removed by vortex evaporation at 40° C. (See Fig. 1, step 3.)

Hydrolysis of tert.-butyldimethylsilyl derivatives

The methyl ester tBDMS ether derivatives purified by HPLC were treated with a solution of tetrabutylammonium fluoride (100 m*M*, 100 μl) at 40° C for 1 h; sodium acetate buffer (0.1 *M*, pH 3.5, 200 μl) was added and the hydrolysis products extracted twice into *tert.*-butylmethyl ether (2 × 500 μl). After evaporation of the pooled organic phase (1 ml) the products were rederivatised as the trimethylsilyl (TMS) ethers (Fig. 1, step 5.)

Alternatively a solution containing acetic acid—methanol—water (2:2:1, 100 μl) was used as the hydrolytic reagent. The reaction was carried out at 40° C overnight. The products were diluted with water (200 μl) and extracted as above. (See Fig. 1, step 4.)

Trimethylsilyl ether formation

Following hydrolysis of the tBDMS ether derivatives, TMS ether derivatives

were formed by addition of BSTFA (25 μl) to the hydroxy fatty acid methyl ester residues. (See Fig. 1, step 5.)

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on neutral silica gel on glass-backed plates (200 × 50 × 0.25 mm) (Anachem, Luton, U.K.) developed to 150 mm using a solvent system consisting of 2,4,4-trimethylpentane—ethyl acetate (1:1). Radioactive samples applied as a spot (5 mm diameter) were removed in sections (5 mm) after development. Standard samples were visualised with iodine vapour.

Scintillation counting

Liquid scintillation counting was carried out using an LKB 1012 Liquid Scintillation Counter. Radioactive samples (^{14}C) were added to scintillant (3 ml) and the dpm values were obtained after correction for quenching using the automatic external standard channels ratio method and a quench calibration curve. Silica gel sections from TLC plates were added directly to the scintillation vials and counted as described.

Gas chromatography

Packed column chromatography. GC was performed on either a Pye Model 104 Series gas chromatograph with flame ionisation detection or a Pye Model 204 Series gas chromatograph interfaced via a jet separator to the mass spectrometer described below.

Glass columns (1.5 m × 2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh) were employed, except where hydrogen was used as the carrier gas (80 kPa head press), when a narrower glass column (1.5 m × 1.2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh) was used.

Capillary column chromatography. GC was carried out on a fused silica OV-17 coated capillary column (25 m × 0.3 mm) attached to an all-glass dropping needle injector (Chrompack, London, U.K.) using hydrogen as carrier gas (33—167 kPa head press). GC—MS was carried out using the gas chromatograph described above. Where flame ionisation detection was employed GC was carried out on a Packard 428 instrument also attached to a dropping needle injector. In this case helium was used as carrier gas (115 kPa head press).

Hydrogenation

Liquid phase hydrogenation. Hydroxy fatty acid methyl esters (100 μg each) were dissolved in methanol (50 μl). To this a suspension of platinum(IV) oxide (10 mg/ml, 200 μl) was added and after purging with nitrogen the compounds were reduced by bubbling with hydrogen for 2 min. The catalyst was then rapidly sedimented by centrifugation in an Eppendorf 5412 centrifuge at maximum speed for 2 min. The supernatant was removed and evaporated at 40° C prior to formation of either TMS or tBDMS ether derivatives.

Vapour phase hydrogenation. The catalysts were prepared as reported previously [12]. Palladium(II) chloride was dissolved in methanol with the addition of a few drops of conc. hydrochloric acid. A weighed quantity of the

support phase, either Gas-Chrom Q (100—120 mesh) or Chromosorb G HP (Phase Sep, 100—120 mesh) was then added to give the final degree of palladium coating required. Batches of catalyst were prepared with coatings between 1 and 10% by weight. The solvent was removed by rotary evaporation under reduced pressure at 70°C followed by vacuum desiccation. The catalyst was then packed into an empty column, placed in the gas chromatograph oven and activated with hydrogen gas at 250°C for 30—60 min, the effluent being combusted in the flame ionisation detector. The support phase was then deactivated by injection of BSTFA or Col Treet.

Catalytic reduction on the packed column

On-column hydrogenation did not necessitate any reconstruction of the GC apparatus. Reduction was achieved using hydrogen as the carrier gas and a plug of the catalyst was situated at a chosen site on the GC column.

(A) Pre-chromatographic reduction. A plug of catalyst (4 cm) contained by glass wool was placed at the top of the glass column contained in the injector port enabling the temperature of catalysis to be controlled.

(B) Post-chromatographic reduction. In this case the plug of catalyst (10 cm) was placed at the end of the GC column just prior to the interface with the mass spectrometer. (See Fig. 1, step 6.)

Capillary column reduction

On-column hydrogenation employed a catalytic chamber packed with the prepared catalyst which was deactivated with BSTFA before use. The catalytic chamber consisted of a length of glass-lined steel tubing (15 cm × 1.6 mm I.D.) with union fittings (1.6 mm) (SGE, Milton Keynes, U.K.) connecting chromatographic and interface capillary columns. A number of configurations were investigated for interfacing the catalytic chamber and the mass spectrometer two of which are described below:

Configuration 1: introduction of the effluent from the catalytic chamber directly into the source of the mass spectrometer;

Configuration 2: addition of helium make-up gas prior to the catalytic chamber and jet separator interface.

Addition of helium make-up gas at a wide range of flow-rates prior to the catalytic chamber had no detrimental effect on the efficiency of reduction and eliminated any dead space in the catalytic chamber, thus improving peak shape while also providing the neccessary flow through the jet separator. Use of configuration 2 (Fig. 2) enabled optimum conditions of chromatography, recovery and reduction to be achieved. (See Fig. 1, step 7.)

Mass spectrometry

MS was performed on a VG Analytical Model 305 single focusing magnetic sector mass spectrometer. Mass spectra were acquired at an ionising energy of 40 eV with a trap current of 500 μA operating at a source temperature of 200°C. Selected ion monitoring was carried out by switching the accelerating voltage focusing masses between *m/z* 207 and *m/z* 385. The source tuning was relatively unaffected over this range, however at the higher masses monitored the signal was attenuated due to the decrease in ion energy.

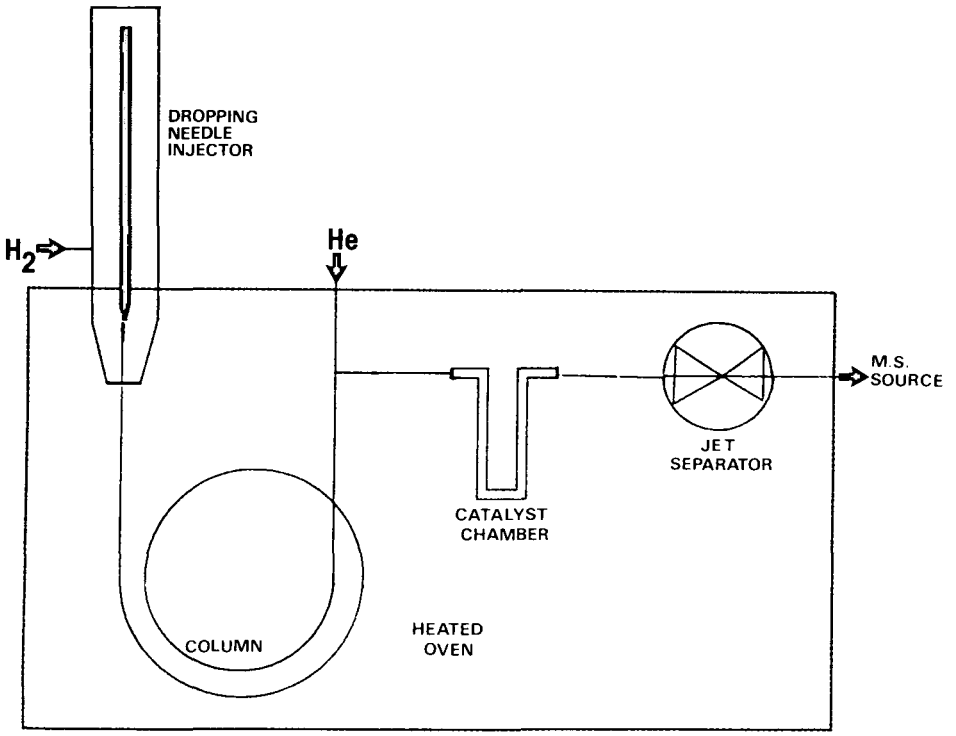

Fig. 2. Scheme of capillary column GC—MS incorporating post-chromatographic reduction (configuration 2).

RESULTS AND DISCUSSION

The electron impact mass spectra of the TMS and tBDMS ether derivatives of hydroxy fatty acids are stongly characterised by the presence or absence of unsaturation within the molecule [13]. Both derivatives of the unsaturated methyl esters predominantly yield a single intense ion in their mass spectra due to α-cleavage at the ether group, as illustrated by these derivatives of 12-HETE (Fig. 3a and b), however two ions could be expected to be produced by this fission. That only one of the α-cleavage fragments is usually present is a reflection of the greater resonance stability gained by formation of that ion in conjugating with the diene in the hydrocarbon chain.

On hydrogenation of the molecule however, the methyl ester TMS ether derivatives yield both α-cleavage fragment ions (Fig. 3c), widely used determinants for the position of the hydroxyl substitution in the parent molecule. In contrast, the methyl ester tBDMS ether derivatives of the saturated compounds exhibit characteristically intense high mass ions where the $[M-(C_4H_9 + CH_3OH)]^+$ ion is usually the base peak (Fig. 3d) [13]. We therefore decided that reduction of the methyl ester TMS ether derivatives would allow specific analysis of fatty acids substituted at any position on the carbon chain.

Such a procedure has previously been demonstrated [14], where reduction was carried out prior to GC—MS. However, when a number of unsaturated

fatty acid precursors are available, as occurs in human skin, it is important to eliminate the possibility that hydroxy fatty acids with differing degrees of unsaturation will be reduced to form identical compounds.

Sample preparation

Conversion of the polar lipid fraction derived from psoriatic scale as described above, into the methyl ester tBDMS ethers, transformed both fatty acids and their hydroxylated metabolites into non-polar derivatives which were stable to the reversed-phase HPLC system used.

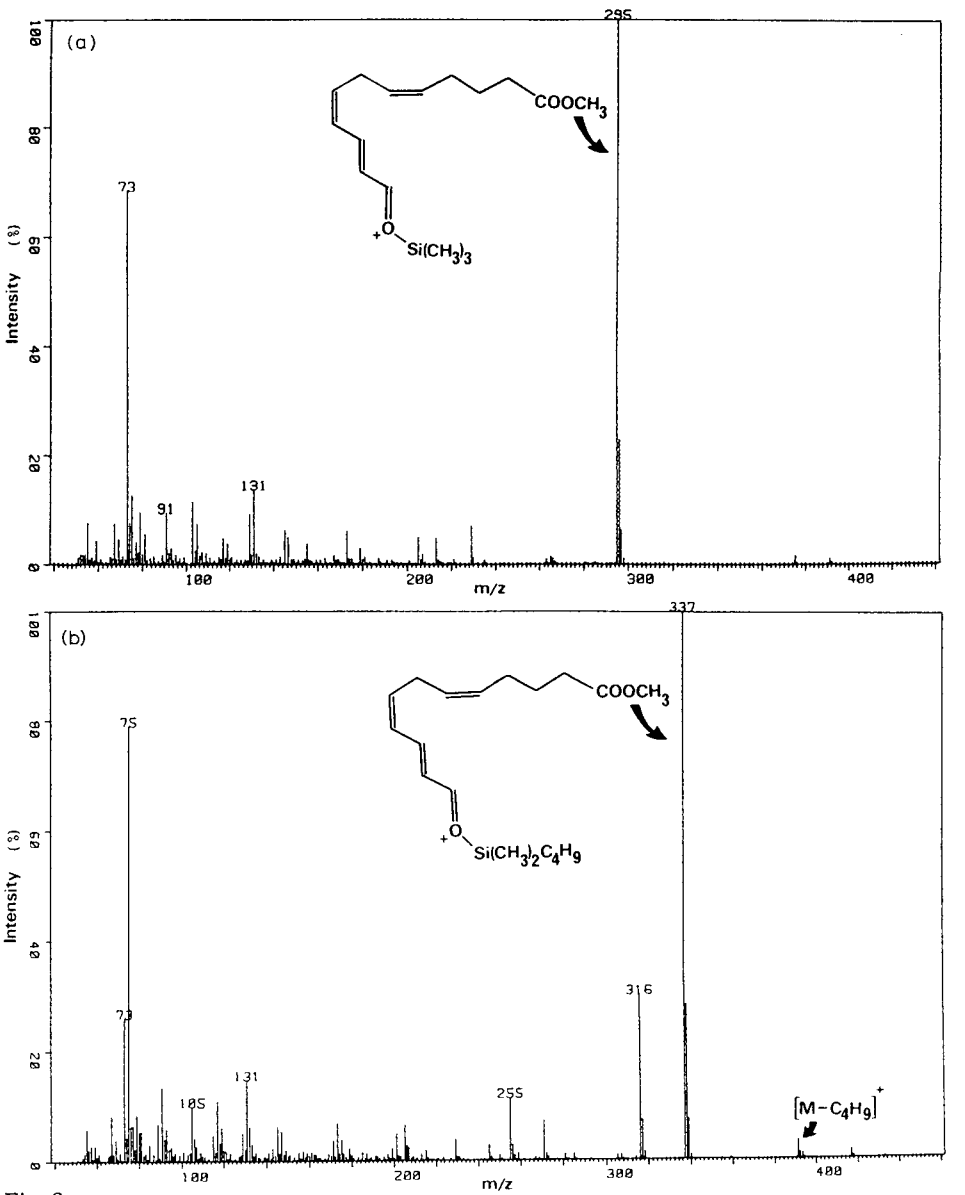

Fig. 3.

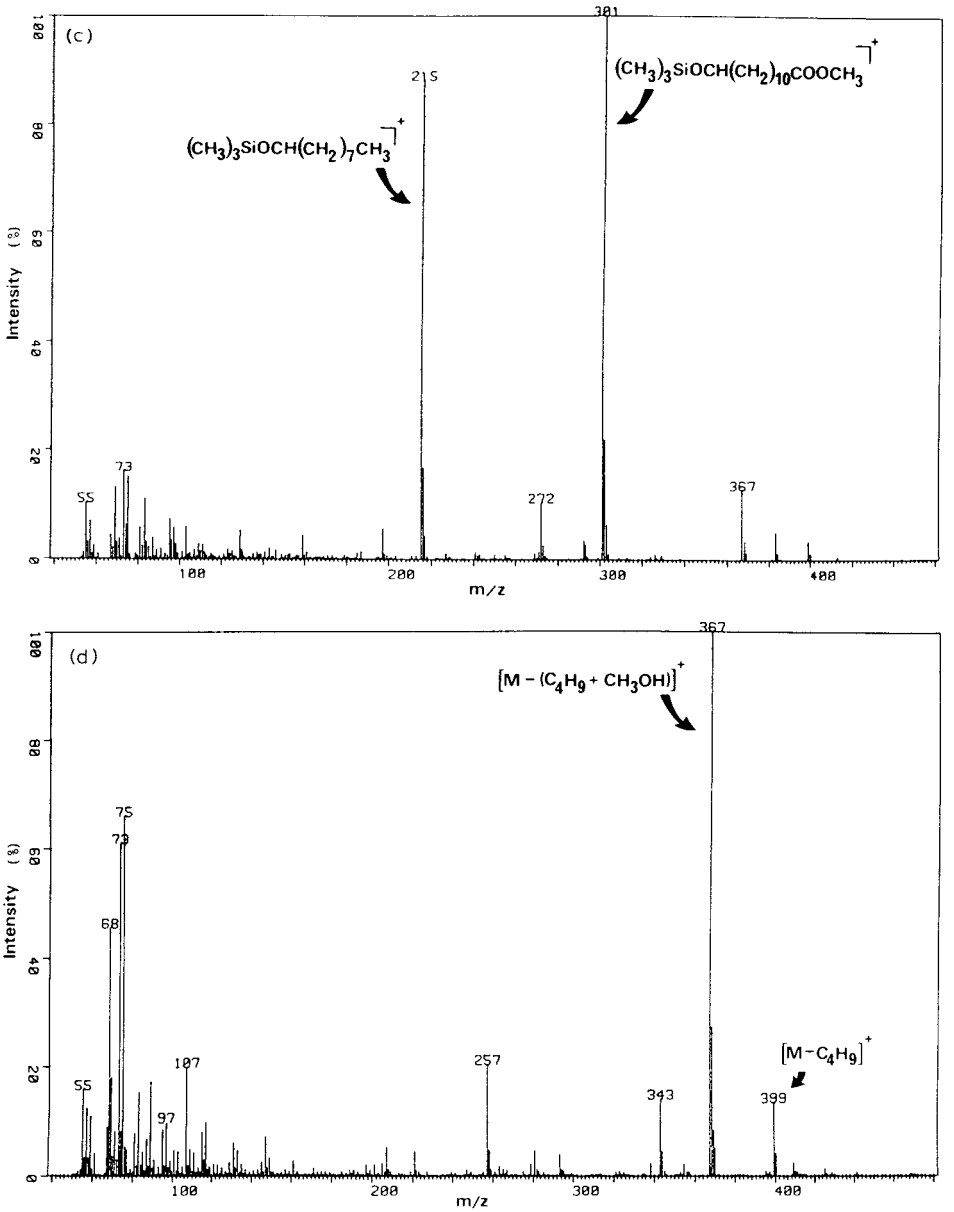

Fig. 3. Mass spectra of 12-HETE derivatives: (a) methyl ester TMS ether, (b) methyl ester tBDMS ether; and after hydrogenation of the double bonds, (c) methyl ester TMS ether, (d) methyl ester tBDMS ether.

Subjection of the derivatives obtained from 25 mg of scale to reversed-phase HPLC yielded UV absorbance profiles, one of which is shown in Fig. 4. The retention characteristics of the methyl ester tBDMS ether derivatives were influenced only by those factors affecting their polarity and were based therefore on three criteria: (1) the number of carbon atoms in the fatty acid

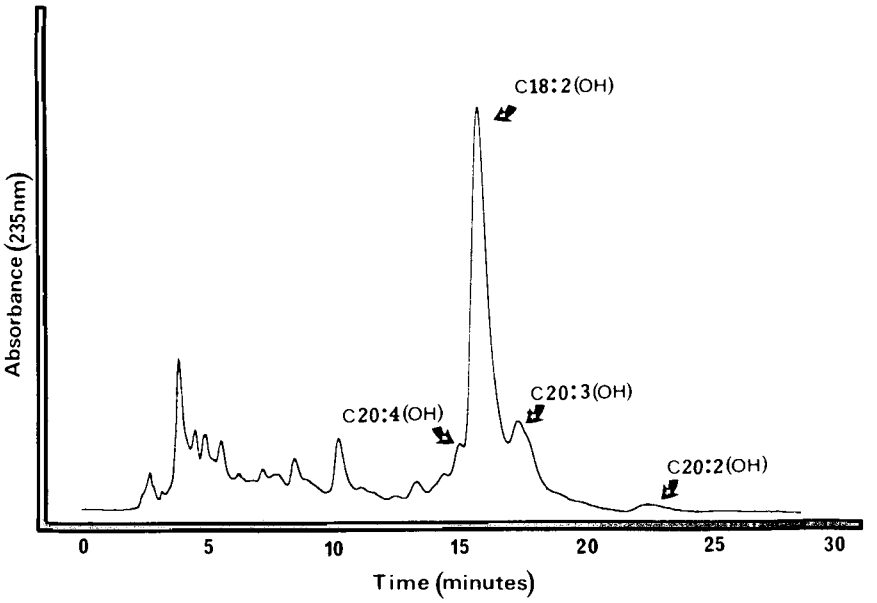

Fig. 4. HPLC profile of the methyl ester tBDMS ether derivatives of the organic extract from 25 mg of lesional psoriatic scale. UV absorbance was monitored at 235 nm. The labelled peaks indicate the identity of the mixture of derivatised hydroxy fatty acid positional isomers (OH) differing in carbon chain length (C_{20} and C_{18}) and number of double bonds (:2, :3, :4). HPLC was carried out on an analytical Spherisorb S5 ODS column using as the mobile phase methanol—water (925:75).

skeleton; (2) the number of double bonds in the fatty acid skeleton; (3) the number of substituted hydroxyl groups present.

The position of the substituted hydroxyl group had no significant effect on the retention time. This enabled collection of all the monohydroxylated metabolite derivatives from any particular fatty acid precursor in a single fraction. Separation based on the degree of unsaturation was especially important where the oxidation products of $C_{20:4}$, $C_{20:3}$ and $C_{20:2}$ fatty acids were present. In addition the separation of the double bond congeners was governed by an exponential relationship between the number of double bonds and the retention time (Fig. 5). From this relationship the retention time of any chosen monohydroxylated derivative could be predicted. The derivatives of the 8,11,14- and 11,14,17-eicosatrienoic acid metabolites, differing only in the position of their double bonds were partially separated by HPLC and were collected in the same fraction. It was also convenient to collect in one fraction the metabolite derivatives of arachidonic and linoleic acids.

Analysis of high-performance liquid chromatography fractions

GC—MS analysis of the effluent HPLC fractions could be carried out on the methyl ester tBDMS ether derivatives or after removal of the tBDMS group using tetrabutylammonium fluoride and re-derivatisation as TMS ethers. In either case the use of psoriatic scale led to the formation of mixed mass spectra as shown for the methyl ester tBDMS ether derivatives of 9- and 13-hydroxy-octadecadienoic acid (HODD) (Fig. 6). The C_{20} hydroxy fatty acid derivatives,

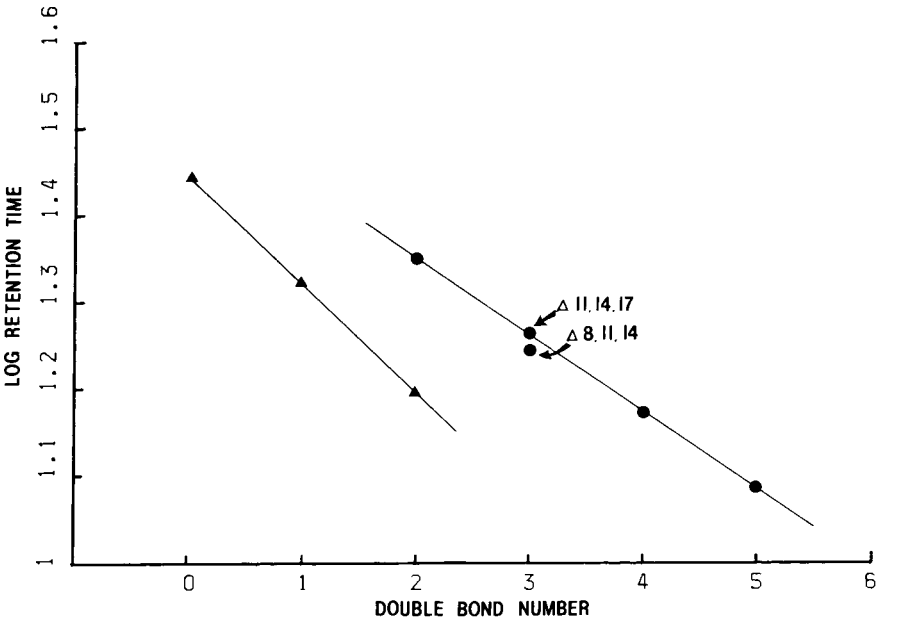

Fig. 5. Relationship between the log retention time (HPLC) and the number of double bonds in the carbon chain of the methyl ester tBDMS ether derivatives of C_{18} (▲) and C_{20} (●) hydroxy fatty acids. Derivatives containing conjugated double bonds were monitored for UV absorbance at 236 nm. Ricinoleic acid ($C_{18:1}$) and 12-hydroxy stearic acid ($C_{18:0}$) derivatives were detected by GC analysis of the HPLC fractions.

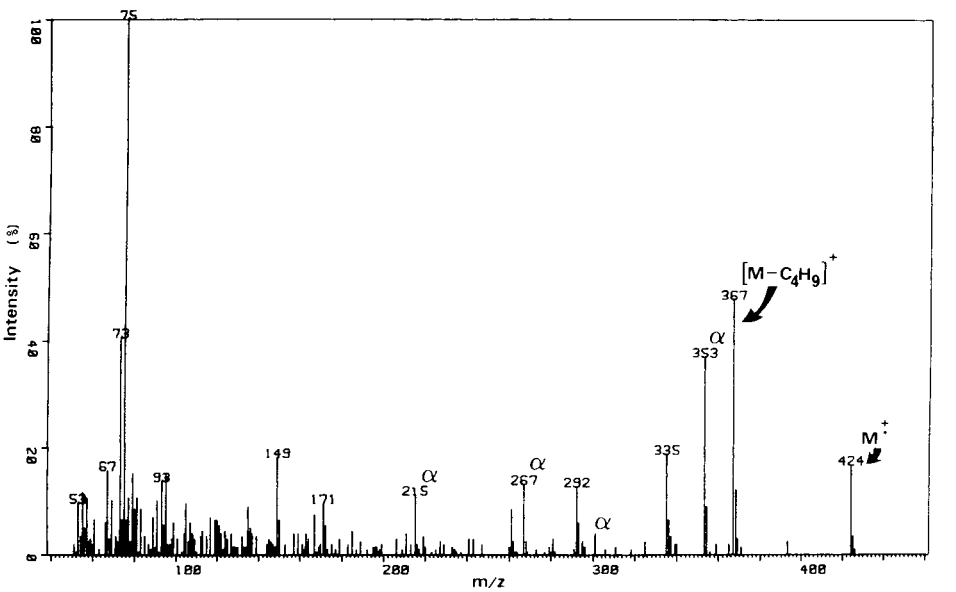

Fig. 6. Mixed mass spectrum of the $C_{18:2}$ (OH) fraction (Fig. 4) containing the methyl ester tBDMS ether derivatives of 9- and 13-hydroxy octadecadienoic acid (HODD). α-Cleavage ions are present at *m/z* 267 and *m/z* 301 (9-HODD) and *m/z* 215 and *m/z* 353 (13-HODD).

however, were generally present in a concentration too low for the acquisition of their full mass spectra.

An HPLC fraction containing the precursor fatty acids as their methyl esters could also be obtained, but its analysis will not be reported in this paper.

Hydrolysis of tert.-butyldimethylsilyl derivatives

The necessary conditions for hydrolysis of the tBDMS group were dependent on the presence of unsaturation in the hydrocarbon chain. Tetrabutylammonium fluoride in tetrahydrofuran (100 m*M*) achieved rapid quantitative removal of the tBDMS group in all of the $C_{20:4}$ ether derivatives except for 5-HETE where the reaction resulted in the formation of an unstable unidentified product. The ease of hydrolysis was ranked as follows: 8- and 12-HETE > 9- and 11-HETE > 14- and 15-HETE, with the reaction for all HETE compounds being complete within 45 min at 40°C. The removal of the tBDMS group of 12-hydroxystearic acid methyl ester was considerably less effective with this reagent, while increasing the concentration of tetrabutylammonium fluoride (1 *M*) caused concomitant removal of the methyl ester group. The use of acid hydrolysis at 40°C overnight with the reagent described was more successful for the saturated derivative with no apparent losses occurring, however for the $C_{20:4}$ ether derivatives hydrolysis was not quantitative. (See Fig. 1, step 4.)

Recovery of hydroxy eicosatetraenoic acids

Psoriatic scale samples (25 mg) spiked in duplicate with [1-^{14}C]12-HETE (23 ng, 58 mCi/mmol), [1-^{14}C]8-HETE (32 ng, 58 mCi/mmol), and [1-^{14}C]5-HETE (31 ng, 58 mCi/mmol) were processed through the assay system and analysed after HPLC (Fig. 1, step 3). One sample of each HETE derivative was subjected to scintillation counting, while the duplicate was hydrolysed with tetrabutylammonium fluoride (12-HETE and 8-HETE) or acetic acid reagents (5-HETE) (Fig. 1, step 4). These hydrolysates were then subjected to TLC and subsequent scintillation counting. Total percentage recovery of radioactivity for each compound was as follows: 12-HETE (49.1%, n = 2), 8-HETE (54.9%, n = 2), and 5-HETE (47.8%, n = 2).

Hydrolysis of 8-HETE and 12-HETE tBDMS derivatives was shown to be virtually quantitative by TLC, less than 0.5% of the silyl derivatives remaining. For the 5-HETE derivative only 58% of the recovered radioactivity was hydrolysed under acid conditions. In addition, hydrolysis was studied using GC—flame ionisation detection (FID). Amounts (approx. 2.5 μg each) of [1-^{14}C]HETE methyl ester tBDMS ether derivatives (40.6—87 μCi/mmol) were hydrolysed under both conditions described, before subjection to scintillation counting or reaction with BSTFA. Recovery of radioactivity was greater than 93% (average 97.8 ± 1.3%, n = 12, mean ± S.E.M.) for all HETEs when hydrolysed with tetrabutylammonium fluoride. GC—FID analysis after reaction of the hydrolytic products with BSTFA showed no tBDMS derivative remaining and indicated quantitative formation of the TMS ethers, except for that of the 5-HETE derivative. Under acid conditions however, hydrolysis of all the hydroxy fatty acid derivatives was shown to be incomplete.

On-column hydrogenation

For all compounds reduced, whether unsaturated fatty acid methyl esters or their hydroxylated TMS and tBDMS ether derivatives complete vapour phase reduction was possible during GC. No significant differences were detected between their mass spectra and those obtained by reduction prior to silylation and GC—MS. In addition vapour phase reduction could be carried out using both packed and capillary columns.

Introduction of the catalyst at the top of the column resulted in immediate reduction upon injection and hence chromatographic separation of the reduced components. No significant increase in retention times was detected whether reduction was carried out on-column or the previously reduced compounds were chromatographed. Thus reduction of the double bonds of the hydroxy fatty acid derivatives removed their effect on the retention characteristics.

By incorporation of the catalyst at the detector end of the column separation of the parent derivatives occurred before their reduction. This was advantageous in that all the chromatographic properties of the parent derivatives were conserved until MS itself. Hydrogenation of a mixture of compounds prior to GC may result in the reduction of more than one component to an identical compound or the formation of complex chromatograms. However, by using GC analysis with and without post-chromatographic reduction, both parent and reduced components can be easily matched from the chromatograms without complication.

The characteristics of vapour phase reduction

Using GC—MS it was possible to monitor simultaneously reduction efficiency, catalyst inactivation, hydrogenolysis, peak shape and absolute sensitivity. Several different configurations of catalyst position, chromatography column and MS interface were used (see Experimental). It was evident from these that the amount of hydrogen present in the catalytic chamber was always in large excess during chromatography. The catalyst, however, was susceptible to coating by chromatographic phase and subsequent inactivation at low palladium coverage (1%). With greater catalyst coverage (up to 10%) the chamber could be re-used many times without loss of catalytic efficiency, especially in combination with the capillary column.

Estimation of catalytic efficiency was carried out by monitoring the α-cleavage ion for 8-HETE methyl ester TMS ether at *m/z* values between *m/z* 265 (totally unreduced) and *m/z* 271 (totally reduced). Post-chromatographic reduction using a wide range of carrier flow-rates and catalyst concentrations resulted in almost total reduction (maximum signal at *m/z* 271) with a constant residual 3% signal remaining at *m/z* 269 (one double bond remaining intact).

Employment of palladium as catalyst kept hydrogenolysis of the derivatives to a minimum and mass spectra obtained after post-chromatographic reduction showed insignificant breakdown of the compounds. Similarly pre-chromatographic reduction led to the formation of single peaks without evidence of hydrocarbon products. In contrast, hydrogenation of the HETE methyl esters using PtO_2 in methanol gave 3—10% conversion to arachidic acid methyl ester. Where rhodium (5% coverage) was employed as the vapour phase catalyst, breakdown of the derivatives was considerable.

Gas chromatography—mass spectrometry with selected ion monitoring

Hydroxy fatty acids produced by photo-oxidation were subjected to HPLC as their methyl ester tBDMS ether derivatives. Subsequent GC—MS-selected ion monitoring (SIM) analysis of the $[M-(C_4H_9 + CH_3OH)]^+$ ion after pre-chromatographic reduction gave no distinction between the isomers, but yielded a trace which was the sum of the individual components (Fig. 7). For samples derived from psoriatic skin this information was similar to that obtained after HPLC, however greater sensitivity was obtained from the GC—MS—SIM analysis.

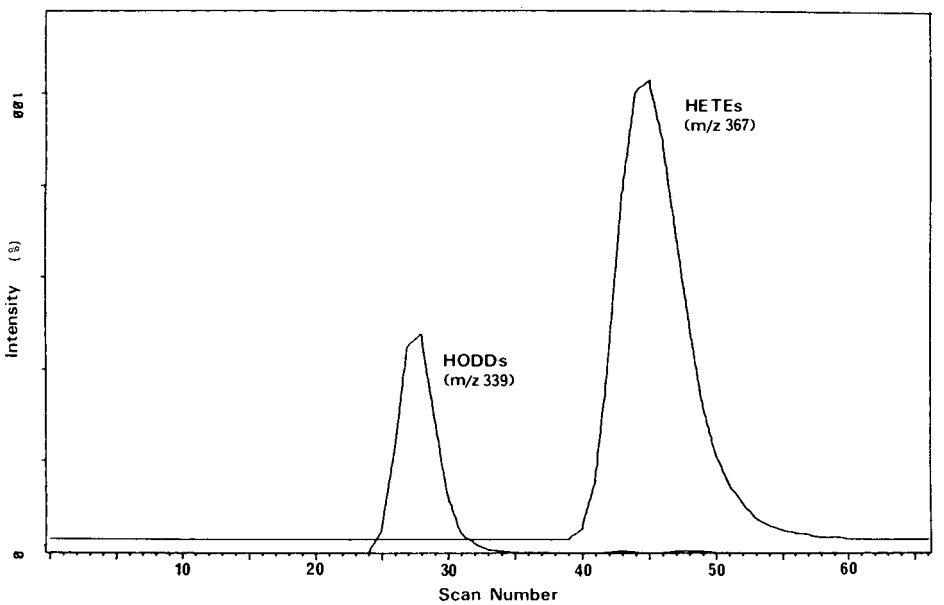

Fig. 7. Ion chromatogram of the $[M-(C_4H_9 + CH_3OH)]^+$ ion obtained for 20 ng of each component: 9- and 13-HODD (*m/z* 339) and 5-, 8-, 9-, 11-, 12-, 14- and 15-HETE (*m/z* 367) after pre-chromatographic reduction. GC was carried out on a packed column (1.5 m × 1.2 mm I.D.) containing 3% OV-1 on Gas-Chrom Q (100—120 mesh). Hydrogen was used as the carrier gas.

On the other hand, information on each of the components was obtained by GC—MS—SIM analysis with post-chromatographic reduction if the methyl ester TMS ether derivatives were used. Identification of the hydroxy fatty acids synthesised from a single precursor was possible simply by monitoring the *m/z* values corresponding to either α-cleavage fragment ion for each derivative (Table I). Scale samples from ten patients with psoriasis were then processed and analysed in the same manner. GC—MS—SIM analysis of the HPLC fraction containing the HODD and HETE compounds is illustrated in Fig. 8. Ion traces for up to ten derivatives could be obtained from a single injection and in almost every case noise levels were very low and chromatograms were free of interfering peaks.

Semi-quantitative data obtained from this analysis were similar to that obtained from an earlier study [6]. 13-HODD was present at highest concentrations (13.8 ± 5.3 ng/mg scale, n = 10; mean ± S.E.M.) with lower 9-HODD

TABLE I

EQUIVALENT CHAIN LENGTHS OF THE METHYL ESTER TMS ETHER DERIVATIVES OF HYDROXY FATTY ACIDS

Hydroxy fatty acid	Equivalent chain lengths		Ions monitored (m/z)
	OV-1 packed	OV-17 capillary	
12(OH) C20 Δ5,8,10,14	21.3	21.8	301, 215
14(OH) C20 Δ5,8,11,15	21.2	21.4	329, 187
15(OH) C20 Δ5,8,11,13	21.3	21.8	343, 173
11(OH) C20 Δ5,8,12,14	21.3	21.8	287, 229
9(OH) C20 Δ5,7,11,14	21.3	21.8	259, 257
8(OH) C20 Δ5,9,11,14	21.3	21.8	271, 245
5(OH) C20 Δ6,8,11,14	21.4	21.8	313, 203
12(OH) C20 Δ8,10,14	21.5	21.9	301, 215
14(OH) C20 Δ8,11,15	21.3	21.5	329, 187
15(OH) C20 Δ8,11,13	21.5	21.9	343, 173
11(OH) C20 Δ8,12,14	21.6	21.9	287, 229
8(OH) C20 Δ9,11,14	21.6	21.9	271, 245
12(OH) C20 Δ10,14,17	21.6	21.8	301, 215
15(OH) C20 Δ11,13,17	21.8	22.3	343, 173
14(OH) C20 Δ11,15,17	21.8	22.2	329, 187
17(OH) C20 Δ11,14,18	21.8	22.0	371, 145
18(OH) C20 Δ11,14,16	22.2	22.6	385, 131
11(OH) C20 Δ12,14,17	21.9	22.2	287, 229
12(OH) C20 Δ10,14	21.7	21.7	301, 215
14(OH) C20 Δ11,15	21.7	21.7	329, 187
15(OH) C20 Δ11,13	21.9	22.0	343, 173
11(OH) C20 Δ12,14	21.9	22.0	287, 229
12(OH) C18 Δ9,13	19.7	19.7	301, 187
13(OH) C18 Δ9,11	19.9	20.0	315, 173
9(OH) C18 Δ10,12	19.9	20.0	259, 229
12(OH) C18 Δ9	19.8	—	301, 187
12(OH) C18 —	20.1	—	301, 187

concentrations (5.5 ± 1.8 ng/mg scale, n = 10; mean ± S.E.M.). 12-HETE was the most abundant metabolite of arachidonic acid (0.73 ± 0.3 ng/mg scale, n = 10; mean ± S.E.M.) and the rank order of concentration was as follows: 12-HETE > 8-HETE > 15-HETE ≈ 9-HETE > 11-HETE.

Quantification

Stable isotope labelled compounds have frequently been used as internal standards in the quantification of the metabolites of arachidonic acid. Deuterium-labelled HETE analogues are conveniently synthesised by photo-oxidation of [5,6,8,9,11,12,14,15-octadeutero]-arachidonic acid [15];

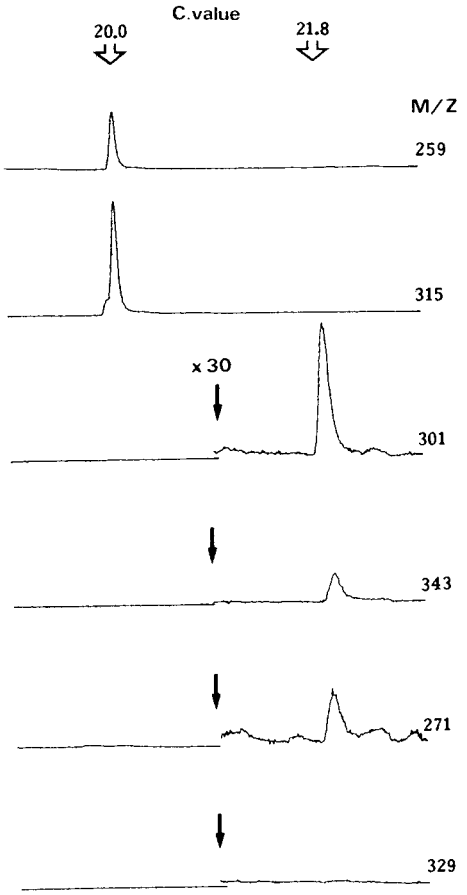

Fig. 8. GC—MS—SIM analysis of the methyl ester TMS ether derivatives of the HODD [$C_{18:2}$ (OH)] and HETE [$C_{20:4}$ (OH)] HPLC fraction (Fig. 4), obtained from 25 mg of psoriatic scale. Post-chromatographic reduction was carried out using a capillary column in configuration 1 (Experimental) with hydrogen as carrier gas. Equivalent chain lengths (C value) of peaks are indicated and ions monitored correspond to one α-cleavage fragment for each derivative: *m/z* 259, 9-HODD; *m/z* 315, 13-HODD; *m/z* 301, 12-HETE; *m/z* 343, 15-HETE; *m/z* 271, 8-HETE; *m/z* 329, 14-HETE (absent). Data were obtained from a single injection.

however where catalytic reduction is employed they are not suitable owing to exchange of the deuterium atoms during hydrogenation of the double bonds.

Given that the hydroxy fatty acid derivatives were purified by HPLC in a batchwise fashion it was desirable to choose a single internal standard for each fraction collected. For the HETE and HODD fraction, the choice of 14-HETE as a suitable internal standard was made after the scrutiny of scale samples from the ten patients studied. In none of these samples was 14-HETE detected and it is therefore unlikely to be produced in lesional skin (Fig. 8). Further the lack of a conjugated diene and thus the absence of the UV chromophore (λ_{max} 236) monitored during HPLC enabled all quantitative information from

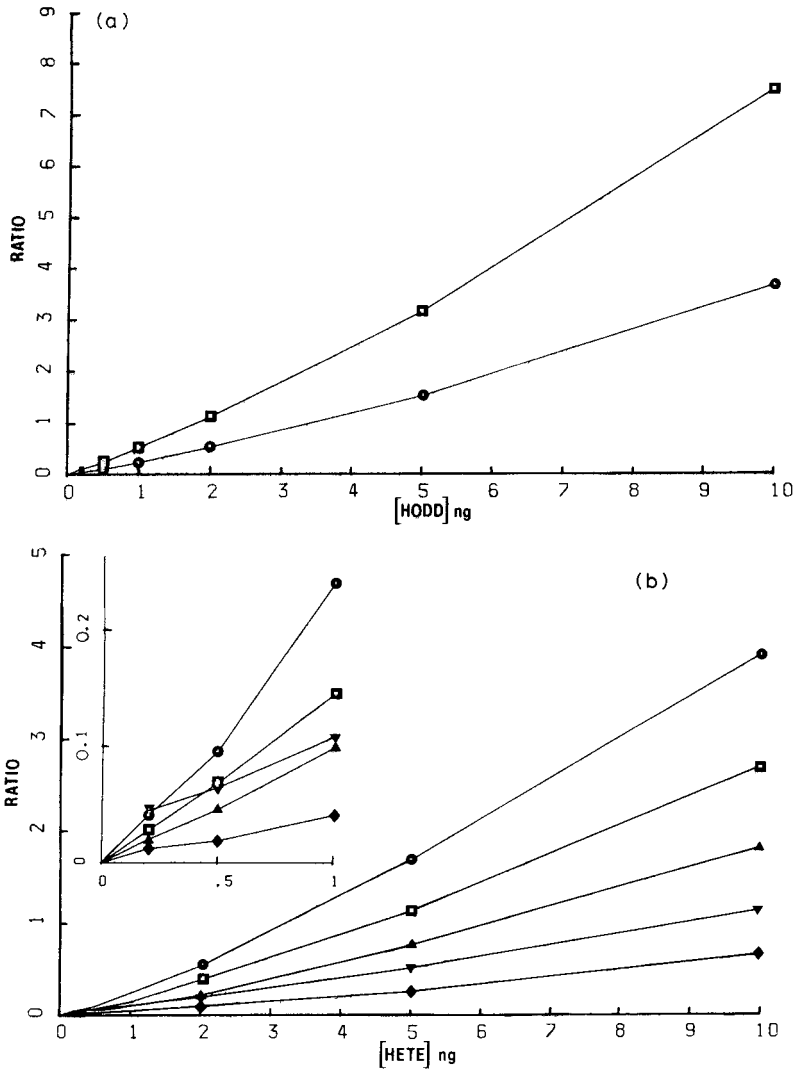

Fig. 9. GC—MS—SIM calibration curves obtained using post-chromatographic reduction (Fig. 2, configuration 2). Curves were obtained from duplicate solutions containing the methyl ester TMS ether derivatives of 14-HETE (10 ng) per injection (3 μl) against (a) 9- (■) and 13-HODD (●), 0.2—10 ng and (b) 5- (◆), 8- and 12- (▲), 9- (●), 11- (■) and 15-HETE (▼), 0.2—10 ng. The ions were monitored after a single injection of each solution as indicated in Table I. A trace amount of 15-HETE in the internal standard accounts for the interception of the curve for 15-HETE on the *y* axis.

this step to be retained. We therefore constructed calibration curves relating responses to varying amounts of each HODD and HETE derivative with responses to a constant amount of 14-HETE derivative (Fig. 9a and b).

Selectivity

The selectivity of the assay is improved by HPLC purification of the metabolites after derivatisation, as this procedure tends to select only those com-

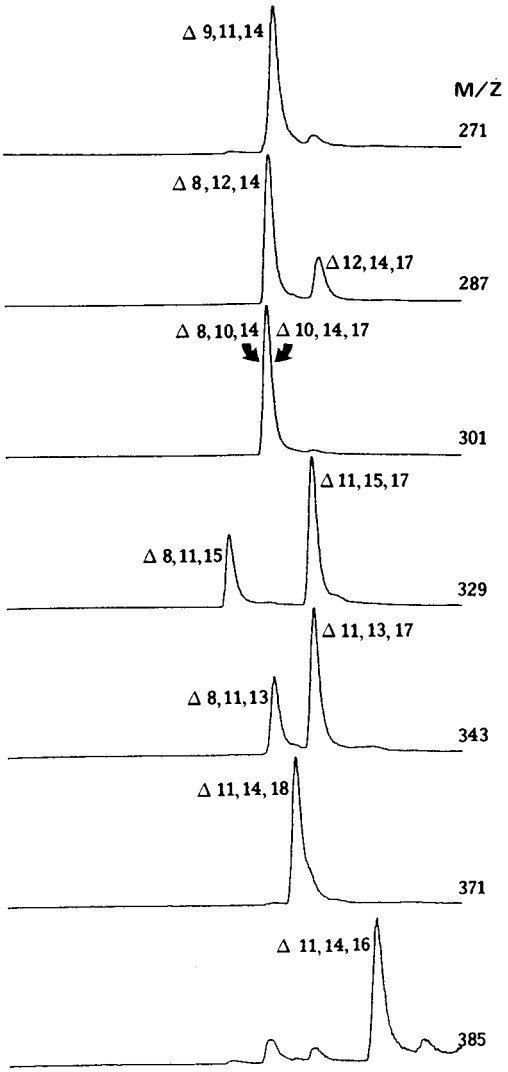

Fig. 10. GC—MS—SIM of the double bond isomers of $C_{20:3}$ hydroxy fatty acid, methyl ester TMS ether derivatives (single injection). GC with post-chromatographic reduction was carried out in configuration 1 (Experimental). The ions monitored correspond to the α-cleavage fragments of compounds in Table I and the labelling of the peaks indicates the positions of the double bonds.

pounds able to form methyl esters and tBDMS ethers. While the HPLC and GC—MS—SIM steps are complimentary in differentiating between degree of unsaturation and positional hydroxyl isomers respectively, the discrimination between double bond isomers hydroxylated in an identical position is dependent on their separation by GC and therefore on the equivalent chain length of their derivatives (Table I). Pre-chromatographic reduction therefore, would not distinguish between them.

Post-chromatographic reduction of those hydroxy fatty acid derivatives

resulting from photo-oxidation of 8,11,14- and 11,14,17-eicosatrienoic acids, as described (Experimental), is illustrated (Fig. 10). In a single injection all the ether derivatives substituted in identical positions were separated by GC with one exception. In this instance, separation was not achieved between the 12-hydroxy-8,10,14- and 12-hydroxy-10,14,17-eicosatrienoic acid derivatives.

Limits of detection

For any given recovery during sample preparation the minimum possible amount of analyte monitored will depend on the signal-to-noise (S/N) ratio for the final detection. As nine or ten ions were monitored in any one injection the signal was attenuated to approximately 1/3 of that value obtained had only one

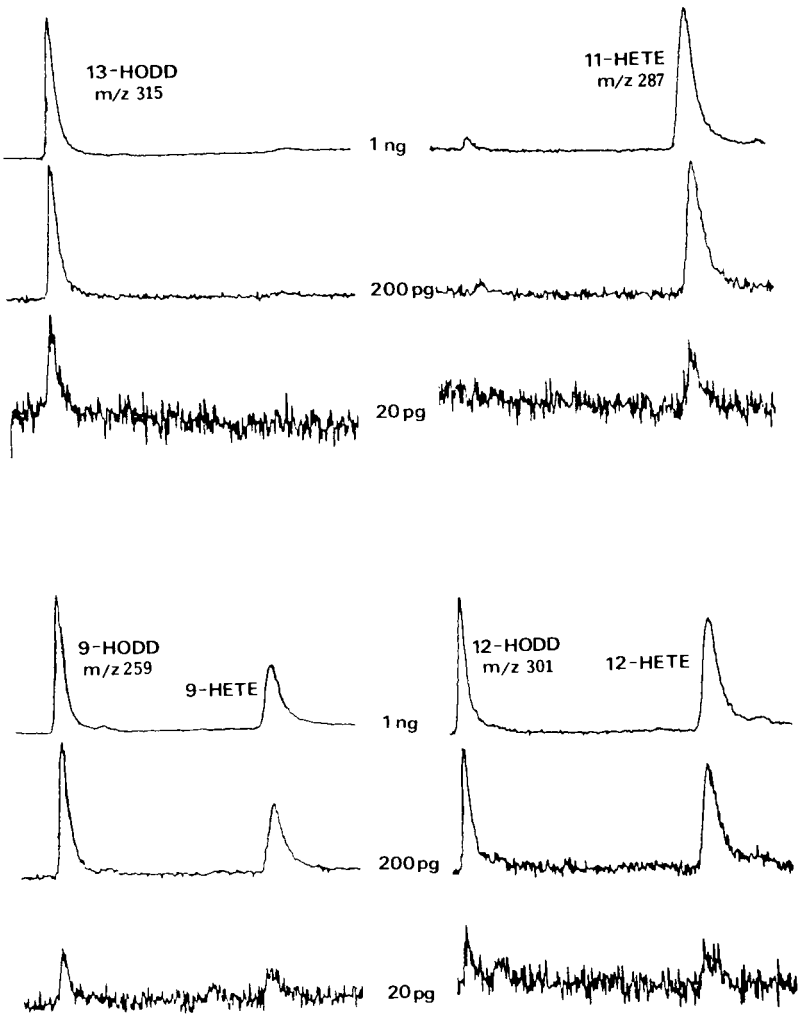

Fig. 11. GC—MS—SIM analysis of the methyl ester TMS ether derivatives of the compounds indicated. GC with post-chromatographic reduction was carried out as in Fig. 2 (configuration 2).

ion been monitored. However, all the compounds examined were detectable at amounts below 200 pg per injection while S/N ratios of approximately 3:1 were obtained for injection of 20 pg of 9- and 13-HODDs and 9-, 11- and 12-HETEs (Fig. 11).

CONCLUSIONS

The ability of the assay to measure the in vivo concentrations of a wide range of metabolites may provide a means of assessing the specificity and efficacy of potential lipoxygenase inhibitors. The predictability inherent in the HPLC purification of the methyl ester tBDMS ether derivatives facilitates screening for particular monohydroxy metabolites or lipoxygenase pathways. In addition, the batchwise collection of groups of derivatives allows the analysis, from a single biological sample, of both the precursor fatty acids and dihydroxy metabolites, such as leukotriene B_4.

This pathway however, presents special problems as demonstrated by the anomolous behaviour of the 5-HETE derivative. The application of this method, in our hands, to the measurement of this compound awaits the successful hydrolysis of the tBDMS ether group. The 5-lipoxygenase pathway is particularly important due to the potent leukocyte stimulating properties of leukotriene B_4 [16], which is derived from 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid, and its association with the disease, psoriasis [17].

Vapour-phase reactions in combination with GC have been exploited in the past as a means of identification. For quantification, this technique may find uses in the analysis of an increasing number of unsaturated compounds, especially where hydrogenation simplifies the mass spectra yielding useful structural information or intense ions suitable for selected ion monitoring detection. In addition on-column catalytic reactions are not limited to hydrogenation, as demonstrated by the vapour-phase oxidation of alcohols to aldehydes [18].

The identity and concentrations of hydroxy fatty acids present in psoriatic skin are at present under investigation. Whereas it has been shown that some monohydroxy eicosatetraenoic acids have chemokinetic and chemotactic properties, it will be of interest to evaluate the biological activity of other monohydroxylated metabolites.

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CHROMATOGRAPHIC PROFILING OF URINARY VOLATILE AND ORGANIC ACID METABOLITES OF NORMAL AND DIABETIC C57BL/Ks MICE

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SUMMARY

Capillary gas chromatography and gas chromatography—mass spectrometry were used to examine the urinary volatile and organic acid metabolites in normal and diabetic C57BL/Ks male mice. Quantitative differences in the excretion of these metabolites were assessed in the animals from 5 to 24 weeks of age. Statistically significant differences were examined with respect to known metabolic abnormalities in the diabetic animals and to the possible toxic effects of elevated levels of certain metabolites. A number of aldehyde metabolites and aromatic acids, as well as most other organic acids, were found at consistently higher levels in diabetic urine. Several ketone metabolites, linalool, and 2-*sec.*-butylthiazoline were found at consistently low levels throughout the study.

INTRODUCTION

Considerable research has been conducted concerning the chromatographic profiling of volatile and acidic metabolites in urine with association to diabetes mellitus [1—9]. Various aspects of the relationship between the production of these metabolites and diabetes have been investigated in humans and in suitable animal models. Such research has explored differences in the excretion of urinary acid and volatile metabolites [1—3], including alcohols [4], the application of computerized pattern recognition algorithms in distinguishing normal and diabetic volatile urinary profiles [5], as well as the effect of the induction of alloxan and streptozotocin diabetes in the rat on volatile urinary metabolites [6, 7]. In addition, the possibility of a link between the excretion of certain ketones and diabetic polyneuropathy [8] has also been examined.

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In certain cases, the observed differences in the excretion of organic acid or volatile metabolites have been associated with specific alterations in metabolic pathways due to the diabetic condition. For example, the increased ω-oxidation of long-chain fatty acids [10] and the enhanced degradation of branched-chain amino acids [11] observed in ketoacidotic subjects are reflected by higher urinary levels of several dicarboxylic acids and additional acids, such as 3-hydroxyisovaleric and 3-hydroxyisobutyric acids. In many other cases, however, the metabolic origins of the acidic and volatile urinary metabolites remain obscure.

Attempts to elucidate the more subtle differences in the composition of normal and diabetic urine and to better define the relationships between the excretion of the volatile and acidic metabolites and diabetes in humans are hampered by the considerable inter-individual variability encountered with human subjects and the influence of such factors as diet, medication, physical state, etc., which are often not easily controlled. The potential importance of uncovering these subtle differences is underscored by recent reports suggesting a connection between the excretion of certain ketones and diabetic neuropathy, as well as indications that certain metabolic processes, such as lipid peroxidation, which are increased in diabetics may yield toxic metabolites [8, 12]. Thus, the use of animal models of diabetes mellitus, such as the db/db mouse and alloxan or streptozotocin rats, provides the opportunity for greater control over experimental variables, allowing a greater potential for assessing slight differences in the excretion of urinary metabolites.

The present study examines differences in the excretion of acidic and volatile urinary metabolites in normal (m^+/m^+ or db/m^+) and diabetic (db/db) mice of the C57BL/Ks strain. The diabetes in this strain is caused by an autosomal recessive gene with full penetrance in the homozygote [13] and is characterized by abnormal early obesity followed by hyperglycemia, polyuria and glycosuria [13, 14]. Chromatographic profiling of the urinary metabolites of the mice began at 5 weeks of age and continued until the mice were 24 weeks of age. The study was designed to examine consistent differences in the urinary profiles of the diabetic and normal animals with the twin objective of (a) relating the observed differences to known metabolic abnormalities in the diabetic animals; and (b) examining metabolites which appear at significantly higher levels in the diabetic animals with regard to the possible toxicity of such compounds.

EXPERIMENTAL

Sample collection

Ten normal (m^+/m^+ or db/m^+) and ten diabetic (db/db) male mice of the C57BL/Ks strain were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) at weanling age (4 weeks). The mice were housed in smooth-bottomed plastic cages (two animals) with adequate bedding materials and were allowed free access to food and water.

Urine samples (24-h) were collected from the animals weekly from 5 to 10 weeks of age and biweekly from 10 to 24 weeks of age. During urine collection, the mice were housed in standard metabolism cages and the urine was collected

over dry ice. Urine columns and animal weights were recorded at the end of each collection period; blood glucose values were also monitored. Following urine collection, the samples were quickly thawed, filtered, diluted to a standard volume, divided into suitable aliquots, and refrozen until analysis. No preservatives were used.

Analysis of volatile metabolites

Analysis of the volatile constituents of the diabetic and normal urine samples was accomplished using a headspace concentration method followed by capillary gas chromatography (GC) as previously described [15]. In this method, volatile compounds are purged from the heated urine headspace (100°C) with high-purity helium and absorbed onto the porous polymer Tenax-GC (2,6-diphenyl-*p*-phenylene oxide polymer; Applied Science Labs., State College, PA, U.S.A.). The porous polymer was contained in platinum micro-baskets which were subsequently encapsulated for injection [15].

Separation of the volatile compounds was performed on an automated capillary GC system [15]. Reproducibly prepared glass capillary columns (60 m × 0.25 mm I.D.) coated with 0.2% UCON-50-HB2000 (Applied Science Labs.), a polar stationary phase, containing 0.015% benzyltriphenylphosphonium chloride (BTPPC) as surfactant were employed for chromatographic analysis. A Perkin-Elmer 3920 gas chromatograph equipped with an automatic injector (Perkin-Elmer, AS41), a flame ionization detector, a mass-flow controller (Brooks, Model 5840) and a data acquisition system (PEP-2, Perkin-Elmer) was utilized for chromatographic analysis. All samples were run at a flow-rate of 2 ml/min and the columns were temperature-programmed from 28 to 160°C.

Analysis of organic acid metabolites

Chromatographic profiles of the urinary organic acids were obtained using a two-step solvent extraction method and two derivatization techniques. In this procedure a 2-ml aliquot of urine is first made basic (pH 10) with sodium hydroxide (6 *M*) and 200 μl of a 20 mg/ml solution of methoxylamine hydrochloride (Applied Science Labs.) are added, as well as 10 μl of the 2 mg/ml solution of *p*-chlorobenzoic acid used as internal standard. The sample is then heated to 60°C for 45 min to allow formation of the methoxime derivatives of the urinary ketoacids. Following this derivatization, the basic sample is extracted once with a 4-ml portion of ethyl acetate. The organic layer is discarded and the aqueous sample is saturated with sodium chloride and acidified with 6 *M* hydrochloric acid. The urinary acids are extracted into an organic solvent using two 4-ml extractions with ethyl acetate, followed by one extraction using 4 ml of diethyl ether. Th organic extracts are combined and evaporated to dryness using a gentle nitrogen stream. Trimethylsilyl derivatives are formed by adding 50—200 μl of bis-trimethylsilyltrifluoroacetamide (BSTFA, Pierce, Rockford, IL, U.S.A.) and heating in tightly sealed vials for 45 min at 60°C. Following derivatization, 0.1—0.2 μl aliquots of each sample are analyzed by capillary GC.

A Varian Model 1400 gas chromatograph, modified for use with capillary columns and employing flame ionization detection was used in the analysis of

the derivatized acids. Peak area and retention time data were provided by a Sigma 10 (Perkin-Elmer) chromatographic data acquisition system. Separation of the sample constituents was accomplished using a glass capillary column (40 m × 0.25 mm I.D.) coated with SE-30 (Applied Science Labs.), a non-polar methylsilicone stationary phase. Following an initial 2-min isothermal period at 50°C, temperature programming was employed from 50 to 250°C at 4°C/min.

Identification of profile constituents

Structural identification of urinary volatile and acidic metabolites was accomplished using capillary GC—mass spectrometry (MS). Low-resolution electron impact ionization spectra were obtained at 70 eV with a Hewlett-Packard Model 5982 combined gas chromatograph—dodecapole mass spectrometer—computer system. The glass capillary column (SE-30- or UCON-coated) was directly interfaced with the mass spectrometer ion source (maintained at 220°C). All spectra were run at a scan-rate of 100 a.m.u./sec using the same chromatographic conditions which were employed in the analysis of the volatile or acidic fractions of urine. Whenever possible, proposed structures were verified through comparison of mass spectra and retention times of authentic compounds.

Data analysis

Quantitative comparisons of diabetic and normal urinary metabolites were carried out using peak area values obtained with the computerized data acquisition system. The average peak area of each measured peak in the diabetic profiles was calculated as a percent of the corresponding normal value plus or minus the standard error. Statistically significant differences in the excretion of any metabolites were assessed using the Student's *t*-test.

RESULTS AND DISCUSSION

Upon arrival at 4 weeks of age, the diabetic (db/db) and heterozygote (db/m^+) normal mice were barely distinguishable on the basis of size and weight. By 7 weeks of age, the average diabetic weight was 7 g (ca. 30%) more than that of the normal average. The 24-h urine excretion for the diabetic animals averaged 15—20 ml at 7 weeks compared with 0.5—1.0 ml per 24 h for the normal mice. The diabetic animals reached their maximum weights between 12 and 16 weeks of age followed by a slow decline in body weight and in some cases, death. Four diabetic animals died prior to the end of the study; one at 16 weeks, two at 18 weeks and one at 24 weeks of age. Blood glucose values for the diabetic mice ranged from 586 to 772 mg per 100 ml of blood during the acute later stages of the syndrome. The normal values ranged from 106 to 143 mg per 100 ml of blood.

The use of the C57BL/Ks db/db mouse in this study has provided a valuable opportunity to evaluate alterations in the excretion of acidic and volatile urinary metabolites due to the development of a condition similar to the maturity-onset diabetes. Since many of the morphological and metabolic abnormalities of this mutant diabetic strain have been well characterized [14], it is of note that the alterations in physiological parameters (body weight, urine

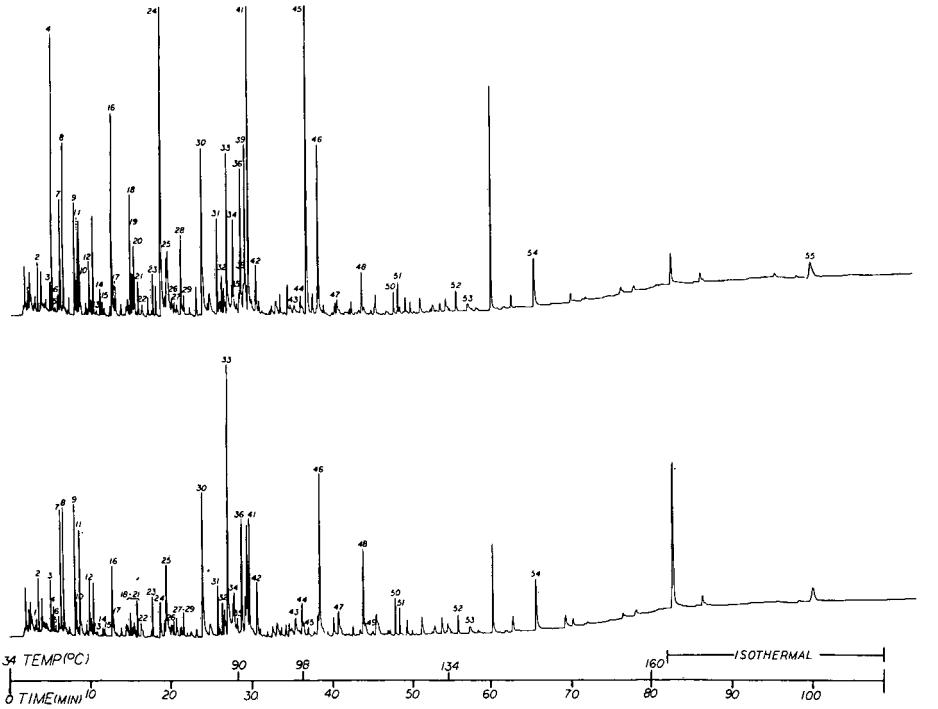

Fig. 1. Urinary volatile metabolic profiles of normal (top) and diabetic (bottom) mice at 5 weeks of age. Peak numbers correspond to the metabolite identifications of Table I.

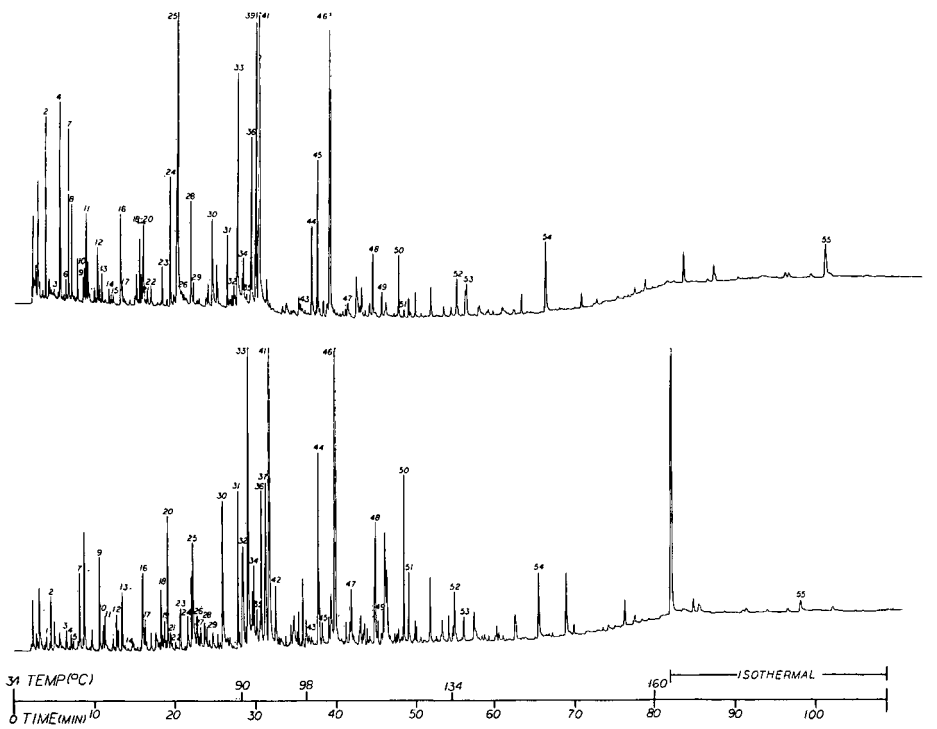

Fig. 2. Urinary volatile metabolite profiles of normal (top) and diabetic (bottom) mice at 10 weeks of age. Peak numbers correspond to the metabolite identifications of Table I.

TABLE I

SIGNIFICANT DIFFERENCES IN VOLATILE METABOLITE EXCRETION IN DIABETIC MICE AS PERCENTAGE OF MEAN AGE-MATCHED CONTROL VALUE

Values reported as percentage of mean age-matched control value ± standard error. Only significant ($p \leq 0.05$ Student's *t*-test) differences are reported. Peak numbers correspond to those of Figs. 1 and 2.

Peak No.	Structural identification	Age (weeks)								
		5	6	7	8	10	12	14	20	24
3	Pentanal			219 ± 96	327 ± 117					
4	Dehydration product I from 6-hydroxy-6-methyl-3-heptanone	16 ± 36		14 ± 60	34 ± 54			20 ± 70		
9	Hexanal		164 ± 49	282 ± 100		230 ± 31		270 ± 67	277 ± 130	293 ± 93
11	Dehydration product II from 6-hydroxy-6-methyl-3-heptanone	25 ± 39	32 ± 60	9 ± 25	8 ± 53	16 ± 23		12 ± 33		34 ± 43
13	2,4-Pentadienal*	238 ± 74	305 ± 171	345 ± 160	201 ± 59	284 ± 76	215 ± 80	160 ± 45	297 ± 84	368 ± 215
16	2-Heptanone	49 ± 16				175 ± 22	180 ± 67	77 ± 10	292 ± 64	173 ± 41
18	Styrene + *trans*-5-hepten-2-one	34 ± 24		39 ± 23	46 ± 37					
20	*trans*-4-Hepten-2-one	20 ± 36	14 ± 19	20 ± 8	23 ± 54	39 ± 35		14 ± 15		
28	6-Methyl-6-hepten-3-one	33 ± 32	33 ± 17	24 ± 51	28 ± 62	52 ± 40		46 ± 18		34 ± 50
30	Furfural					141 ± 21		176 ± 30		
33	Nonanal	248 ± 55	330 ± 95	160 ± 37	138 ± 19	134 ± 23	202 ± 39	215 ± 41	289 ± 67	242 ± 83
35	2-Octenal	42 ± 44			192 ± 51	163 ± 30	237 ± 100		190 ± 54	
38	N-Acetylpyrrole			748 ± 199	632 ± 163	582 ± 240	631 ± 238	898 ± 320	990 ± 510	468 ± 52
41	2-*sec.*-Butylthiazoline	25 ± 43	30 ± 29	35 ± 26	31 ± 56	31 ± 34	61 ± 23	36 ± 28	61 ± 30	53 ± 38
42	5-Methylfurfural*	201 ± 57	235 ± 93	231 ± 83	199 ± 63	350 ± 90	265 ± 79	264 ± 48	388 ± 130	276 ± 59
44	2-Nonenal	194 ± 37	197 ± 43	200 ± 54	231 ± 42	203 ± 37	216 ± 52	325 ± 82	408 ± 87	317 ± 68
45	Linalool	6 ± 14	17 ± 35	25 ± 10	21 ± 49	30 ± 32	29 ± 33	19 ± 48	25 ± 36	9 ± 48
46	Acetophenone					63 ± 32		39 ± 36		
48	Phenylacetone	167 ± 32	366 ± 100			159 ± 35	146 ± 38	158 ± 50	197 ± 62	205 ± 42
50	Unidentified	226 ± 77	343 ± 142	204 ± 84	257 ± 77	277 ± 105	264 ± 88	376 ± 170	363 ± 120	
51	Unidentified		177 ± 64	201 ± 73	191 ± 41	191 ± 52			206 ± 67	
53	Indole	430 ± 60	348 ± 155	231 ± 107	221 ± 69		296 ± 143			881 ± 199

Additional peaks exhibiting no significant differences

1	Acetone	19	6-Methyl-3-heptanone	34	2-Acetylfuran
2	Acrolein	21	2-(Methylthio)furan	36	Benzaldehyde
5	2-Methyl-3-buten-2-ol	22	2-Pentylfuran	37	4,6-Octadiene-3-one*
6	Toluene	23	3-Hepten-2-one	39	Unidentified
7	2-Ethyl-5-methylfuran*	24	2,5-Dimethylpyrazine	40	2-Methyl-5-vinylpyrazine*
8	2-Methylbutanenitrile*	25	2-Propylisoxazole*	43	3-Nonen-2-one
10	3-Penten-2-one	26	2-Heptenal	47	4-(Methylthio)-2-butenal
12	4-Heptanone	27	Octanal	49	2-Decenal*
14	3-Heptanone	29	6-Methyl-5-hepten-2-one	52	Benzothiazole
15	Dehydration product III from 6-hydroxy-6-methyl-3-heptanone	31	Dehydrobrevicomin	54	Unidentified
		32	3-Octen-2-one	55	Unidentified
17	Heptanal				

*Tentative identification.

volume, blood glucose values and morbidity) observed in the diabetic animals in this study are in agreement with previous reports [14, 15]. This consonance allows the correlation of observed urinary excretion patterns with an established sequence of metabolic events.

Volatile metabolites

The profiling results involving volatile metabolites reflect the physiological and metabolic differences between the normal and diabetic mice. The representative chromatograms shown in Figs. 1 and 2 visually demonstrate these differences in the two sets of animals at 5—10 weeks of age. This evaluation is confirmed by the data in Table I, which give the average peak area value of the components in the diabetic profiles as a percentage of the corresponding average peak area value in the normal profiles throughout the 24-week study. Only peaks which gave significantly different values ($p \leqslant 0.05$) in the diabetic and normal profiles were included in Table I.

Upon examination of the chromatograms in Figs. 1 and 2 and the data in Table I, it is immediately obvious that large quantitative differences exist in the urinary excretion of volatile metabolites in the normal (m^+/m^+ and db/m^+) and diabetic (db/db) mice, even as early as 5 weeks of age. At this stage in the syndrome, the db/db mice have blood glucose levels which are only slightly above normal, they are hyperinsulinemic, and display elevations in certain hepatic enzymes as well as abnormal fat deposition [13, 14]. These early abnormalities of the diabetic syndrome are reflected in the urinary excretion of volatile metabolites by the significantly higher levels of nonanal, 2-nonenal, 5-methylfurfural, indole, phenylacetone and the unidentified substance represented by peak number 50, as well as the dramatically lower levels of several branched-chain and/or unsaturated ketone metabolites (5-hepten-2-one, 3-hepten-2-one and 6-methyl-6-hepten-2-one) as well as linalool (peak 45) and 2-*sec.*-butylthiazoline (peak 41).

By the seventh week of the study, pentanal, hexanal and the tentatively identified 2,4-pentadienal and 2-octenal have risen to levels that are two to three times normal, while the levels of many ketone metabolites, linalool and 2-*sec.*-butylthiazoline remain low. Insulin levels are peaking at this age in the diabetic [14] and blood glucose levels are generally in the range of 300—400 mg per 100 ml blood [13]. Increased gluconeogenesis is occurring as evidenced by elevated activities of enzymes such as glucose-6-phosphatase, fructose-1,6-diphosphatase and others [14, 16].

Based on available information concerning the alterations in metabolism occurring in the diabetic mice and the results of similar profiling studies in alloxan and streptozotocin diabetic rats [6, 7], it is possible to relate these observed differences in the excretion of volatile metabolites with the metabolic alterations occurring in this early stage of the diabetic syndrome. One of the most striking differences observed between the early diabetic and normal urine volatile profiles is the elevation of several straight-chain aldehyde metabolites. Although the metabolic origin of these compounds has not definitely been established, the structures suggest that they arise through a pathway related to fat metabolism. More specifically, the abnormally high levels of such compounds found in urine may be a result of the increased lipid peroxidation

activity reported in diabetics [12, 17], since similar compounds are known to be metabolic products of this process [18].

In contrast to the observed elevation of the aldehyde metabolites in the early weeks of the diabetic syndrome, the diabetic volatile profiles display drastically lower levels of 5-hepten-2-one, 3-hepten-2-one and 6-methyl-6-hepten-2-one. Similar compounds have been observed in the urine of alloxan and streptozotocin diabetic rats [6, 7]. In long-term studies, these were found to be excreted in significantly lower amounts in the diabetic animals with depleted fat stores [7]. In this case, the production of such metabolites would appear to be related to a deficiency in fat breakdown. Their presence in significantly lower amounts in the urine of diabetic mice probably reflects the hyperinsulinemia and correspondingly reduced fat catabolism occurring in these animals in the early stages of the syndrome [14].

Another metabolite which was found at characteristically lower levels throughout the 24-week study is 2-*sec.*-butylthiazoline. The excretion of this compound has been reported to be sex-related and unique to the mouse [19]; the lower excretion observed in the diabetic males is probably related to the infertility and lack of sexual development in this model [13].

The period between 10 weeks and 16 weeks of age in the diabetic mice is characterized by a gradual decline of insulin levels to normal, coupled with a continued high rate of gluconeogenesis [14]. Many of the aldehyde metabolites were still excreted at elevated levels, while the ketones (with the exception of 2-heptanone) are found at lower-than-normal levels. Other metabolites, such as linalool, 2-*sec.*-butylthiazoline or N-acetylpyrrole were found at consistently lower or higher amounts than normal. The relationships between the altered excretion of linalool and N-acetylpyrrole and the diabetic state have not yet been firmly established, although certain pyrrole derivatives, including N-acetylpyrrole have been reported as increased in alloxan and streptozotocin diabetic rats [6, 7].

Around 16 weeks of age, the drop in insulin secretion in the diabetic mice is pronounced and the animals cease to gain weight. Increased gluconeogenesis and reduced glucose oxidation combine to yield continued severe hyperglycemia and the condition of the animals rapidly deteriorates. The urinary volatile profiles obtained during the last stages of the syndrome retain the characteristic elevation of several aldehydes, phenylacetone, peak 50, and indole. Correspondingly, the excretion of linalool, 2-*sec.*-butylthiazoline, 3-hepten-2-one and 6-methyl-6-hepten-2-one remains depressed. However, it is worthwhile to note that the excretion of some of the previously depressed branched-chain and/or unsaturated ketones (such as 5-hepten-2-one) appeared at near normal levels. This corresponds with the low insulin secretion and increased fat catabolism occurring in this stage [14].

In examining the consistent differences in the excretion of volatile metabolites in the normal and diabetic mice throughout the study, the most striking abnormalities were the increased excretion of a number of aldehyde metabolites, N-acetylpyrrole, 5-methylfurfural and indole in the diabetics. Equally dramatic were the initially lower levels of branched-chain and/or unsaturated ketones and the consistently low levels of linalool and 2-*sec.*-butylthiazoline found in the diabetic animals. The alterations in the excretion of these

compounds appear to be characteristic for the diabetic syndrome in this model.

The possible metabolic relationships between the excretion of most of these compounds and the diabetic syndrome have already been discussed, but it is important to consider possible physiological effects of increased levels of these compounds in the diabetic animals. Long-term diabetes in man is associated with many pathological complications including neuropathy, retinopathy, early development of vascular and circulatory difficulties and renal failure [20]. The metabolic abnormalities underlying these degenerative processes are generally poorly understood and considerable research is currently focussed in these areas. A recent report has suggested that patients with metabolic neuropathies such as diabetic polyneuropathy may produce abnormal neurotoxic metabolites which may be involved in the pathogenesis of the disorder [21]. Other reports suggest that increased lipid peroxidation may account for degenerative changes in the retina of diabetics [22] or contribute to other diabetic complications [12].

In this regard, the elevation of the aldehyde metabolites observed in the diabetic animals deserves further consideration. Such or structurally similar compounds, which have been reported to be products of lipid peroxidation [18], have also been reported as having cytotoxic effects as well as the ability to inhibit S-adenosylmethionine decarboxylase activity [23], and to alter the excitability and conduction properties of isolated nerves [24]. Further investigation is certainly warranted regarding the possible toxic effects of chronically elevated blood levels of such compounds, especially with reference to the later complications of diabetes.

Organic acid metabolites

Alterations in urinary organic acid excretion due to the diabetes in the mouse can be clearly observed in Figs. 3 and 4 (representative chromatograms). In Fig. 3 which presents chromatographic profiles of the urinary organic acids of a normal and diabetic mouse at 5 weeks of age, clear differences in the excretion of these metabolites are already apparent, although the blood glucose values for the diabetics are only slightly above normal. By 10 weeks of age (Fig. 4), the excretion of organic acid metabolites has increased tremendously in the diabetics; insulin levels are elevated and severe hyperglycemia is present. This dramatic increase in the excretion of organic acids continues until at least 24 weeks of age, as can be ascertained by examining the contents of Table II. The data in Table II are presented in the same manner as those of Table I; the average peak area for each of the numbered peaks in the diabetic profiles is given as the percent of the corresponding mean peak area from the normal profiles.

No significant reduction in the excretion of acid metabolites was observed in the diabetic animals. Instead, a general increase in the excretion of most acid metabolites was observed by 6 weeks of age. There are differences in the degree of elevated excretion observed; in some cases, such as with glyoxylic acid, the acid is excreted in amounts two to three times those of the normal animals. Other acids, such as 3-hydroxypropionic acid are found at levels 50—100 times higher than normal throughout the study.

At 5 weeks of age, significantly higher levels of several intermediates of

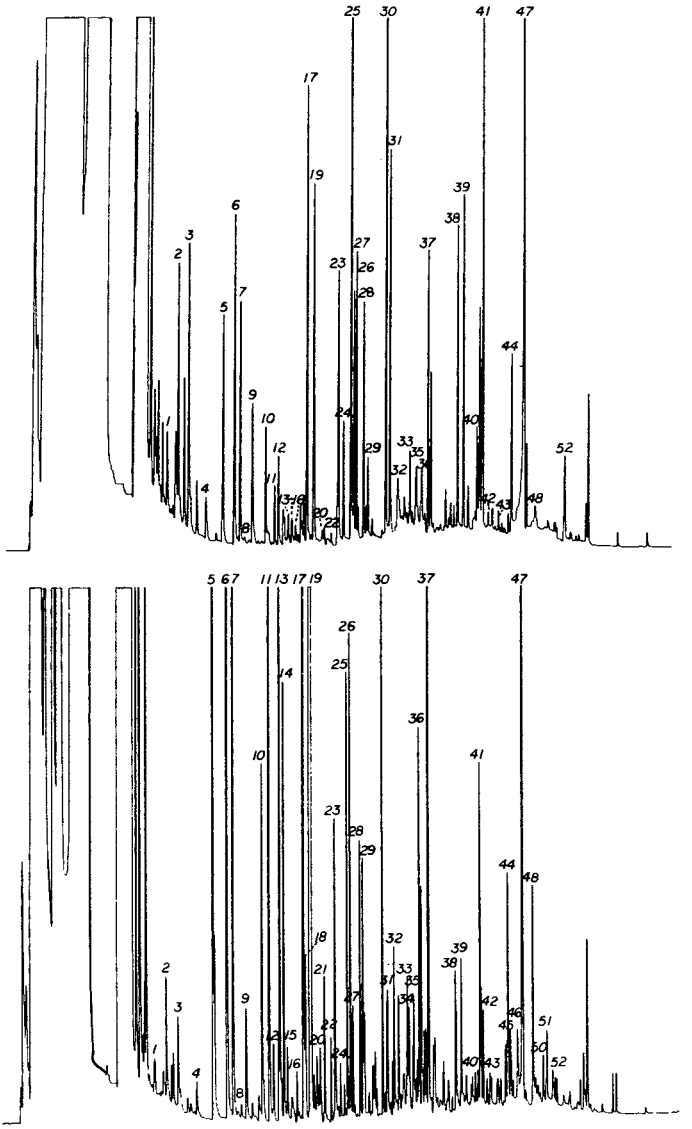

Fig. 3. Urinary organic acid profiles of normal (top) and diabetic (bottom) mice at 5 weeks of age. Peak numbers correspond to the identified metabolites of Table II.

glucose metabolism were observed; lactate, pyruvate, glyceraldehyde, dihydroxyacetone, 3-hydroxypropionate and glycerate are all elevated to levels 10—60 times normal. As blood glucose continues to rise in the diabetics, these metabolites are excreted in continually increasing amounts and, with the exception of lactate, pyruvate and 3-hydroxypropionate, reach peak levels between 7 and 10 weeks of age. Lactate, pyruvate and 3-hydroxypropionate are found at progressively higher levels until 10 weeks of age, reflecting the persistently high rate of gluconeogenesis in the diabetics [14].

A trio of tentatively identified furanoic acids are found among the acids in

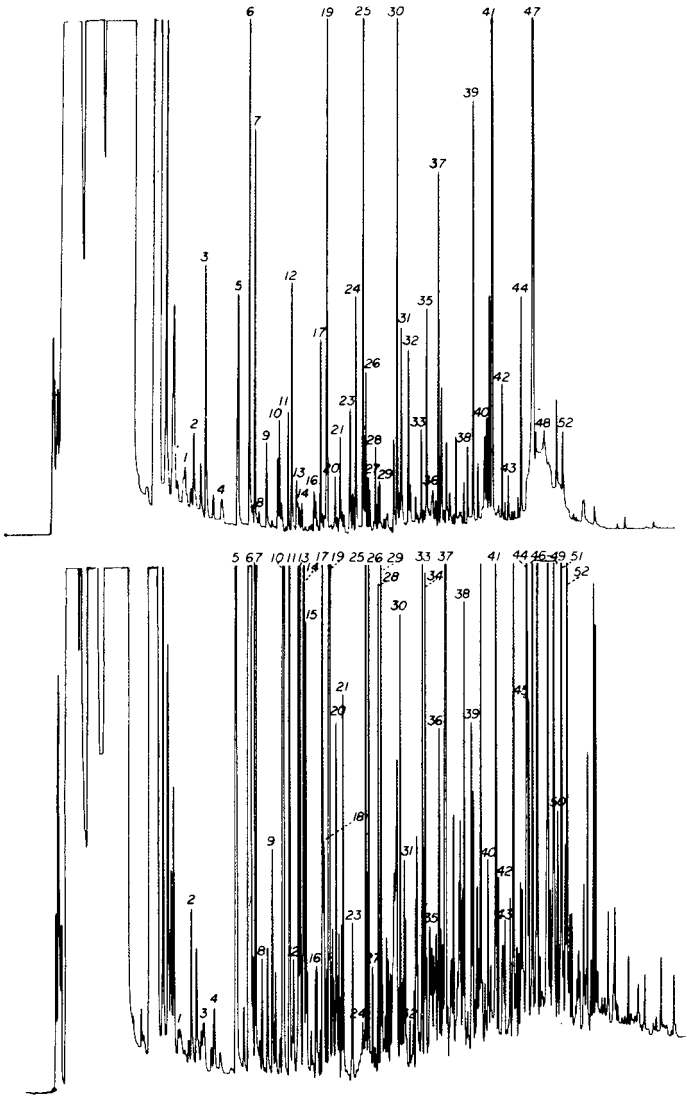

Fig. 4. Urinary organic acid profiles of normal (top) and diabetic (bottom) mice at 10 weeks of age. Peak numbers correspond to the identified metabolites of Table II.

urine (which are only slightly elevated in the diabetics at 5 weeks); however, these were later found in concentrations 40 times greater than normal. These derivatives probably arise from the metabolism of glucuronic acid [23] and ultimately from glucose. Their elevation roughly parallels the rising blood glucose levels in the diabetic animals as would be expected.

Several acids which are elevated during the middle stages of the diabetic syndrome (oxaloacetic, succinic, aconitic, 2-hydroxyglutaric acid), or throughout the entire study (malic, 2-ketoglutaric acid) are intermediates in the tricarboxylic acid cycle. Since the early and middle stages of the diabetic state are associated with increases in the activities of insulin-dependent enzymes such as

TABLE II

SIGNIFICANT DIFFERENCES IN THE URINARY EXCRETION OF ORGANIC ACIDS IN DIABETIC MICE RELATIVE TO AGE-MATCHED CONTROLS

Values presented with diabetic as percentage of mean age-matched control value ± the standard error. Peak numbers correspond to those of Figs. 3 and 4.

Peak No.	Structural identification	Age (weeks)		
		5	6	7
2	Glyoxylate	250 ± 78		780 ± 570
4	Phenol	398 ± 66	205 ± 62	222 ± 83
5	Pyruvate	1740 ± 890	3130 ± 630	3930 ± 640
6	Lactate	921 ± 353	1810 ± 1160	1380 ± 220
7	Glycolate	705 ± 140	1300 ± 527	1950 ± 750
9	Levulinate*	289 ± 64	1820 ± 700	940 ± 570
10	2-Hydroxybutyrate	581 ± 250	2220 ± 410	1360 ± 380
11	3-Hydroxypropionate	6970 ± 2430	8600 ± 1990	8550 ± 1690
13	Unidentified	3320 ± 2200	5270 ± 710	3920 ± 1990
14	Unidentified		2428 ± 920	5790 ± 2600
17	Glyceraldehyde I	3090 ± 780	10312 ± 1700	7480 ± 1600
19	Glyceraldehyde II	5920 ± 1480	9893 ± 1790	8550 ± 2230
20	Unidentified	1290 ± 430	643 ± 140	2303 ± 1180
21	Dihydroxyacetone*	3210 ± 1650	3390 ± 1300	3460 ± 1350
23	Oxalacetate*	463 ± 116		496 ± 187
25	Succinate		186 ± 42	
26	3-Hydroxy-2-furoate*		1360 ± 840	4670 ± 2150
28	4-Hydroxy-2-furoate*	228 ± 98	1930 ± 890	727 ± 310
29	Glycerate	960 ± 470	2740 ± 880	4030 ± 1320
31	Unidentified	168 ± 48	930 ± 320	411 ± 103
32	3-Hydroxy-5-methyl-2-furoate*	270 ± 120	1021 ± 760	406 ± 160
33	2-Methylglycerate	410 ± 145	620 ± 63	575 ± 150
35	Methylcatechol	401 ± 91	981 ± 362	659 ± 300
36	Unidentified	1580 ± 407	3145 ± 1400	1521 ± 390
37	Malate	767 ± 450	3575 ± 380	1000 ± 200
38	2-Ketoglutarate	329 ± 190	2255 ± 1060	1320 ± 610
39	2-Hydroxyglutarate*			
40	*o*-Ethylhydroxybenzoate	211 ± 54	1050 ± 690	1250 ± 900
41	Atrolactate*			394 ± 95
43	3,5-Dihydroxy-2,4-heptadienoate*			733 ± 141
45	Catechol		2250 ± 390	2790 ± 1160
47	Aconitate			499 ± 261

Additional identified peaks exhibiting no significant differences.

1	Acetophenone	34	2,3-Dihydroxybutyrate*
3	2,3-Butanediol	42	2-Ketoglutarate
8	2-Ketobutyrate	44	3-Hydroxypentanedioate*
12	Pyruvate	46	Vanillate
15	3-Furoate*	48	3-Deoxyhexuronic acid derivative*
16	5-Methyl-2-furoate*	49	Pyrogallol
18	Benzoate	50	Hippurate
22	3-Hydroxy-2-hydroxyethylfuran	51	Protocatecheate
24	Glycerol	52	Citrate
27	2-Methylglycerol*		
30	*p*-Chlorobenzoate, internal standard		

*Tentative identification.

glucokinase, glucose-6-phosphate dehydrogenase, citrate lyase and acetyl-CoA synthetase and, hence, glucose oxidation [13, 25], it is not surprising to find abnormally high levels of the Krebs cycle intermediates in the urine. By 18 weeks of age, insulin levels have fallen, and lower levels of glycolytic and pentose-phosphate shunt enzymes are found. Correspondingly, normal urinary levels of oxaloacetate, succinate and 2-hydroxyglutarate were observed at this time.

8	10	12	16	20	24
232 ± 94	287 ± 160	245 ± 105	289 ± 61	330 ± 66	
345 ± 115	429 ± 176			259 ± 87	
3500 ± 1350	3860 ± 950	2790 ± 1000	2250 ± 730	5370 ± 580	1630 ± 440
3900 ± 1600	5280 ± 3160	1200 ± 500	7510 ± 3790	22100 ± 5300	5920 ± 2600
2450 ± 940	4270 ± 2080	1900 ± 420	4748 ± 2200	6150 ± 1050	1750 ± 670
1210 ± 440	741 ± 200	527 ± 146	286 ± 98	288 ± 70	
1770 ± 590	3520 ± 1400	1770 ± 660	5240 ± 1700	9340 ± 4600	1940 ± 1200
8540 ± 1500	8240 ± 3200	5360 ± 1640	4510 ± 1150	9100 ± 710	1280 ± 370
5460 ± 1700	2830 ± 1570	1030 ± 460	2640 ± 500	8490 ± 4210	
11200 ± 6000	22900 ± 9900	5060 ± 2200	11500 ± 3500	25300 ± 5200	
6240 ± 600	4430 ± 2200	3690 ± 2300	1940 ± 860	8820 ± 1600	386 ± 120
5340 ± 400	3530 ± 1800	3000 ± 2100	1420 ± 670	8560 ± 2200	1406 ± 650
3870 ± 2600	2920 ± 1970	464 ± 251	1960 ± 490	3522 ± 2000	
1620 ± 450	793 ± 315	613 ± 320	370 ± 115	1301 ± 240	209 ± 88
470 ± 170	534 ± 186	376 ± 150	288 ± 160		
240 ± 88	264 ± 75	473 ± 258	154 ± 45		
4310 ± 2500	2110 ± 660	1800 ± 670	4960 ± 3200	2530 ± 750	422 ± 180
745 ± 180	1390 ± 727	786 ± 360	815 ± 400	1340 ± 35	354 ± 160
4230 ± 870	4480 ± 1170	1520 ± 420	6810 ± 2200	6760 ± 820	690 ± 250
880 ± 230		490 ± 210	234 ± 107	240 ± 30	197 ± 82
1420 ± 700	237 ± 64	332 ± 105	660 ± 190	1800 ± 1000	
732 ± 250	967 ± 319	602 ± 150	1410 ± 680	1170 ± 200	895 ± 380
351 ± 146	219 ± 55	254 ± 93	185 ± 53	915 ± 35	
1610 ± 230	2480 ± 570	4280 ± 1800	2510 ± 400	4070 ± 620	653 ± 190
2530 ± 540	2700 ± 300	3300 ± 1400	2930 ± 870	3290 ± 310	829 ± 430
1410 ± 390	1350 ± 270	2100 ± 700	1150 ± 570	1060 ± 510	252 ± 100
420 ± 260	556 ± 250	478 ± 190			
1050 ± 760	414 ± 186	847 ± 199		840 ± 300	1144 ± 800
716 ± 150	503 ± 112	710 ± 193	358 ± 115	1030 ± 170	
1160 ± 390	325 ± 128	391 ± 104	2460 ± 1900	1680 ± 300	
1850 ± 430	878 ± 217	1130 ± 700	3780 ± 1000	5400 ± 1800	
353 ± 120		352 ± 186	291 ± 98	442 ± 125	

It should be noted that no ketone bodies or other acids derived specifically from increased fat oxidation were found to be elevated in the diabetic urine. This finding is in agreement with reports of decreased lipolytic acitivity and enhanced lipogenesis in the diabetic mice as a result of high insulin levels [16].

The remainder of the compounds which have been identified and which are elevated in diabetic urine are phenolic compounds (phenols, catechol, methylcatechol) or aromatic acids (O-ethylhydroxybenzoic, atrolactic acid). Addition-

ally, several similar compounds were found exclusively in the urine of diabetic mice, including vanillate, pyrogallol and protocatechuate. These compounds are probably intermediates in the catecholamine metabolism although it is not clear if plasma catecholamines are elevated in the diabetic mouse. Studies of norepinephrine levels in isolated organs innervated by sympathetic nerves revealed significantly reduced concentrations in the heart, kidney and salivary glands of older diabetic mice, but normal concentrations in the spleen and adrenal glands [26]. However, a massive elevation of monoamine oxidase, an enzyme that degrades norepinephrine to inactive products, was found in the kidneys of older diabetic mice [26]. It is possible that the elevated levels of degradation products of catecholamines observed in diabetic urine may be related to enhanced catecholamine catabolism. Alternatively, the inappropriately high glucagon levels found in the diabetic mice [14] may stimulate the metabolism of tyrosine and phenylalanine through the induction of tyrosine dehydroxylase [27], thus contributing to the appearance of phenolic metabolites in the urine.

In either case, a significant consideration in the observation of elevated urinary levels of phenolic metabolites is the possible consequence(s) of correspondingly elevated plasma levels of these toxic compounds. Recently, the elevation of several phenolic and polyphenolic acids has been reported in analyses of uremic hemofiltrate [28, 29]. These studies have commented on the general toxicity of such compounds and their possible involvement in the pathogenesis of such uremic symptoms as central nervous system dysfunction, anemia, and impaired blood coagulation. Curiously, a polyneuropathy virtually identical to diabetic polyneuropathy in its course develops in cases of severe uremia and alcoholism [30]. A toxic assault on the nerve has been implicated in the etiology of both neuropathies, although the toxin(s) responsible have yet to be identified. The further investigations of the elevation of phenolic acids in urine and in plasma of diabetic subjects would seem to be warranted in this regard.

Although further interpretation of the urinary organic acid data was hampered by difficulties in the unambiguous assignment of structures from the mass spectra of trimethylsilyl derivatives, the trends in the excretion of these compounds in the normal and diabetic mouse are fairly evident and will serve to direct future investigations.

CONCLUSIONS

As indicated in the introduction, the intent of this paper has been to go beyond the mere reporting of observed differences in the excretion of certain urinary metabolites among normal and diabetic mice. This investigation has also attempted to relate observed differences in the excretion of certain urinary metabolites to known metabolic abnormalities of the diabetic animals and to examine the data in terms of possible implications of elevated levels of some potentially toxic metabolites. In particular, the observed increased urinary levels of several aldehyde and phenolic metabolites warrant further investigation with regard to possibly elevated blood levels and the toxic effects of such compounds.

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SEMI-MICRO QUANTITATIVE ANALYSIS OF COMPLEX URINARY STEROID MIXTURES IN HEALTHY AND DISEASED STATES

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SUMMARY

A method for the determination of urinary steroid hormones in healthy and diseased states is described. The use of a Van den Berg all-glass injector coupled to a fused silica column has shown a dramatic increase in sensitivity and a significant reduction in gas chromatography analytical time. The increase in sensitivity also eliminates the need for processing large volumes of urine.

The method has proven to be rapid, precise, reproducible and sensitive. Also, column life is increased due to the absence of solvent. This technique has shown to have broad applications in the analysis of such classes of compounds as sugars, steroids, prostaglandins and fatty acids.

INTRODUCTION

Multicomponent analysis of urinary steroids relies on the techniques of gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS).

The early work of Sparagna et al. [1], on urinary steroid extracts showed it necessary, in some instances, to separate certain 17-ketosteroids prior to analysis by GC. They showed that dehydroepiandrosterone (DHA) and androsterone co-eluted on packed columns and quantitative data could only be obtained if these compounds were previously separated by some other chemical method. Alternatively, these compounds could be distinguished by analyzing mixtures collected in different fractions from a primary gas chromatograph and reinjecting selected fractions on a second GC column containing a different phase. Although the overall precision was good the analysis was time consuming and also required large volumes (approx. 100—200 ml) of urine to achieve the desired sensitivity.

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Following the introduction of open tubular columns, many workers [2—9] have demonstrated the ability to analyze urinary steroid extracts containing those compounds previously considered difficult to separate using conventional packed column GC. However, the GC analytical time was still considerable (e.g. 90—120 min) and the GC resolution was inadequate for the separation of some of the urinary steroids. Also, because of the low column capacity and the sensitivity of the column coating to the presence of large solvent loads, the majority of the sample had to be split and vented into the atmosphere (split injection). Therefore, in order to obtain comparable sensitivity, highly concentrated samples were required for analysis and this again involved processing large volumes (approx. 25—50 ml) of urine. Today there are a number of GC inlet systems currently in use with capillary GC columns. Of these split, mentioned above, and splitless have gained most popularity.

Splitless injection is strongly dependent on the "solvent effect" [10]. It requires that the initial column temperature be below the boiling point of the solvent utilized in the analysis. This is necessary to achieve a narrow band of the compounds to be analyzed at the head of the column; a prerequisite for good chromatographic resolution. A variation of splitless injection is the on-column method where the injection port is absent and the solvent and analyte are placed in a fine band at the head of the column in a cool oven.

Using split, splitless, and on-column injection methods; solvent traverses the column. During GC analysis this is represented by a solvent front at the beginning of the chromatogram. The presence of solvent in the GC column can cause premature degradation of column performance. Similarly, during GC—MS analysis, repeated exposure of the ion source of the mass spectrometer to solvent can lead to rapid defocusing of the ion beam caused by contamination of the lenses. To avoid this degradation of performance many investigators use complex valving systems to divert the solvent peak.

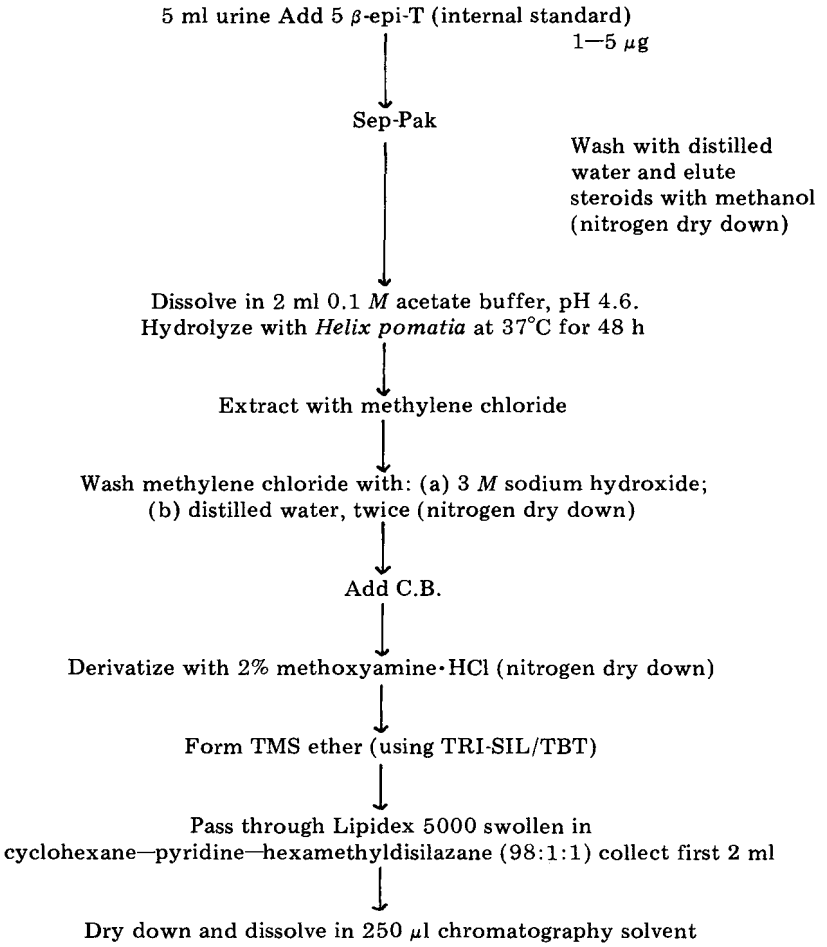
In our laboratory we have used a modified Van den Berg falling needle injector [11, 12]. The modifications include using swagelok connections and needle valves to control helium inlet and outlet gas flows. These allow precise and reproducible gas regulation. Finally, using glass capillary tubing with an inner diameter very close to the outer diameter of the column in the area situated in the injection port decreases dead space and improves GC resolution. This type of inlet system has overcome most of the problems associated with the other forms of inlet systems mentioned above. By eliminating the need to vent the solvent or split the sample there is a dramatic increase in sensitivity, since all the sample is deposited on to the column. It also eliminates the passage of solvent through the column. Because of these two fundamental parameters there is a profound decrease in GC analytical time. Moreover, by using this form of inlet system, relatively small volumes of urine, 1—5 ml, can be assayed with excellent sensitivity and precision.

This technique has shown to be of immense value for the analysis of small volumes of urine. It would also be useful where the compounds of interest are in low concentration or where the total sample volume is small.

EXPERIMENTAL

Twenty-four hour urines were collected and stored at −70°C until the day of analysis. All glassware used throughout the analytical procedure was acid washed and silanized.

Urine was allowed to completely thaw and aliquots of 1—5 ml removed. 5 μg per ml of urine of 5β-dihydro-epitestosterone (Steraloids) were added in ethanol to each as an internal standard for overall recovery. The aliquots were then passed through a Sep-Pak (Waters Assoc.) and a steroid-rich fraction eluted with 8 ml methanol [13]. Solvent was removed under a stream of nitrogen and the steroid conjugates hydrolyzed using the following method. The dried methanolic extracts were dissolved in 2 ml of 0.1 *M* acetate buffer, pH 4.6, followed by 100 μl of *Helix pomatia* digestive juice (Calbiochem-Behring). The mixture was incubated for 48 h at 37°C. Neutral steroids were extracted with 4 ml methylene chloride, this in turn was washed



Scheme 1.

with 1 ml of 3 *M* sodium hydroxide followed by 2 × 2 ml of distilled water. To the organic phase were added 5 μg cholesterol butyrate (CB) as an internal standard for GC. Solvent was removed under a stream of nitrogen and the meth(yl)oxime, trimethylsilyl (TMS) ether derivatives were prepared by the method of Thenot and Horning [14] as follows: 100 μl of 2% methoxyamine·HCl in pyridine were added to the dried extract and allowed to derivatize at 60°C overnight. The pyridine was removed under nitrogen and residual moisture removed in vacuo at 60°C. Trimethylsilyl ethers were prepared by reacting the extract with 100 μl Tri-Sil/TBT reagent [composed of N-(trimethylsilyl)-imidazole (TMSI)—N,O-bis(trimethylsilyl)acetamide (BSA)—trimethylchlorosilane (TMCS) (3:3:2, v/v)] (Applied Science Labs.) for 2 h at 100°C. Excess reagent was removed under a stream of nitrogen and the derivatized extracts suspended in Lipidex 5000 (Packard) slurry swollen in cyclohexane—pyridine—hexamethyldisilazane (98:1:1) [15]. The slurry was added to the top of a 2-cm column of Lipidex 5000 and eluted using the same chromatography solvent. The first 2 ml were collected and evaporated under a stream of nitrogen.

It was found necessary to dilute the derivatized extract equivalent to 5 ml of urine to 250 μl with the chromatography solvent and inject 1 μl.

Gas chromatography

The analysis of urinary steroid extracts was performed on a Hewlett-Packard 3710A gas chromatograph fitted with a modified Van den Berg falling needle injector. This was coupled to a 30 m × 0.25 mm, film thickness 0.25 μm, DB1 fused silica open tubular column (J & W Scientific, Cordova, CA, U.S.A.). The carrier gas was helium, velocity 20 cm/sec and the make-up gas nitrogen with a flow-rate of 20 ml/min. Hydrogen and air flow-rates were 30 ml/min and 300 ml/min, respectively. The injector and flame ionization detector temperatures were 300°C.

GC analysis of urinary steroid extracts was performed with an initial column temperature of 200°C for 4 min, then increased to a final temperature of 300°C at a rate of 4°C/min. The recorder chart speed was 1.25 cm/min.

Gas chromatography—mass spectrometry

Analyses were performed on a Hewlett-Packard 7620A gas chromatograph fitted with a modified Van den Berg injector. The GC column was a 30 m × 0.25 mm, film thickness 0.25 μm, DB1 fused silica column. The carrier gas was helium, velocity 40 cm/sec. Make-up gas was not required since the column was directly inserted into the ion source. The injection block and transfer line temperatures were 300°C.

This was interfaced to a Vacuum Generator (VG) MM-16 low resolution magnetic sector instrument. The multiplier was 1.75 kV, with a gain of $2 \cdot 10^{-6}$. The electron energy was 70 eV, source temperature 200°C and an accelerating voltage of 4 kV. The scan rate was 1 sec per decade. Results were output to a VG 2050 data system.

GC—MS analysis of urinary steroid extracts was performed using the same temperature conditions as stated previously.

RESULTS AND DISCUSSION

Fig. 1 is a typical GC—flame ionization detector trace of a synthetic steroid mixture containing most of those compounds of clinical importance. Each peak is equivalent to 20 ng of steroid. Fig. 2 is a total ion chromatogram of the same mixture obtained from the mass spectrometer data system. Comparison of the two traces indicates almost identical GC resolution. The dif-

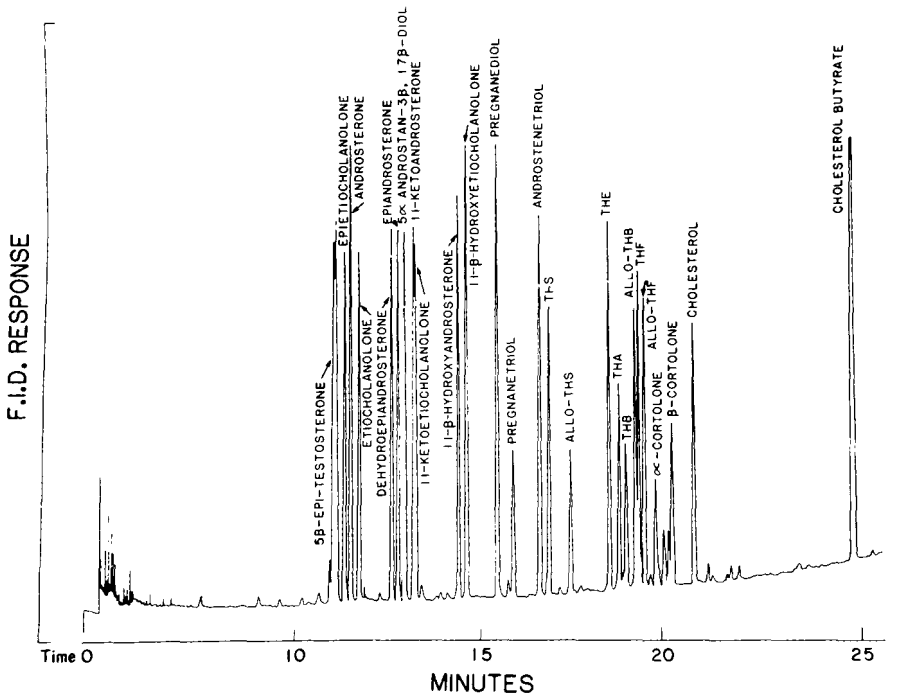


Fig. 1. Gas chromatogram of a synthetic steroid mixture as their meth(yl)oxime TMS ethers. Each peak is equivalent to 20 ng of authentic standard.

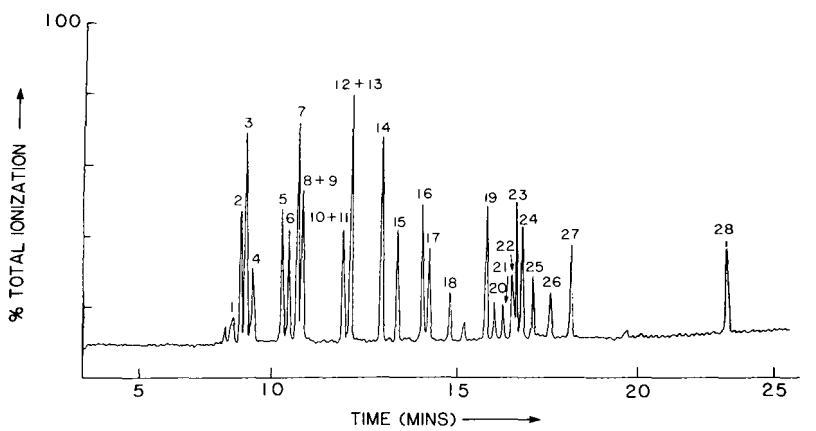


Fig. 2. Total ion current of a synthetic steroid mixture, as their meth(yl)oxime TMS ethers. Each peak is equivalent to 20 ng of authentic standard. See Table I for peak identification.

TABLE I

COMPOUNDS REPORTED IN FIGS. 1—5

Peak No.	Abbreviation	Trivial name	Systematic name	Methylene units
1	5β-dihydro-epi-T	5β-Dihydroepitestosterone	5β-Androstan-17α-ol-3-one	25.31 (25.34)*
2	Epi-E	Epietiocholanolone	5β-Androstan-3β-ol-17-one	25.53
3	A	Androsterone	5α-Androstan-3α-ol-17-one	25.60
4	E	Etiocholanolone	5β-Androstan-3α-ol-17-one	25.71
5	DHA	Dehydroepiandrosterone	Androst-5-en-3β-ol-17-one	26.30
6	Epi-A	Epiandrosterone	5α-Androstan-3β-ol-17-one	26.43
7	And-diol	Androstanediol	5α-Androstane-3β,17β-diol	26.51
8	11 Keto A	11-Ketoandrosterone	5α-Androstan-3α-ol-11,17-dione	26.69
9	11 Keto E	11-Ketoetiocholanolone	5β-Androstan-3α-ol-11,17-dione	26.69
10	17α-OH Preg	17α-Hydroxypregnanolone	5β-Pregnane-3α,17α-diol-20-one	27.35
11	11β-OH-A	11β-Hydroxyandrosterone	5α-Androstane-3α,11β-diol-17-one	27.35
12	Preg	Pregnanolone	5β-Pregnan-3α-ol-20-one	27.51
13	11β-OH-E	11β-hydroxyetiocholanolone	5β-Androstane-3α,11β-diol-17-one	27.51
14	P. diol	Pregnanediol	5β-Pregnane-3α,20α-diol	28.08
15	P. triol	Pregnanetriol	5β-Pregnane-3α,17α,20α-triol	28.31
16	A. triol	Androstenetriol	Androst-5-ene-3β,16α,17β-triol	28.78
17	THS	Tetrahydro substance S	5β-Pregnane-3α,17α,21-triol-20-one	28.89
18	Allo-THS	Allo-tetrahydro substance S	5α-Pregnane-3α,17α,21-triol-20-one	29.28
19	THE	Tetrahydro compound E	5β-Pregnane-3α,17α,21-triol-11,20-dione	29.95
20	THA	Tetrahydro compound A	5β-Pregnane-3α,21-diol-11,20-dione	30.14
21	THB	Tetrahydro compound B	5β-Pregnane-3α,11β,21-triol-20-one	30.27
22	Allo-THB	Allo-tetrahydro compound B	5α-Pregnane-3α,11β,21-triol-20-one	30.46
23	THF	Tetrahydro compound F	5β-Pregnane-3α,11β,17α,21-tetrol-20-one	30.47
24	Allo-THF	Allo-tetrahydro compound F	5α-Pregnane-3α,11β,17α,21-tetrol-20-one	30.56
25	α-Cortolone		5β-Pregnane-3α,17α,20α,21-tetrol-11-one	30.81
26	β-Cortolone		5β-Pregnane-3α,17α,20β,21-tetrol-11-one	31.11
27	Cholesterol		Cholest-5-en-3β-ol	31.56
28	CB	Cholesterol butyrate	Cholest-5-en-3β-ol *n*-butyrate	34.66

*Methylene unit value in parentheses indicates the *syn*- and *anti*-isomers.

ferences observed in their relative intensities are due to the independent means of detection. Table I lists these compounds using the trivial name, systematic name and methylene unit (MU), in order of elution from the GC column.

Quantitation was obtained by analyzing a synthetic steroid mixture of known concentration. The peak areas were integrated by a Hewlett-Packard 3385A system. A response factor (RF) was then computed for each steroid in the mixture relative to the internal standard 5β-dihydro-epitestosterone, using the formula

$$RF = \frac{(\text{Conc. U.K.}) \equiv (\text{Area STD})}{(\text{Conc. STD}) \equiv (\text{Area U.K.})}$$

where: Conc. U.K. = concentration of unknown peak or synthetic standard; Area U.K. = area of unknown peak or synthetic standard concentration; Conc. STD = concentration of internal standard (5β-dihydro-epitestosterone); Area STD = area of internal standard (5β-dihydro-epitestosterone).

These response factors were put into a BASIC program. Using the peak areas and the corresponding internal standard, concentrations were then computed for each steroid in our patient samples. Reproducibility and precision for both concentration and MU were determined on the GC system routinely employed. This was achieved by ten replicate analyses of a normal adult male 24-h urine. A summary of these results is shown in Tables II and III. Also included in Table II is a summary of percentage recoveries of some clinically important steroids. This was performed in model experiments using authentic standards. The values represent the recovery at the final stage of purification.

Except for THA and THB, the overall precision for those steroids indicated is excellent. The discrepancy in quantitating those two compounds may be partly due to their relatively low levels normally excreted in urine. It is there-

TABLE II

TEN REPLICATE DETERMINATIONS OF AN ADULT MALE 24-h URINE

	Mean (mg/24 h)	S.D.	Coefficient of variation (%)	Recovery (%)
Androsterone (A)	4.19	0.058	1.38	54
Etiocholanolone (E)	3.44	0.061	1.77	52
11-keto A + E	0.97	0.07	7.22	63
11β-OH-A	1.63	0.074	4.54	62
11β-OH-E	0.81	0.048	5.87	63
Pregnanetriol	1.08	0.040	3.7	57
THE	1.67	0.134	8.02	68
THA	0.152	0.020	13.16	52
THB	0.33	0.043	13.03	57
Allo-THB	0.50	0.049	9.8	61
THF	0.79	0.063	7.9	64
Allo-THF	0.54	0.039	7.22	65
α-Cortolone	0.24	0.023	9.58	65
β-Cortolone	0.19	0.025	13.16	63
Cholesterol	0.30	0.040	13.33	50

TABLE III

METHYLENE UNIT DETERMINATION ON NINE REPLICATE SAMPLES OF THE STEROID STANDARD USING THE DRY INJECTOR

	Methylene unit reproducibility						
	Andro	Etio	DHA	11β-OH-A	THE	THF	A-THF
$\bar{x}$	25.60	25.71	26.30	26.42	29.95	30.47	30.56
σ_{n-1}	0.007	0.008	0.009	0.006	0.006	0.007	0.007

fore possible that the integrating system employed does not reproducibly quantitate peak areas of this order of magnitude.

The illustration of methylene unit (MU) reproducibility in Table III was calculated for several steroids present in a normal adult male urine. The calculated standard deviation (S.D.) was found to be insignificant for the nine determinations performed. Indeed, the data system employed to identify the MU values for those peaks has been calibrated only once in its several months of use.

The establishment of a normal range from healthy individuals is of prime importance if the evaluation of pathological conditions is to be determined. A number of workers [1, 2, 16] have reported values for their analytical procedures. Since most of their analyses were performed using the more conventional GC inlet systems, it was considered necessary to establish a normal range using the system and techniques we were using.

Twenty-four hour urine was collected from nine healthy, normal adult

TABLE IV

NORMAL ADULT MALE URINARY STEROIDS (mg/24 h)

Steroid	Range 95% population (n=9)	Literature values [8]	Mean [13]
Androsterone	3.09 —6.96	1.69	4.47
Etiocholanolone	3.07 —5.03	2.08	2.99
DHA	0.51 —1.74	0.42	2.17
11-Keto (A and E)	0.16 —1.82	0.53	0.49
11β-OH-A+17α-OH preg.	1.36 —3.84	—*	0.77
11β-OH-E+preg.	0.56 —1.52	—	0.47
Pregnanediol	0.04 —1.51	0.80	—
Pregnanetriol	0.65 —1.90	1.64	—
Androstenetriol	2.05 —5.06	—	—
THE	1.13 —3.58	6.06	—
THA	0.72 —1.40	—	—
THB	0.006—0.45	—	—
Allo-THB	0.11 —0.84	—	—
THF	0.60 —1.33	—	—
Allo-THF	0.57 —1.35	—	—
α-Cortolone	0.21 —0.45	—	—
β-Cortolone	0.19 —0.43	—	—
Cholesterol	0.12 —1.16	—	—

*Dash (—) indicates these compounds were not quantitated.

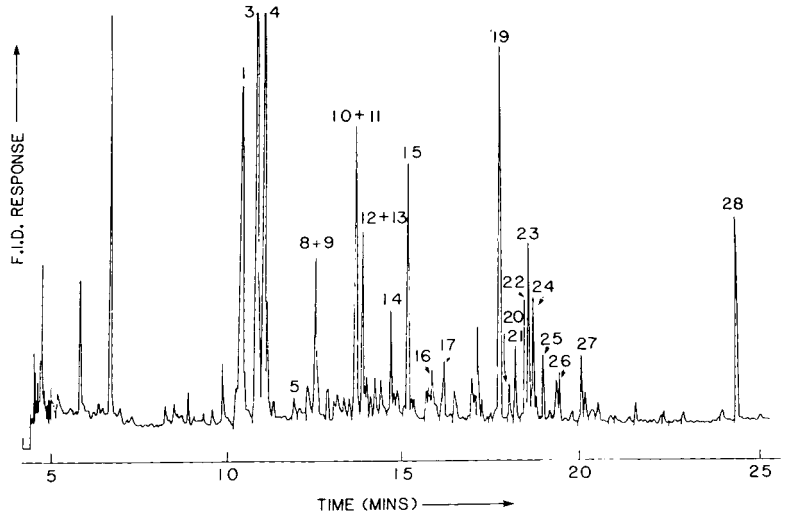


Fig. 3. Gas chromatogram of a normal adult male 24-h urinary extract, as the meth(yl)oxime TMS ethers. The extract is 1/250 of a 5-ml aliquot of urine. See Table I for peak identification.

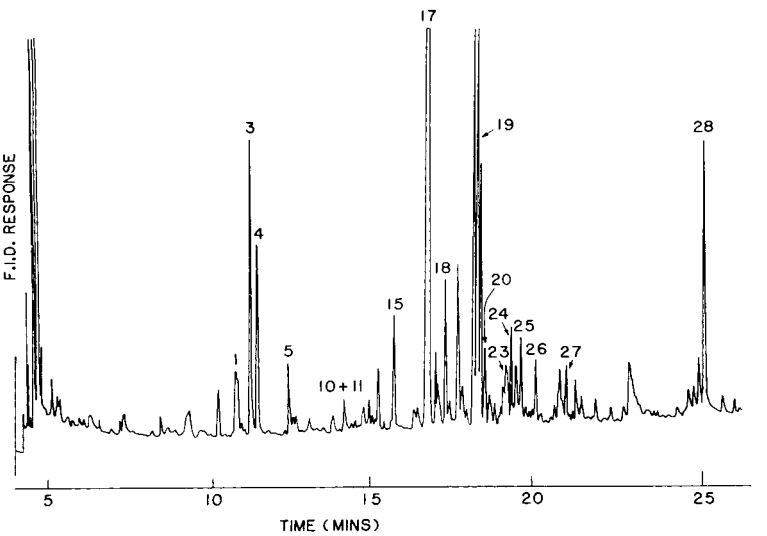


Fig. 4. Metabolic profile of a urinary extract from a patient treated with metyrapone for Cushings syndrome. See Table I for peak identification.

male volunteers working in the laboratory. Their ages ranged between 25 and 37 years, with a mean age of 31 years. Aliquots of 5 ml were removed and analyzed as described previously. Results were expressed as mg per 24 h and are summarized in Table IV. The range was calculated as ±2 S.D. of the normal mean for each steroid.

Comparison of these data with results reported by other investigators can often be misleading due to differences in methodology. Many authors include hot acid hydrolysis in their procedure. However, because of both the similarity

of our data to those of other investigators and the precision and reproducibility, we feel that the data reported in this paper indicate that the acid hydrolysis step may not be necessary.

Fig. 3 is an example of a GC metabolic profile from a normal adult male. It is dominated by the major urinary androgens, androsterone and etiocholanolone, peaks 3 and 4, respectively. Cortisol metabolites at this period of life are greatly diminished. Comparison of this profile with one from a patient being treated with metyrapone, Fig. 4, for Cushings syndrome, shows an elevated level of THS, peak 17. In this instance, THS has been further metabolized to its corresponding 20α- and -β-pregnanetetrols. These are the un-numbered peaks eluting either side of THE (peak 19). Metyrapone acts by inhibiting the enzyme 11β-hydroxylase, which is responsible for the conversion of 11-deoxycortisol (substance S) to cortisol (compound F). Consequently, excessive amounts of 11-deoxycortisol are built up in plasma and are excreted in the urine as tetrahydro substance S. Another example of disease profiling using this technique is shown in Fig. 5. The urinary steroid profile is that from a patient suffering from adrenogenital syndrome. In this condition the enzyme responsible for 21-hydroxylation is genetically absent. Biochemically, the pathway to the formation of cortisol is blocked at the conversion of 17α-hydroxyprogesterone to substance S due to the absence of the 21-hydroxylase enzyme; 17α-hydroxyprogesterone in turn is excreted in considerable amounts as its urinary metabolite of pregnanetriol, peak 15.

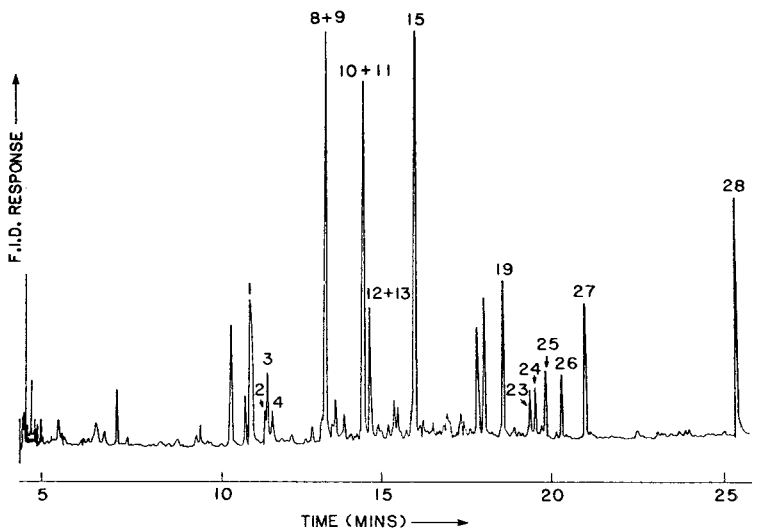


Fig. 5. Metabolic profile of a urinary extract from a patient with 21-hydroxylase deficiency. Patient was being treated with prednisone. See Table I for peak identification.

CONCLUSION

Routine analysis of urinary metabolic profiles is still hindered by the lengthy enzymatic hydrolytic procedure. The method described in this paper still incorporates this stage. However, incorporating fused silica capillary columns coupled to a Van den Berg injector has greatly reduced the GC analysis time,

with little or no sacrifice in GC resolution. Because of the excellent resolution and sensitivity achieved with the use of these columns it is possible to analyze significantly reduced volumes of urine. This has eliminated the cumbersome extraction procedures with large volumes of toxic solvents that are necessary when large volumes of urine are used and has made it possible to rapidly prepare larger numbers of samples prior to hydrolysis.

The stability and reproducibility of this method is exemplified in the Results section. Standard deviations for MU reproducibility were shown to be very small. Indeed, even after several months of use the agreement of these values to their original are still excellent.

The precision of this method is quite remarkable considering the small volumes of urine analyzed. The majority of the analytes having a coefficient of variation of less than 10%, and most of the major metabolites less than 5%.

The normal ranges presented in Table IV are in very good agreement with those methods that incorporate hot acid solvolysis [1, 13]. This could be indicative of the efficiency of the enzyme system employed in this method, since the mixture does contain both the arylsulfatase and β-glucuronidase. An alternative explanation would be that the enzyme cleaves the conjugates reproducibly but incompletely. However, studies where we have extended the incubation time to 75 and 96 h have not changed the reproducibility obtained with 48-h incubations. Furthermore, preliminary results using radiolabeled steroid sulfate conjugates has indicated that the enzymatic hydrolysis is more than 95% complete. However, further investigations into this area are still in progress to confirm this aspect.

The flexibility of fused silica columns has enabled the column to be directly coupled to the mass spectrometer. Also the absence of solvent has circumvented the need for the conventional type of separator usually incorporated into the system. Using this system aliquots previously analyzed by GC can now be examined by GC—MS without further manipulation of the derivatized extract, for example eliminating the need to further concentrate the sample or change solvent systems.

ACKNOWLEDGEMENTS

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DETERMINATION OF THE STABLE ISOTOPE OF NITRITE FLUX IN THE BLOOD OF MICE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY WITH SELECTED ION MONITORING

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SUMMARY

A simple and reliable method for the determination of the stable isotope of nitrite ($^{15}NO_2$) flux in the blood of mice is described. It is based on the reaction of $^{15}NO_2$ with 1-hydrazinophthalazine in acidic solution to form [^{15}N]tetrazolophthalazine, a stable compound which can be extracted with an organic solvent and then determined by gas chromatography—mass spectrometry with selected ion monitoring using the pentafluorobenzoyl ester of 2,4-dinitro-6-*sec.*-butylphenol as an internal standard. Amounts of 0.2—10 μg of $^{15}NO_2$ can be determined. The detection limit of $^{15}NO_2$ was 0.1 μg/ml. This is a specific method for $^{15}NO_2$. The procedure for determining $^{15}NO_2$ in the blood of mice involves extraction with solvent, followed by further clean-up by alumina column chromatography; the detection limit is 0.05 μg. With the new technique we were able to perform a metabolic fate study of nitrite in the blood of mice following a single oral dose of $^{15}NO_2$ or the stable isotope of nitrate ($^{15}NO_3$).

INTRODUCTION

The determination of nitrite in blood may need to be carried out in cases of intoxication by nitrite-containing drugs or ingestion of food containing nitrite or nitrate. Ingested nitrates may be reduced by intestinal flora to nitrite. Nitrite, when absorbed into the bloodstream, oxidizes haemoglobin to

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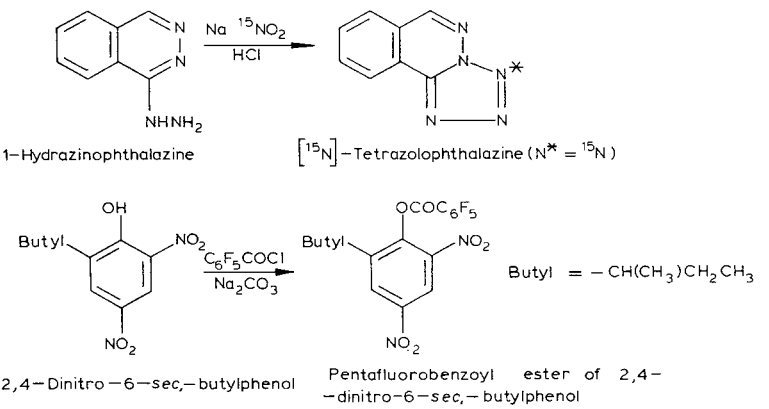
methaemoglobin, and if sufficient haemoglobin is converted the oxygen-carrying capacity of the blood may be markedly reduced. Therefore, the metabolic fate of nitrite in blood is of great interest. Nitrite in blood is usually determined by a colorimetric method based on the formation of an azo dye produced by diazotization of sulfanilic acid and subsequent coupling with 1-naphthylamine or N-(1-naphthyl)ethylenediamine [1—4]. However, these colorimetric methods are limited by the fact that occasionally turbid and slightly coloured sample extracts can affect the colour of the azo dye and, consequently, the accuracy of the nitrite determination. We found that tetrazolophthalazine [5] can be quantitatively prepared by reaction with nitrite and 1-hydrazinophthalazine in acidic solution and can be extracted with several organic solvents [6—9]. This is a specific reaction for nitrite (see Scheme 1, top).



Scheme 1.

In this paper, we have monitored the in vivo disappearance of nitrite in the blood of mice by following a single oral dose of $^{15}NO_2$ or $^{15}NO_3$. In general, there are various methods for determining nitrogen-15, including mass spectrometry (MS) [10], chemical ionization MS [11] and emission spectrography [12]. However, these methods cannot distinguish whether $^{15}NO_2$ or $^{15}NO_3$ is being determined because they determine total nitrogen-15. Furthermore, the procedures are complex [13, 14]. We describe here an accurate, reliable, reproducible and selective determination of $^{15}NO_2$. In the present study deproteinized mice blood was analyzed by gas chromatography (GC) MS with selected ion monitoring after reaction and clean-up by alumina column chromatography.

EXPERIMENTAL

Reagents and standards

Sodium nitrite (95 atom% ^{15}N) and potassium nitrate (99.5 atom% ^{15}N), obtained from Merck Sharp and Dohme (Montreal, Canada), were dried at 110°C for 1 h under vacuum immediately before use. A stock nitrite solution was prepared by dissolving 0.493 g of $Na^{15}NO_2$ in 100 ml of distilled water to give a concentration of 1 mg/ml of $^{15}NO_2$. A stock nitrate solution

was prepared by dissolving 0.718 g of $K^{15}NO_3$ in 100 ml of distilled water to give a concentration of 1 mg/ml $^{15}NO_3$. 1-Hydrazinophthalazine (Tokyo Kasei Kogyo, Tokyo, Japan) was of a special high grade and was used without further purification. A solution (1.0%, w/w) of it was prepared by dissolving 1.0 g in 100 ml of distilled water. The pentafluorobenzoyl (PFB) ester of 2,4-dinitro-6-*sec.*-butylphenol (2,4-dinitro-6SBP) as internal standard for GC—MS with selected ion monitoring was prepared as follows. A 0.1 g portion of 2,4-dinitro-6-*sec.*-butylphenol (ICN Pharmaceuticals, Plainview, NY, U.S.A.) was dissolved in 10 ml of 5% sodium carbonate solution; then 0.1 g of pentafluorobenzoyl chloride (Aldrich, Milwaukee, WI, U.S.A.) was added (see Scheme 1, bottom). After reaction at room temperature with shaking for 5 min, the reaction mixture was extracted with 50 ml of benzene and the benzene layer was separated and washed with 50 ml of distilled water. The benzene layer was dried with an adequate amount of anhydrous sodium sulphate and concentrated to 5 ml. The 5-ml solution was applied to the column (30 cm × 1.0 cm I.D., prepared with 10 g of activated alumina) and eluted with benzene. The 100 ml of effluent was collected, and then evaporated at 50° C. The substance obtained as mentioned above was prepared by dissolving 3 μg in 1 ml of ethyl acetate. Deproteinizing solution A was prepared by dissolving 90 g of ammonium thiocyanate and 80 g of mercuric chloride in 1000 ml of distilled water. Deproteinizing solution B was prepared by dissolving 125 g of zinc acetate in 500 ml of distilled water. All water used was triple distilled and deionized. All other reagents and solvents were of high purity and were obtained from Wako (Osaka, Japan).

Animals

Male ddY mice weighing 20—25 g were used for the experiments. They were housed in plastic cages on soft-wood bedding at room temperature (25° C) under a 12 h dark—light rhythm. The animals had free access to standard laboratory diet (Funabashi Farm, Chiba, Japan) and tap water.

Treatment of animals

A 100- or 500-μg amount of $^{15}NO_2$ was orally given to mice by gavage in 0.1 ml or 0.5 ml of the stock nitrite solution; also, $^{15}NO_3$ was given orally to mice in a similar manner. The mice were anesthetized with diethyl ether and killed by cervical dislocation 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after the dose had been given. Blood samples were collected in heparinized containers and were stored at 0—4° C for about 15 min.

Preparation and analysis of mice blood extracts

A 1-ml blood sample was placed in a 10-ml test-tube (11.5 cm × 1.5 cm I.D.). A 1-ml volume of each deproteinizing solution A and B was added and then the mixture was shaken for 1 min followed by centrifugation at 1400 *g* for 5 min. A 2-ml aliquot of the supernatant was placed in a 10-ml test-tube and 1 ml of 1-hydrazinophthalazine solution and 2 ml of 2 *M* hydrochloric acid were added. The mixture was heated at 70° C in a water-bath with occasional shaking for 20 min. After cooling to room temperature, the reaction mixture was extracted with 5 ml of toluene with shaking for 3—5 min. The toluene

extract was pipetted into a 10-ml test-tube and then the procedure was repeated. After drying with an adequate amount of anhydrous sodium sulphate, the combined extracts were subjected to alumina column chromatography as described previously [6] to remove interfering substances from the blood. The effluent was then evaporated in a stream of nitrogen gas at 40°C. The residue was dissolved in an adequate volume of internal standard solution. A 5-μl volume of the final solution was analyzed by GC—MS with selected ion monitoring.

Gas chromatography—mass spectrometry

GC—MS was carried out using a Shimadzu LKB-9000 combined gas chromatograph—mass spectrometer equipped with a multiple ion detector-peak matcher. The column was a glass tube (0.5 m × 3 mm I.D.) packed with 3% OV-225 on Chromosorb W HP (80—100 mesh) and was conditioned at 230°C; injector temperature was 180°C. The flow-rate of helium carrier gas was 30 ml/min. MS conditions were as follows: separator temperature 235°C; ion source temperature 290°C; trap current 60 μA; electron energy 70 eV; accelerating potential 3.5 keV. For selected ion monitoring, the following ions were used: *m/e* 171 for tetrazolophthalazine, *m/e* 172 for [^{15}N]tetrazolophthalazine, and *m/e* 167 for the PFB ester of 2,4-dinitro-6SBP as internal standard.

Preparation of calibration curve

A series of working standard $^{15}NO_2$ solutions was prepared by diluting the stock solution with distilled water. Aliquots were placed in 10-ml test-tubes. After the addition of 1-hydrazinophthalazine and hydrochloric acid and subsequent reaction, the reaction mixtures were extracted with toluene and the solvent removed by evaporation according to the procedure described above. The residues were dissolved in 1 ml of internal standard solution and 5-μl aliquots of the resulting solutions were injected for GC—MS with selected ion monitoring. The concentration range of the $^{15}NO_2$ standard was 0.2—10.0 μg/ml. As shown in Fig. 1, the retention time of [^{15}N]tetrazolophthalazine

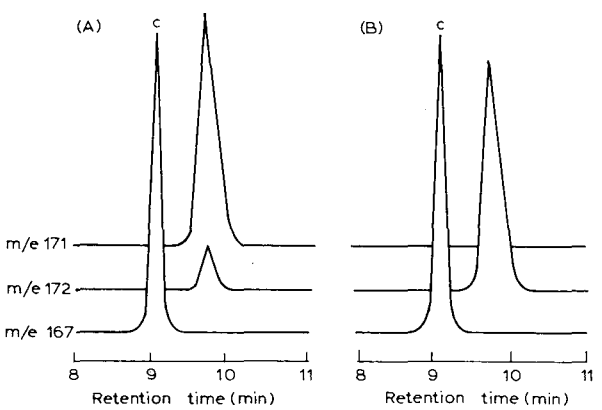


Fig. 1. Selected ion chromatograms of tetrazolophthalazine (A) and [^{15}N]tetrazolophthalazine (B) with the pentafluorobenzoyl ester of 2,4-dinitro-6-*sec.*-butylphenol as internal standard (C). Aliquots of 5 μl of a mixture of the internal standard solution and tetrazolophthalazine or [^{15}N]tetrazolophthalazine were directly injected for GC—MS with selected ion monitoring. For conditions, see text.

relative to that of internal standard was 1.07. The peak height ratios of [^{15}N]-tetrazolophthalazine to internal standard were plotted against the amount of $^{15}NO_2$ analyzed. The ratio (R) of the peak height of [^{15}N] tetrazolophthalazine to internal standard was calculated as follows:

$$R = (a - 0.219b)/c \quad (1)$$

where a and b are the peak heights, of [^{15}N] tetrazolophthalazine at m/e 172 derived from $^{15}NO_2$ and that of tetrazolophthalazine at m/e 171 derived from $^{14}NO_2$, and c is the peak height of internal standard at m/e 167. The typical standard curve was linear.

RESULTS AND DISCUSSION

In order to investigate an obvious difference between tetrazolophthalazine derived from $^{14}NO_2$ and [^{15}N] tetrazolophthalazine derived from $^{15}NO_2$, each product was analyzed by GC—MS. As shown in Fig. 2, the parent peak (m/e 171) for tetrazolophthalazine and that of m/e 172 for [^{15}N] tetrazolophthalazine correspond to the molecular weight of each compound. However, the shift of the peaks from m/e 115 to 76 for [^{15}N] tetrazolophthalazine was in agreement with that of tetrazolophthalazine (Fig. 2C). Therefore, for GC—MS with selected ion monitoring, we adopted m/e 171 for tetrazolophthalazine and m/e 172 for [^{15}N] tetrazolophthalazine.

Columns containing DC-200 (5%, w/w), SE-30 (3%, w/w), OV-1 (5%, w/w), OV-101 (2%, w/w) and OV-225 (3% w/w) on Chromosorb W HP were tested. Except for OV-225, all the columns caused peak tailing of [^{15}N] tetrazolophthalazine. Good peak characteristics and sensitivity were achieved with OV-225 under the conditions described above. A high temperature and a short column were preferable for the GC—MS with selected ion monitoring of [^{15}N] tetrazolophthalazine.

At 235°C a 0.5-m column containing OV-225 on Chromosorb W HP gave a good ion chromatogram (see Fig. 1). On the other hand, the PFB ester of 2,4-dinitro-6SBP was chosen as internal standard since it had favourable properties for the OV-225 column. Furthermore, as blood extract contents interfered with the base peak (m/e 195), we adopted m/e 167 as the monitoring ion for the internal standard. The retention time of [^{15}N] tetrazolophthalazine relative to that of internal standard was 1.07 (Fig. 1).

$^{14}NO_2$ is widespread in nature. Therefore, to investigate its influence on the determination of $^{15}NO_2$ 1.0—10.0 μg of $^{15}NO_2$ were added to 1.0—10.0 μg of $^{14}NO_2$ as shown in Table I, and each mixture was analyzed by GC—MS with selected ion monitoring after reaction and extraction according to the described procedure. The peak height ratio of [^{15}N] tetrazolophthalazine to internal standard increased with an increase in the amount of tetrazolophthalazine added, and, at the same time, the accuracy decreased (Table I). On the other hand, tetrazolophthalazine derived from $^{14}NO_2$ gave a peak at m/e 172 beside the parent peak at m/e 171 (Fig. 1). Therefore, by using the ratio (k) of the peak height at m/e 172 to that at m/e 171 of tetrazolophthalazine, the determinable peak height of [^{15}N] tetrazolophthalazine (x) in the blood extract of mice can be calculated by the equation $x = a - kb$, where a and b are the peak heights at m/e 172 and m/e 171, respectively, in the analysis

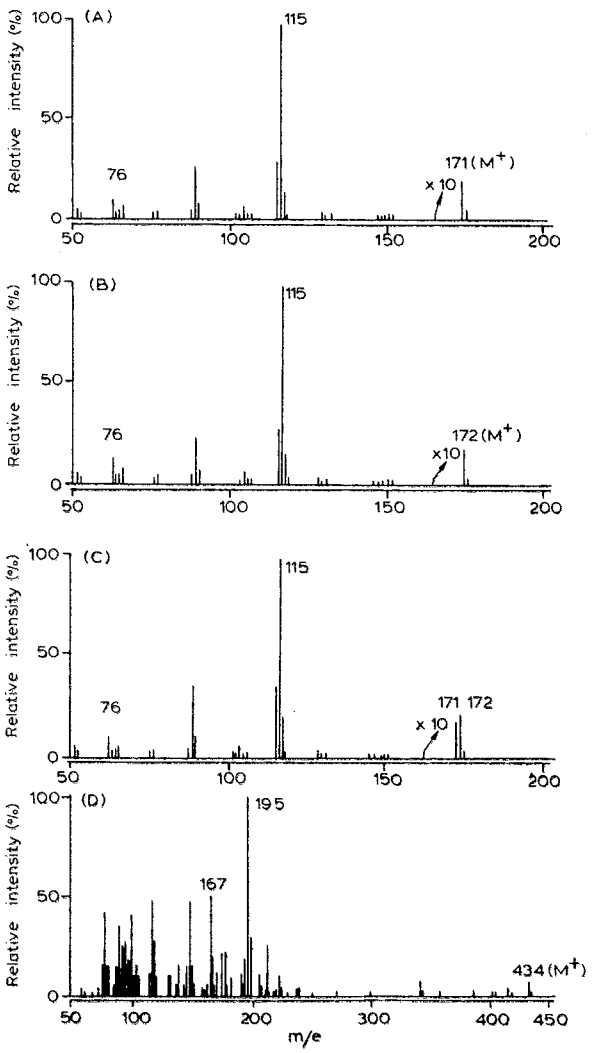


Fig. 2. Mass spectra of tetrazolophthalazine (A), [^{15}N]tetrazolophthalazine (B), a mixture of tetrazolophthalazine and [^{15}N]tetrazolophthalazine (C), and the pentafluorobenzoyl ester of 2,4-dinitro-6-*sec.*-butylphenol (D) as internal standard.

of the sample digest by GC—MS with selected ion monitoring. Therefore, in order to measure the k value, aliquots were placed into test-tubes to give amounts of 0.5, 1, 3, 5, 7 and 10 μg of $^{14}NO_2$. After reaction and extraction according to the described procedure, each extract was evaporated in a stream of nitrogen gas at 40°C. The residues were dissolved in 1 ml of ethyl acetate and dried with a small amount of anhydrous sodium sulphate, then analyzed by GC—MS with selected ion monitoring. As shown in Table II, the k value for each amount of $^{14}NO_2$ ranged from 0.201 to 0.221 with an average of 0.219. Thus, in practice 0.219 was adopted. Since the presence of $^{14}NO_2$ did affect the determination of $^{15}NO_2$, as shown in Table II, the results were re-calculated using eqn. 1. These results are shown in Table I with values in parentheses, and are in fair agreement with the additional amounts of $^{15}NO_2$.

TABLE I

INFLUENCE OF $^{14}NO_2$ ON THE DETERMINATION OF $^{15}NO_2$

For each amount of $^{15}NO_2$ were added various amounts of $^{14}NO_2$ and 1% 1-hydrazinophthalazine. The reaction procedure and GC—MS with selected ion monitoring conditions are as described in the text. The amounts of $^{15}NO_2$ were determined by comparing with the calibration curve obtained from eqn. 1 with $b = 0$; the amounts of $^{15}NO_2$ in parentheses were determined in the same way, after recalculation using eqn. 1 as in the text.

$^{15}NO_2$ added (μg)	$^{14}NO_2$ added (μg)				
	1.0	3.0	5.0	7.0	10.0
1.0	1.21 (0.98)	1.68 (1.01)	2.13 (1.03)	2.46 (0.93)	3.11 (0.92)
3.0	3.19 (0.98)	3.68 (3.01)	4.14 (3.04)	4.50 (2.97)	5.19 (3.00)
5.0	5.16 (4.94)	5.64 (4.98)	6.15 (5.03)	6.54 (5.01)	7.15 (4.95)
7.0	7.26 (7.04)	7.63 (6.96)	8.10 (7.00)	8.49 (6.96)	9.20 (7.00)
10.0	10.15 (9.93)	10.64 (9.98)	10.95 (9.85)	11.45 (9.92)	12.25 (10.04)

TABLE II

STUDY OF SET OF k VALUES

Each amount of $^{14}NO_2$ was added to 1.0 ml of 1% 1-hydrazinophthalazine. The reaction procedure and analysis by GC—MS with selected ion monitoring conditions are as described in the text. Each value is the mean ± standard deviation of five replicate determinations.

$^{14}NO_2$ added (μg)	k (peak height at m/e 172 / peak height at m/e 171)
0.5	0.225 ± 0.011
1.0	0.219 ± 0.018
3.0	0.212 ± 0.019
5.0	0.220 ± 0.010
7.0	0.215 ± 0.014
10.0	0.221 ± 0.013

On the other hand, analysis of the blood digest by the proposed method after the formation of [^{15}N]tetrazolophthalazine is shown in Fig. 3. Besides the peak of tetrazolophthalazine at m/e 171, there was another peak, the retention time of which relative to that of internal standard was 0.98. The intensity of the peak was sufficient to interfere in the determination of tetrazolophthalazine. Therefore, a clean-up stage was needed. However, use of the alumina column as described previously [6] was effective in removing this interfering substance (Fig. 3).

A calibration curve for $^{15}NO_2$ was prepared by adding to mice plasma (2 ml) known amounts of $^{15}NO_2$ (0.2—10 μg) followed by GC—MS analysis with selected ion monitoring, and plotting the ratio of the peak height of [^{15}N]-tetrazolophthalazine to that of internal standard against concentration. The calibration curve was linear with a linear regression equation $y = 0.932x - 0.281$ (correlation coefficient of 0.9962). The average relative standard devia-

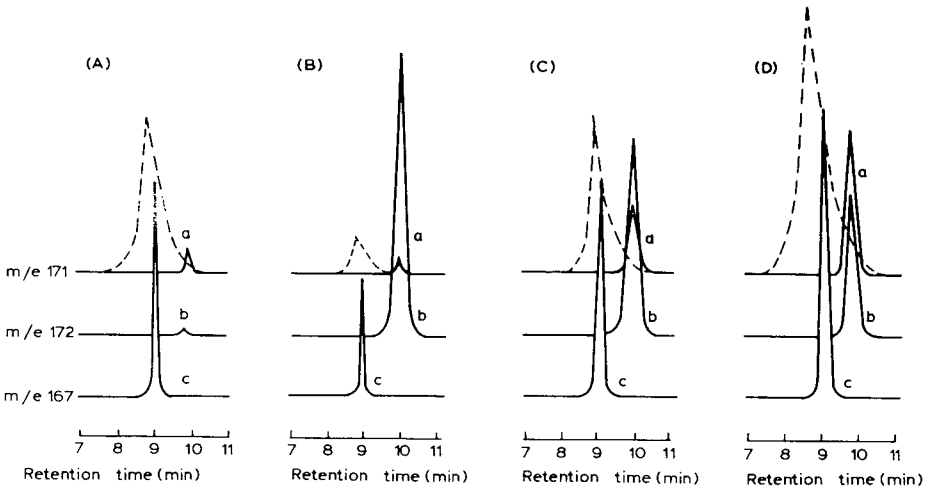


Fig. 3. Selected ion chromatograms of ethyl acetate extracts of mice blood. The sample size was 5 μl. Chromatogram A is control; B and C are obtained with a single oral dose of 500 and 100 μg of $^{15}NO_2$, respectively; D is obtained with a single oral dose of 500 μg of $^{15}NO_3$. Peaks: a = tetrazolophthalazine; b = [^{15}N]tetrazolophthalazine; c = pentafluorobenzoyl ester of 2,4-dinitro-6-*sec.*-butylphenol. The dotted lines show the shape of chromatograms obtained for blood before clean-up by alumina chromatography. For GC—MS with selected ion monitoring conditions, see text.

TABLE III

RECOVERY OF NITRITE FROM MOUSE BLOOD PLASMA

Results represent the mean of five determinations at each level

$^{15}NO_2$ added (μg)	Mean $^{15}NO_2$ found (μg)	C.V. (%)	Mean recovery (%)	Recovery range (%)
0.5	0.46	5.3	92.8	87.6—94.3
1.0	0.96	4.1	95.7	91.7—96.1
3.0	2.92	1.7	97.2	96.3—98.0
5.0	4.88	2.0	97.6	95.8—98.1
7.0	6.78	1.3	96.8	96.0—97.3
10.0	9.70	1.5	97.0	96.4—98.0

tion of five determinations was 2.1% for 0.5 μg or 1.0 μg and 3.7% for 5.0 μg or 10 μg of $^{15}NO_2$ and the reproducibility was considered satisfactory.

Recovery data from whole blood were not evaluated because haemoglobin reacts immediately with nitrite. However, the residual nitrite in blood is also present in plasma as reported by Shechter et al. [4]. In order to check the validity of the proposed procedure for the determination of $^{15}NO_2$ in mice plasma, recoveries of $^{15}NO_2$ added to plasma (2 ml) were measured at each concentration; the results are presented in Table III. The mean recovery varied between 92.8% and 97.6% (the coefficient of variation, C.V., varied between 1.3 and 5.3%). The detection limit was 0.05 μg of $^{15}NO_2$.

The utility of the proposed method was demonstrated by using it in a metabolic fate study of nitrite in mice blood. Mice were injected with a single

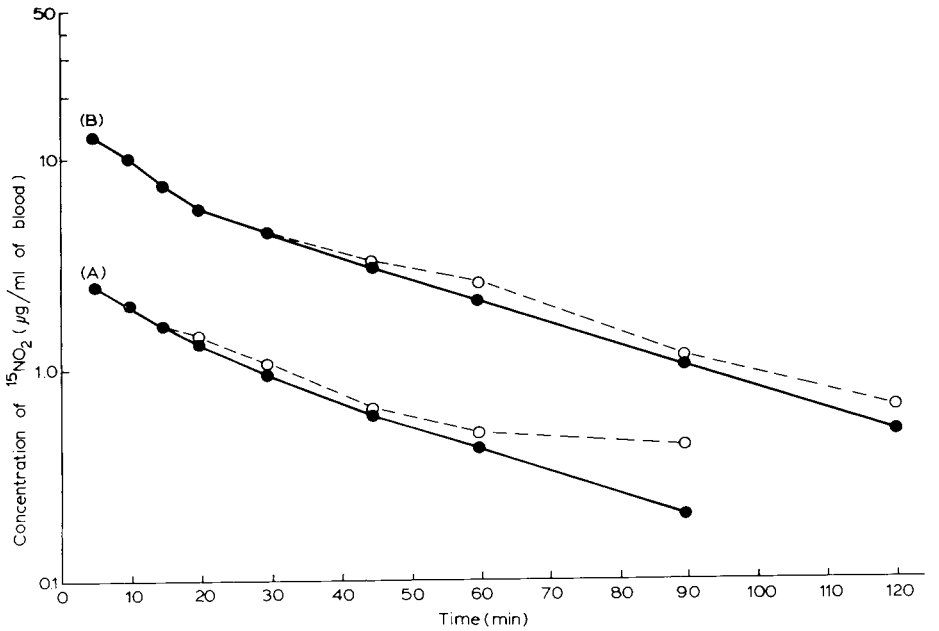


Fig. 4. Concentration of $^{15}NO_2$ in blood of mice vs. time after a single oral dose of 100 μg (A) or 500 μg (B) of $^{15}NO_2$. Each point represents the mean value for five mice. The dotted lines show data obtained using the GC method [6].

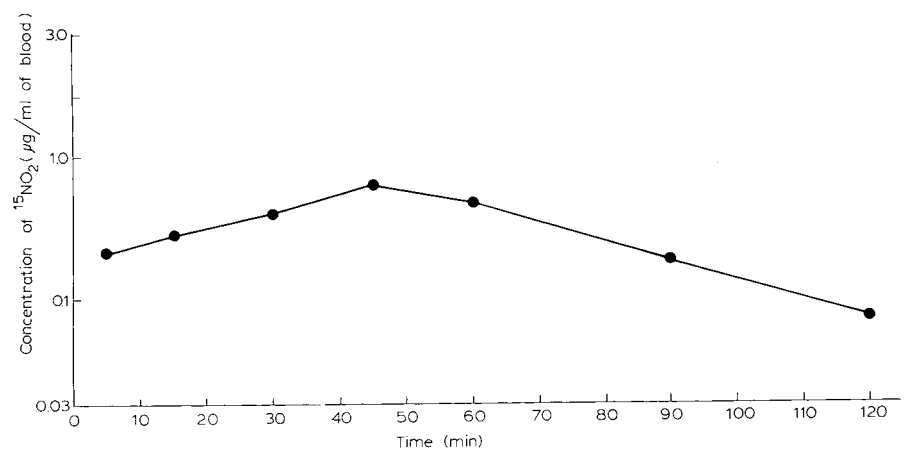


Fig. 5. Concentration of $^{15}NO_2$ in blood of mice vs. time after a single oral dose of 500 μg of $^{15}NO_3$. Each point represents the mean for five mice.

oral dose of 100 μg or 500 μg of $^{15}NO_2$. The mice were killed as described above and blood samples analyzed by the described procedure. The data were used to construct semilogarithmic plots of concentration vs. time as shown in Fig. 4. The in vivo disappearance of nitrite in mice blood was rapid, the essential reaction being completed within 30—45 min and showed an almost linear regression. Although in the case of the 100-μg dose the residual $^{15}NO_2$ disappeared at 90 min, with the 500-μg dose it was still detected in blood at 120 min

after injection. On the other hand, both the proposed method and the GC method [6] were approximately in agreement with the data for 15—20 min after injection. However, the data obtained by the GC method were higher than those obtained with the proposed method at 20—120 min after injection (Fig. 4). It is assumed that the GC method determines both $^{14}NO_2$ and $^{15}NO_2$. Therefore, the proposed method is specific for $^{15}NO_2$. On the other hand, nitrate is hardly absorbed in the stomach compared with nitrite [15]. However, the formation of nitrite may occur if nitrite-reducing bacteria are present in the stomach; also, it has been reported that nitrate is converted to nitrite by nitrate-reducing bacteria in saliva [16]. Therefore, to investigate the presence of $^{15}NO_2$ in mice blood in cases of $^{15}NO_3$ intake, mice were injected with a single oral dose of 100 μg or 500 μg of $^{15}NO_3$ and the subsequent experiments were carried out as for $^{15}NO_2$. The results obtained are shown in Fig. 5. The detectable amount of $^{15}NO_2$ derived from $^{15}NO_3$ was very low. The formation of $^{15}NO_2$ reached a maximum 45 min after $^{15}NO_3$ injection (Fig. 5) and then gradually decreased. From these results the in vivo formation of nitrite from ingested nitrate is clearly demonstrated in mice. For the 100-μg dose, however, it was impossible to determine $^{15}NO_2$ because of the lower limit of detection.

CONCLUSION

The determination of trace levels of nitrite in biological samples such as blood has posed many difficulties. In this study, $^{15}NO_2$ was determined as [^{15}N]tetrazolophthalazine by GC—MS with selected inon monitoring after sample clean-up with alumina column chromatography. The method is simple, and has been successfully applied to a metabolic fate study of nitrite in mice blood using $^{15}NO_2$ or $^{15}NO_3$.

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QUANTITATIVE GAS—LIQUID CHROMATOGRAPHY OF TAURINE

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SUMMARY

A method has been developed for the determination of taurine by gas—liquid chromatography. The method involves the conversion of taurine into its N-isobutyloxycarbonyl di-*n*-butylamide derivative and chromatography on a 1.5% OV-17 column. The derivative can be prepared in quantitative yield, having good chromatographic properties. The calibration curve for taurine in the range 5—500 nmol was linear and sufficiently reproducible for quantitative determination. Complex biological material such as urine can be analysed accurately and precisely by this method without prior clean-up of the sample.

INTRODUCTION

Taurine (2-aminoethanesulphonic acid) is one of the most abundant and ubiquitous free amino acids in the fluids and tissues of animals [1], and much evidence suggests that it plays an important role in the body [2].

The determination of taurine in biological material has been carried out by colorimetric [3—8], fluorometric [9, 10], radiometric [11] and enzymatic [12] assay methods. However, these methods usually require time-consuming pretreatment of the sample [13]. Chromatographic procedures utilizing an amino acid analyzer [13—19] or high-performance liquid chromatographic system [20—24] have also been used for the assay, but when applying them directly to the biological sample there appears to be difficulty in resolving taurine from interfering components [17—19, 23]. On the other hand, the development of a satisfactory gas—liquid chromatographic (GLC) procedure for the analysis of taurine has been hindered by the difficulty of preparing a suitably volatile derivative of this compound. Although several investigators have reported that taurine can be chromatographed as its trimethylsilyl derivative [25, 26], to our knowledge no report has so far appeared on the actual GLC analysis of taurine contained in biological samples.

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This paper reports the development of a GLC method for the determination of taurine, based on the preparation of the N-isobutyloxycarbonyl (N-isoBOC) di-*n*-butylamide derivative.

EXPERIMENTAL

Reagents

Taurine and 3-amino-1-propanesulphonic acid (APS) as an internal standard were purchased from Nakarai Chemicals (Kyoto, Japan) and Sigma (St. Louis, MO, U.S.A.), respectively, and each was dissolved in water to make a stock solution at a concentration of 1 m*M*. Isobutyl chloroformate (isoBCF) stabilized with calcium carbonate was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Thionyl chloride and di-*n*-butylamine (DBA) were purchased from Nakarai Chemicals and used after distillation. Tetrahexylammonium hydroxide (THA-OH) was prepared as follows: 10 g (0.02 mol) of tetrahexylammonium iodide (Eastman Kodak) were dissolved in 60 ml of 80% methanol and to this solution was added 0.07 mol of freshly precipitated silver oxide. The mixture was shaken for 1 h at room temperature. After centrifugation, the supernatant was evaporated at 50°C, and the residue was reconstituted in methanol to prepare a 10% (w/v) solution. Other alkylammonium hydroxide solutions were prepared in the same fashion as described above except for tetraethylammonium hydroxide and tetrabutylammonium hydroxide, which were obtained as 10% methanolic solutions from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were of analytical grade.

Instruments

A Shimadzu 4CM gas chromatograph equipped with a hydrogen flame ionization detector, an on-column injection port and a linear temperature programmer was used. The column packing, 1.5% OV-17 on 100—120 mesh Uniport HP (Gasukuro Kogyo, Tokyo, Japan), was prepared using toluene as a coating solvent according to the solution coating technique [27], and was poured into a silanized glass column (1.5 m × 3 mm I.D.). The packed column was conditioned at 290°C for 24 h with a nitrogen flow-rate of 30 ml/min. The operating conditions were as follows: oven temperature, programmed at 5°C/min from 200°C to 280°C; injection and detector temperatures, 285°C; nitrogen flow-rate, 45 ml/min. A Shimadzu-LKB 9000 gas chromatograph—mass spectrometer with the same type of column as used for GLC analysis was employed under the following conditions: trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion source temperature, 290°C; separation temperature, 285°C; helium flow-rate, 30 ml/min.

Derivatization

The chemical reactions involved in the derivatization procedure proceed as shown in Fig. 1. Pyrex glass screw-top culture tubes (10 cm × 1.0 cm I.D.) with PTFE-lined caps were used as reaction vials. An aliquot of the taurine solution (corresponding to 5—500 nmol) or 0.1 ml of 24-h human urine was pipetted into a 10-ml reaction vial. After addition of 0.2 ml of the internal standard solution, 0.1 ml of 0.5 *M* sodium hydroxide was added and then the total

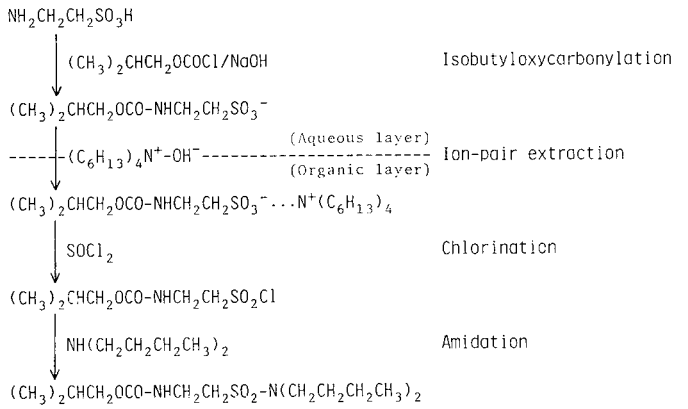

Fig. 1. Derivatization process of taurine.

reaction volume was made up to 1 ml with distilled water if necessary. Immediately after addition of 0.1 ml of isoBCF, the mixture was shaken with a shaker set at 300 rpm (up and down) for 5 min at room temperature. The reaction mixture was washed twice with 3 ml of diethyl ether after adjustment to pH 1- 2 with 0.5 *M* hydrochloric acid. Subsequently, 0.1 ml of 10% THA-OH was added to the aqueous layer, and the ion-pair compounds formed were extracted into 2 ml of methylene chloride by shaking for 3 min. After centrifugation for 1 min, the organic layer was transferred to another reaction vial and the solvent was evaporated at 60° C under a stream of nitrogen. To the residue was added 0.2 ml of thionyl chloride, and the vial was tightly capped and heated at 80° C for 15 min. The excess thionyl chloride was removed at 80° C under a stream of dry nitrogen. To the residue was added 0.2 ml of 2 *M* DBA in acetonitrile, and the mixture was allowed to stand for 5 min at room temperature after tightly capping. The reaction mixture was acidified with 1 ml of 20% orthophosphoric acid and then extracted three times with 3 ml of *n*-pentane. After the solvent was evaporated to dryness at 60° C, the residue was dissolved in 0.1 ml of ethyl acetate and 2—4 μl of this solution were injected into the gas chromatograph. The peak height ratios relative to the internal standard were calculated, and they were used to construct the calibration curve and for the quantitation of taurine in urine.

Preparation of reference compound

A reference sample of the N-isoBOC di-*n*-butylamide derivative of taurine was prepared from 400 mg of taurine in essentially the same manner as the analytical derivatization procedure. The data for elemental analysis are as follows. Calc. for $C_{15}H_{32}N_2O_4S$: C, 53.54; H, 9.59; N, 8.33. Found: C, 53.21, H, 9.74; N, 8.11.

RESULTS AND DISCUSSION

Derivatization

On the basis of the experience gained in our previous studies [28, 29], the isoBOC group was selected as the blocking substituent for the amino function

TABLE I

INFLUENCE OF THE NATURE OF THE COUNTER-ION ON ION-PAIR EXTRACTION OF N-isoBOC DERIVATIVE OF TAURINE

Reactants: counter-ion 1%, taurine 0.2 μmol, internal standard (9-bromophenanthrene) 0.2 μmol. GLC conditions are given in Experimental.

Counter-ion	Number of C atoms	PHR*
Tetraethylammonium hydroxide	8	0
Tetrabutylammonium hydroxide	16	0.49
Trimethylstearylammonium hydroxide	21	0.58
Tetrahexylammonium hydroxide	24	1.07
Trioctylmethylammonium hydroxide	25	0.91

*Peak height ratios are given relative to internal standard.

of taurine. The isobutyloxycarbonylation proceeded rapidly and quantitatively under the conditions described in Experimental. However, the presence of free sulphonic acid precluded the resulting N-isoBOC taurine in the aqueous medium being directly extracted into an organic solvent. In order to solve this problem, ion-pair extraction [30] was used. Of several alkylammonium ions tested, THA proved to be the most satisfactory counter-ion for the purpose (Table I), and with it rapid and quantitative ion-pair extraction of the N-isoBOC taurine into methylene chloride was achieved.

Experiments were conducted to find suitable reaction conditions for the chlorination of small amounts of N-isoBOC taurine; reaction at 80° C for 15 min with 0.2 ml of thionyl chloride proved to be adequate for maximal formation, as shown in Fig. 2. Excess reagent was removed under a stream of dry nitrogen to eliminate possible interference in the subsequent reaction.

A number of amines were evaluated to produce a sulphonamide derivative which is satisfactory in respect to reaction yield and GLC behaviour from the

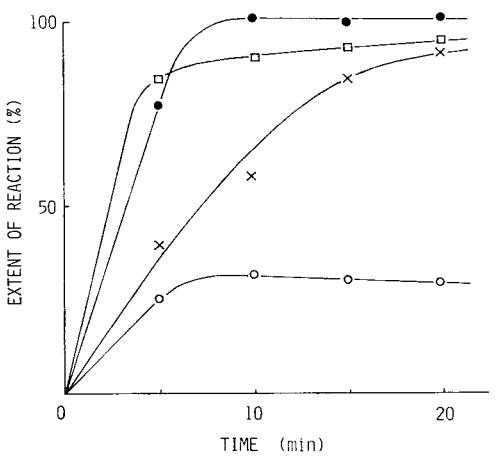

Fig. 2. Time dependence of chlorination of N-isoBOC taurine derivative at various temperatures: 40° C (○), 60° C (×), 80° C (●), 100° C (□).

N-isoBOC taurine sulphonyl chloride. The best result was obtained by DBA dissolved in anhydrous acetonitrile; the reaction proceeded rapidly without heating.

For extraction of the product, the N-isoBOC di-*n*-butylamide derivative, *n*-pentane was found to be a preferable solvent to others such as diethyl ether, ethyl acetate and benzene, the use of which resulted in the appearance of a large front peak on chromatograms, presumably derived from co-extracted THA.

The mean derivatization yield throughout the procedure established above was determined to be 97.3% (n = 5) by comparison with the synthetic reference derivative.

Structure and stability of derivative

Structure of the derivative was confirmed both by gas chromatography—mass spectrometry (GC—MS) and by elemental analysis. Although a molecular ion peak with the postulated *m/e* 336 was not observed, the highest fragment ion peak with *m/e* 263 [$M^+ - (CH_3)_2CHCH_2O^+$] as well as other prominent fragment ion peaks [$M^+ - (CH_3)_2CHCH_2OCONH$, $M^+ - N(C_4H_9)_2$, $M^+ - (CH_3)_2CHCH_2$ and $N(C_4H_9)_2$] were useful for structure elucidation. The values of elemental analysis agreed with the theoretical values calculated for the structure expected. These results supported the structure for the derivative shown in Fig. 1. The derivative was found to be very stable under normal laboratory conditions; no decomposition was observed even after standing in ethyl acetate for three weeks at room temperature.

Quantitative aspects

The derivative of taurine gave an excellent peak on an OV-17 column (Fig. 3). The calibration curve for taurine was conducted using APS, which showed a similar behaviour to taurine during the chemical reactions and was well separated from taurine on a chromatogram as the internal standard. Its linearity was observed in the range 5—500 nmol of taurine, and the relative standard deviation at each point ranged from 1.5 to 5.2% (n = 5), indicating that the reproducibility of analysis throughout the procedure is satisfactory.

Application to the determination of urinary taurine

In order to demonstrate the applicability of the method to biological materials, the content of taurine in human urine was analysed. A preliminary study indicated that the urinary components containing the carboxylic acid function such as amino acids and phenolic acids also provided respective peaks when they were converted into the corresponding isoBOC di-*n*-butylamide derivatives. However, these compounds could be clearly excluded as their isoBOC derivatives at the diethyl ether washing step of the analytical derivatization. Fig. 4 demonstrates the effectiveness of diethyl ether washing. Hippuric acid was also excluded by this washing. GC—MS analysis of the peaks of taurine and the internal standard from urine samples confirmed that each peak was almost uniform.

The recovery rates of taurine added to 0.1 ml of urine in the range 50—500 nmol were 98.0—105.8%, and their relative standard deviations were 1.2—

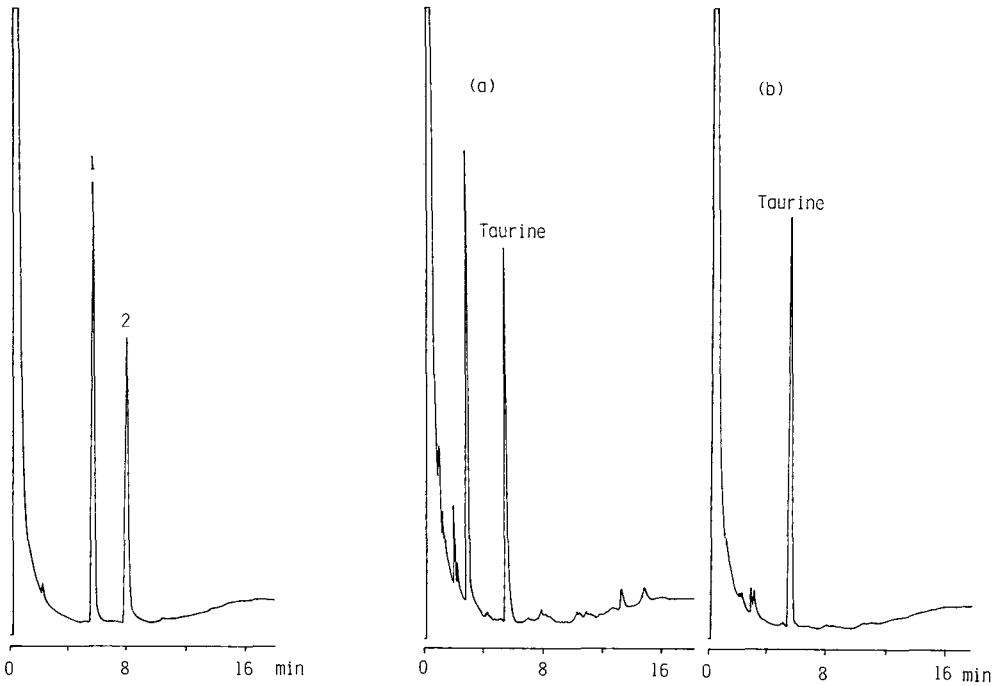

Fig. 3. Gas chromatogram obtained from a standard solution containing 250 nmol each of taurine and APS (internal standard). GLC conditions are given in Experimental. Peaks: 1 = taurine, 2 = APS.

Fig. 4. Gas chromatograms showing the effectiveness of diethyl ether washing on removal of interfering components in human urine: (a) without washing; (b) with washing. In each case, the injection aliquot is equivalent to 2 μl of the original urine and contains approximately 5 nmol of taurine.

TABLE II

RECOVERY OF TAURINE ADDED TO HUMAN URINE

Taurine added (μmol/ml)	Amount found (μmol/ml), mean ± S.D.* (n = 5)	R.S.D.** (%)	Recovery (%)
0	2.03 ± 0.10	4.9	
0.5	2.53 ± 0.03	1.2	100.0
1.0	3.01 ± 0.09	3.0	98.0
2.0	4.01 ± 0.12	3.0	99.0
5.0	7.32 ± 0.15	2.0	105.8
Mean ± S.D.*			100.7 ± 3.5

*Standard deviation.
**Relative standard deviation.

TABLE III

URINARY EXCRETION OF TAURINE IN NORMAL SUBJECTS

Sample number	Age (years)	Sex*	Taurine (μmol per 24 h)**
1	9	M	499
2	21	F	2357
3	22	F	506
4	22	F	801
5	23	M	625
6	27	M	1260
7	36	F	1682
8	41	M	2147
9	43	M	1733
10	52	M	809

*M = male, F = female.
**Each value represents an average of three analyses.

4.9% (n = 5), indicating that this method is very accurate and precise (Table II). The 24-h urinary concentrations obtained from ten healthy volunteers are shown in Table III.

In conclusion, these experiments have conclusively demonstrated that taurine can be accurately and precisely determined by GLC as its N-isoBOC di-n-butylamide derivative. Complex biological material such as urine can be analysed by this method without prior clean-up of the sample. We believe that this method provides a useful tool in biochemical and biomedical research requiring taurine assay.

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CHROMBIO. 1948

SENSITIVE GAS—LIQUID CHROMATOGRAPHIC PROCEDURE FOR URINARY Nτ-METHYLIMIDAZOLE ACETIC ACID, AN INDEX OF HISTAMINE TURNOVER

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SUMMARY

A simple and specific gas—liquid chromatographic procedure, compatible with both nitrogen—phosphorous and electron-capture detection, and employing conventional packed columns, has been devised for urinary 1-methylimidazole-4-acetic acid (Nτ-MeImAA), an index of whole-body histamine turnover. Nτ-MeImAA is isolated by ion-exchange on Dowex 1 (CH_3COO^-), esterified by reaction with chloroethanol—boron trichloride and, depending upon detection employed, chromatographed on base-deactivated SP-2401 or SP-2250. [^{3}H]Nτ-MeImAA serves as internal recovery standard.

INTRODUCTION

In those species, including man, in which ring N-methylation is the predominant route for the catabolism of histamine [1—3], the major urinary product is 1-methylimidazole-4-acetic acid (Nτ-MeImAA). (For nomenclature of N-substituted imidazoles, see ref. 4.) Its positional isomer 1-methylimidazole-5-acetic acid (Nπ-MeIAA) also occurs in urine, but is probably of dietary origin. Nτ-MeImAA comprises a remarkably constant fraction of urinary radioactivity following a bolus of labeled histamine [2]. While the fate of exogenous histamine may not necessarily reflect that of the biogenic material, quantitative measurements of endogenous metabolites [5, 6] support the view that Nτ-MeImAA is the most valid of several available indices of histamine formation/release in man.

The advent of α-fluoromethylhistidine (α-FMH), a specific, irreversible inhibitor of histidine decarboxylase [7], provides a therapeutic approach to histamine-related disease states alternative or adjunctive to histamine receptor

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antagonism. In animals, direct measurement of tissue histamine demonstrates that α-FMH effects acute reductions in the nascent or rapidly turning-over pools of brain [8, 9] and stomach [8, 10], and upon chronic treatment, time-dependent decreases in the relatively inert mast-cell pools (unpublished). As a non-invasive methodology for assessing effects of α-FMH in man, urinary Nτ-MeImAA appears to be a feasible alternative.

A number of procedures for urinary Nτ-MeIAA have been described, all but the last are too cumbersome for routine application. Gas chromatographic methods with flame-ionization detection of Nτ- and Nπ-MeIAA's as methyl esters [6, 11] and ethyl esters [12] require large urine volumes and long run times. Photometric methods employing thin-layer chromatographic separations [13, 14] are semi-quantitative. The capillary gas chromatographic method with nitrogen detection of the isopropyl ester described by Keyzer et al. [15] is not, in our hands, adaptable to the more convenient and accessible packed columns.

The present report describes a procedure, suitable for routine analysis of urinary Nτ-MeImAA as its chloroethyl ester, using packed columns and either of two sensitive detection systems, nitrogen—phosphorous detection (NPD), or electron-capture detection (ECD).

EXPERIMENTAL

Reagents

Nτ-Methylimidazole hydrochloride was obtained from Calbiochem; [^{3}H]-histamine from New England Nuclear; AG1-X8, 100—200 mesh was purchased in the Cl^- form from Bio-Rad Labs. and successively converted through the OH^- and CH_3COO^- forms as per manufacturer's instructions; boron trichloride—2-chloroethanol reagent (10%, w/v) was purchased from Applied Science Labs.; and ethyl acetate, nanograde, from Mallinkrodt.

Preparation of [^{3}H] Nτ-methylimidazole acetic acid.

Each of three mice were injected intravenously with ca. $6 \cdot 10^7$ dpm of [^{3}H(G)] histamine, 8.5 mCi/μmol, and urine collected through 24 h. This was adjusted to pH 8 and chromatographed on AG1-X8 (CH_3COO^-) (see below), 58% of total count being recovered in the acetic acid eluate. This was evaporated to dryness and streaked on Whatman K6 silica gel plates, which were developed with *n*-butanol—toluene—methanol—5% ammonium hydroxide (2:1:1.3:1). The major band corresponding to [^{3}H] Nτ-MeImAA was located by autoradiography at R_F = 0.47 and eluted with methanol to yield a radiochemically pure stock solution ($12 \cdot 10^6$ dpm/ml) of indeterminate but high specific activity, such that spiking of urine samples with 20,000 dpm thereof introduces a negligible mass of the compound of interest.

Isolation and derivatization of MeImAA

All manipulations through injection into the gas chromatograph are carried out in a single 125 × 16 mm disposable culture tube. To a 2.0-ml aliquot of urine are added 20,000 dpm of internal standard, a drop of 0.1% thymol blue indicator, and 2 *M* ammonium hydroxide to pH 8—9. The sample is decanted onto a 2.0-ml bed of AG1-X8 (CH_3COO^-) in a Bio-Rad disposable poly-

propylene column (No. 731-1550), followed by a wash of ca. 10 ml of distilled water, the perchlorate and wash then being discarded. The columns are positioned onto the original tubes and eluted with 5 ml of 0.75 *M* acetic acid. The tubes are transferred to a Model 3-2200 Buchler Vortex evaporator fitted with the 56-place heating block at 80° C and evaporated to dryness in vacuo. To the dried residue is added 200 μl of the chloroethanol—boron trichloride reagent, the block covered, and temperature raised to maximum setting while maintaining slight negative pressure to insure sealing. After 60 min, agitation and full vacuum are applied to reduce the reaction mixture to dryness.

After cooling, the residue is dissolved in 1.0 ml of 0.2 *M* hydrochloric acid, which is twice washed by vortexing with 5-ml portions of ethyl acetate, made alkaline with 100 μl of 6 *M* ammonium hydroxide, and extracted with 5 ml of ethyl acetate. A 0.5-ml aliquot of the final extract is taken for scintillation counting, and a second aliquot promptly sealed in an injection vial for gas–liquid chromatography (GLC).

If the samples cannot be chromatographed within 24 h, the bulk of the final ethyl acetate extract is transferred to a clean tube, evaporated to dryness in vacuo, and reconstituted shortly before analysis in a volume of ethyl acetate that is one-fifth of the original. A corresponding reduction is made in the aliquot taken for counting and for injection into the gas chromatograph.

For construction of a standard curve, a pooled urine sample was constituted by mixing equal volumes of every fifth urine specimen, and 2.0-ml aliquots thereof spiked in duplicate with unlabeled N_7-MeImAA at 0, 0.5, 1, 2, 3 and 5 μg/ml. These were thereafter treated in an identical manner with the unknown samples.

GLC conditions

An HP-5840A instrument fitted with a 36-place automatic sampler and both nitrogen—phosphorous and electron-capture detectors was employed. The following refers to use of NPD. For ECD, the same operating conditions apply except for column packing (SP-2250-DB), carrier gas (argon—methane, 95:5), and less stringent conditioning of the base-deactivated packing.

An 180 cm × 2 mm column of 3% SP-2401-DB on Supelcoport 100—120 mesh was conditioned at 250° C for three days with helium flow-rate at 30 ml/min, with periodic injections of 25 μl ethyl acetate, and a final injection of 20 μl Silyl-8®.

For analyses, helium flow-rate was at 20 ml/min, injector temperature 250° C, detector at 300° C, and oven temperature programmed for 5 min at 205° C then 5° C/min to 225° C, plus 3 min at the final temperature. Each sample was injected twice, with the automatic sampler adjusted to deliver 3 μl. Thus, a 24-h run will accommodate a standard curve series plus thirty unknown specimens, the preparation of which can readily be accomplished by one person in the course of a single working day.

RESULTS

In Fig. 1 are presented specimen chromatograms for a pure N_7-MeImAA sample, a representative urine, and the same urine spiked with a known concen-

tration of Nτ-MeIAA. That the urine peak at retention time, t_R = 2.72 min represents Nτ-MeImAA is confirmed by superimposition of it and the reference compound in the spiked sample. By analogy to the respective retention times of the Nτ- and Nπ-isomers of MeImAA in other systems [11, 15], it might be assumed that the urine peak at t_R = 4.42 min represents Nπ-MeIAA. This is confirmed by results of the GLC—mass spectrometric (MS) analyses described below.

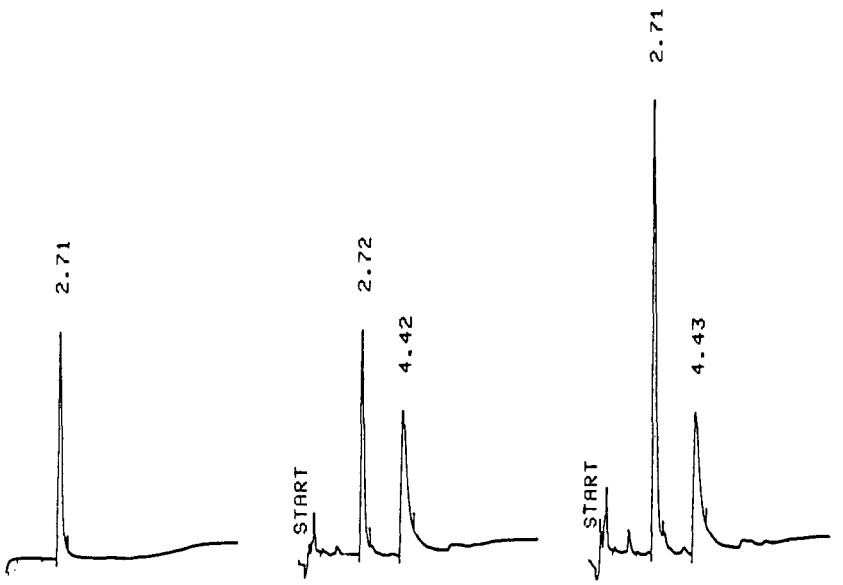

Fig. 1. Gas chromatograms (NPD) for, left to right: reference Nτ-MeIAA, 1.0 μg/ml in buffer; representative urine; and urine spiked with 1.0 μg/ml Nτ-MeIAA. Values above peaks denote retention times in minutes.

The same urine sample was analyzed by capillary GLC—MS employing an on-line HP-5710 gas chromatograph with a 25-m OV-17 fused silica capillary column, temperature programmed from 60°C to 230°C at 8°C/min, with helium as carrier gas at a flow-rate of 1 ml/min and interfaced to a VG—MM7035 mass spectrometer with dedicated 2035 data system. MS operating conditions were: ionization energy, 20 eV; accelerating voltage, 4 kV; and scan rate, 0.3 sec/decade.

A plot of total ionization current revealed surprisingly few peaks, major ones being centered at scan Nos. 1051 (22.4 min) and 1081 (22.8 min) (Fig. 2a). The mass chromatogram constructed for m/z = 202 (the molecular ion for either isomer of MeIAA) shows the same two peaks (Fig. 2b). Their respective mass spectra (Fig. 3a and b) show the same molecular ion at 202 with characteristic chlorine isotope cluster, and base peak at m/z = 95 without it, corresponding to removal of $-CO \cdot OCH_2CH_2Cl$. The pronounced tailing of the t_R = 4.42 min peak in the gas chromatogram is also evident in the mass chromatogram peak at t_R = 22.8 min, lending further support to its identification as Nπ-MeIAA.

A typical standard curve for pooled urine spiked with authentic Nτ-MeIAA

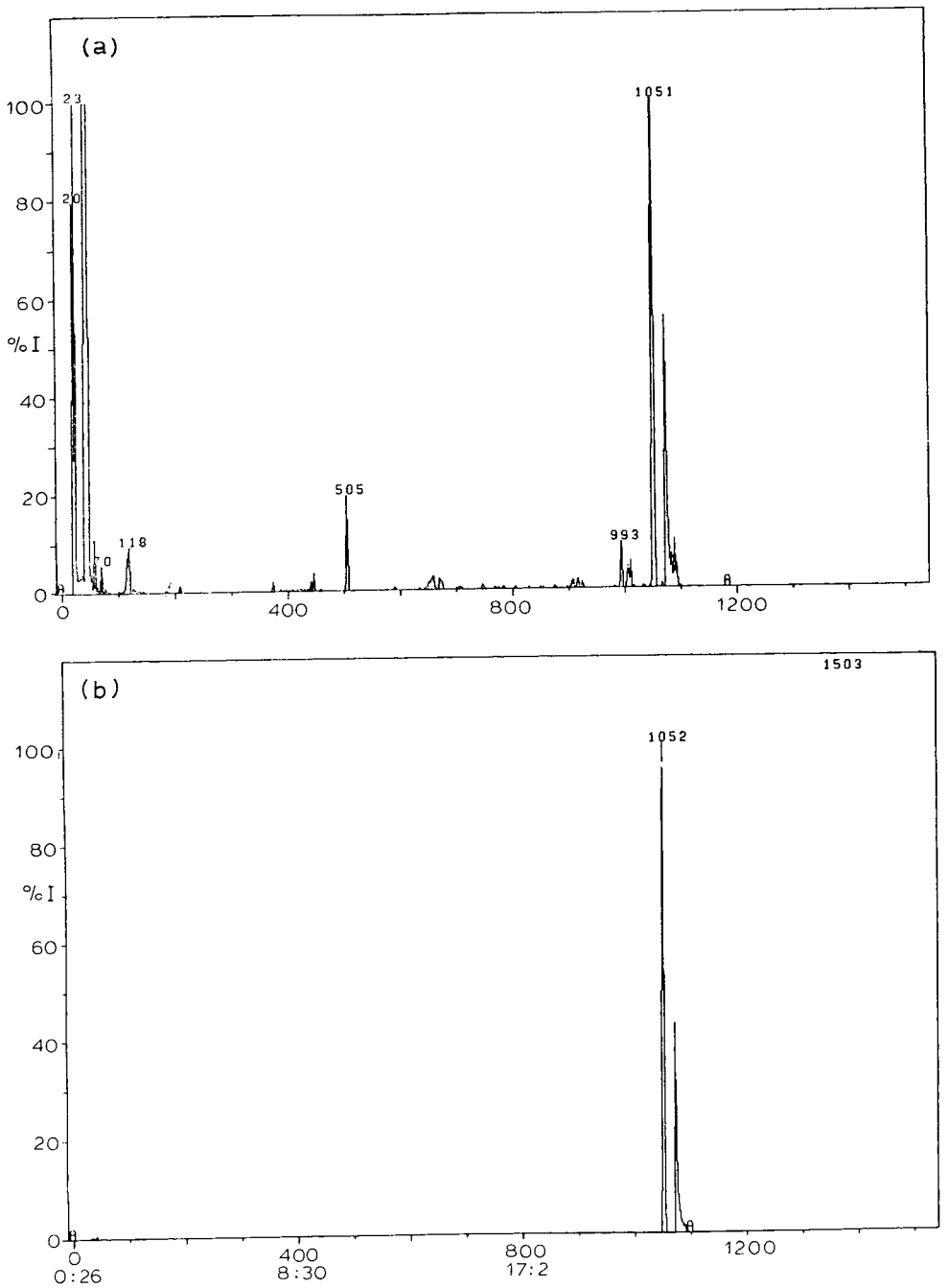

Fig. 2. Capillary gas chromatograms with MS detection for same urine as in Fig. 1. (a) total ionization current; (b) mass chromatogram constructed for molecular ion m/z = 202.

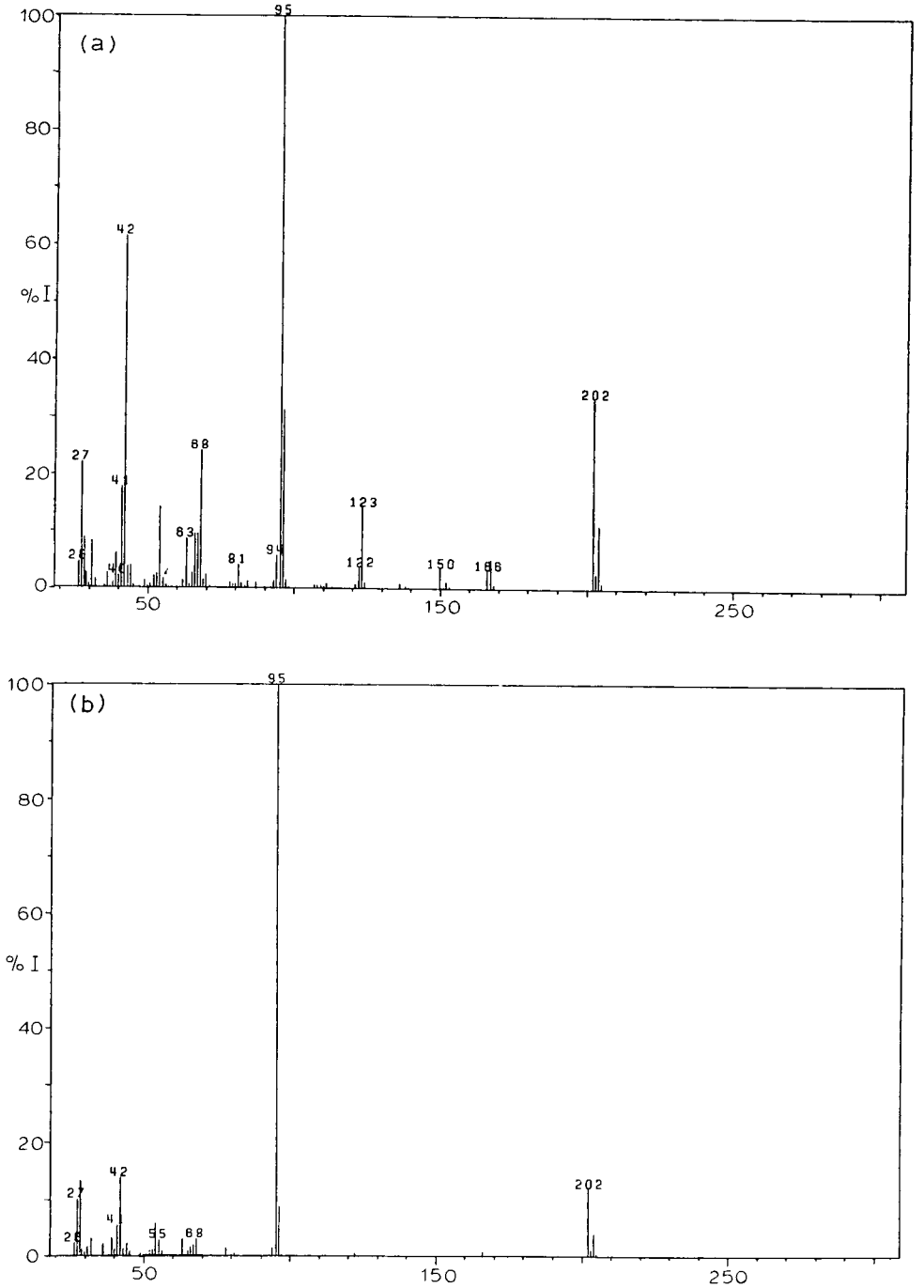

Fig. 3. Mass spectra for scans (a) No. 1052 (Nτ-MeImAA) and (b) No. 1081 (Nπ-ImAA).

(Fig. 4) demonstrates linearity between 2.87 μg/ml (the calculated endogenous level) and 7.87 μg/ml, the upper limit of which is in excess of all but one urine analyzed to date. For pure samples of Nτ-MeIAA in water, significant negative deviation from linearity occurs below 0.25 μg/ml.

Precision and reproducibility of the method were assessed by ten replicate analyses of two pooled urine samples on each of two occasions. For this purpose, as in routine use, standard curves were constructed from fresh dilutions of a frozen reference standard of Nτ-MeIAA (1 mg/ml), and detector sensitivity normalized with respect to fresh dilutions of a solution of Nτ-MeImAA chloroethyl ester, 830 μg/ml (by ^{14}C label). Intra-assay precision averaged 4.9%, and reproducibility between assays was < 1% (Table I), insuring the validity of storing of biological samples in the frozen state until assay.

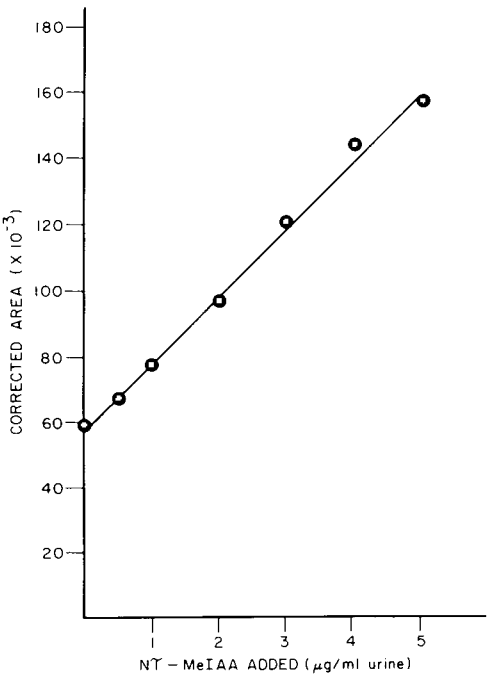

Fig. 4. Standard curve for Nτ-MeImAA added to pooled normal urine. Ordinate values are area at t_R = 2.7 min corrected for recovery of [^{3}H]Nτ-MeImAA[^{3}H].

TABLE I

PRECISION AND REPRODUCIBILITY OF ASSAY

Sample	Day	(μg/ml)			Relative S.D. (%)
		Range	Mean	S.D.	
1	3	1.69—1.95	1.83	0.10	5.5
	18	1.66—1.95	1.85	0.085	4.6
2	3	1.15—1.36	1.23	0.058	4.7
	18	1.17—1.34	1.25	0.055	4.6

TABLE II

DAILY URINARY EXCRETIONS OF $N\tau$-MeIAA AND ITS $N\pi$-ISOMER IN NORMAL MALE SUBJECTS

Subject No.	Day	Urine volume (ml)	Creatinine (mg/24 h)	$N\tau$-MeImAA (μg/24h)	$N\tau$-MeImAA/ creatinine (× 1000)	$N\pi$-MeImAA (μg/24 h)
1	1	1200	2724	2485	0.91	581
	7	990	1881	1926	1.02	nil
2	1	635	1829	1594	0.87	375
	7	1270	2718	3014	1.10	138
3	1	930	2348	1934	0.90	280
	7	1405	4468	2066	0.46	270
4	1	2180	2660	8590	3.23	735
	7	1630	2266	8095	3.57	nil
5	1	2265	2423	2537	1.05	nil
	7	2220	2775	2087	0.75	nil
6	1	1130	1808	5955	3.29	670
	7	1675	2260	8800	3.89	870
7	1	1062	2040	1485	0.73	87
	6	1835	2055	2605	1.26	691
	13	1815	2270	1580	0.70	154
	20	1950	1755	1365	0.78	263
	27	828	1610	1805	1.12	516
	34	910	2510	1765	0.70	235
8	1	985	1595	2445	1.53	3580
	6	1032	2350	16800	7.15	1090
	13	1440	2520	3010	1.19	4125
	20	530	1810	1675	0.92	1740
	27	832	2340	2413	1.03	2865
	34	2065	2975	3263	1.09	nil

The urines employed in development of this procedure were single voidings supplied on an ad libitum basis. In Table II are summarized 24-h excretion data for urines obtained in a controlled clinical environment from eight normal male volunteers; two subjects on six separate occasions, and six others on two occasions. Mean daily excretion of $N\tau$-MeImAA for all collections was 3720 ± 3590 μg/24 h, and excluding the one very atypical sample (subject No. 8, day 6), 3152 ± 2320 μg/24 h. Normalization of these data on the basis of corresponding creatinine excretion minimized intra-individual variations, but had no material effect upon inter-individual variation which, for this limited panel, is greater than the reported coefficients of variation imply for previous studies.

DISCUSSION

With the notable exception of the capillary GLC procedure recently described by Keyzer et al. [15], the present method offers obvious advantages

in terms of efficiency, sensitivity and precision, and with respect to the last, the relative convenience of using conventional packed columns. These, however, must be deactivated to minimize absorption to active sites typical of basic imidazoles. Each of the several base-deactivated packings available from Supelco was investigated, and SP-2401-DB and SP-2250-DB (compatible with NPD and ECD, respectively) were found to provide linear standard curves for Nτ-MeImAA to 0.25 μg/ml; i.e.: for an overall recovery of ca. 50% through the isolation and derivatization procedure, and an injection volume of 3 μl, 750 pg equivalents injected. By employing the pre-conditioning procedure specified above, no detrimental effects upon either NPD or ECD have been observed*

The chromatograms in Fig. 1 are typical of those for some fifty urines examined to date with respect to the absence of significant peaks other than those of the compound of interest, and in most samples its Nπ-isomer. That this is not solely a function of detector selectivity is evident in the total ionization current chromatogram (Fig. 2) which unlike, for example, gas chromatograms obtained with flame-ionization detection (see refs. 6, 11, 12), contains no signals of intensity comparable to that of the compound of interest. This apparent enhancement in specificity of the isolation procedure is at the expense of recovery of the compound of interest, overall recoveries being low, but fairly reproducible, at 45 ± 9% for the [^{3}H] Nτ-MeImAA spike.

The present procedure yields values for urinary Nτ-MeImAA for normal man in substantial agreement with previously published ones [6, 12, 15]. Also consistent with prior reports is the high variability in excretion of its Nπ-isomer, both inter- and intra-individually. This variation, independent of Nτ-MeImAA or creatinine excretion lends further credence to the assumption that the Nπ-MeImAA is of dietary origin.

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*Base-deactivated packings are not recommended by manufacturer for use with NPD without extensive pre-conditioning.

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DETERMINATION OF FREE TRIMETHYLLYSINE IN PLASMA AND TISSUE SPECIMENS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the determination of 6-N-trimethyllysine in tissues and plasma is described. Trimethyllysine and the chemically analogous 6-N-triethyllysine (internal standard) were isolated from acid-soluble fractions of tissue homogenates or plasma by combined ion-exchange—ion-exclusion chromatography. Trimethyllysine and triethyllysine were separated from other sample constituents by reversed-phase ion-pair high-performance liquid chromatography, derivatized post-column by reaction with *o*-phthalicdicarboxaldehyde and 2-mercaptoethanol, and detected fluorometrically. Standard curves were linear over a sample concentration range of 0.5—4 nmol/ml. The detection limit corresponded with 25 pmol trimethyllysine injected into the chromatograph. The procedure was used for the determination of trimethyllysine in plasma, liver, kidney, and mixed skeletal muscle of rat.

INTRODUCTION

The amino acid 6-N-trimethyllysine occurs in numerous proteins, including histones [1] and myosin [2]. Trimethyllysine is formed in mammals by the action of protein methylase III and S-adenosylmethionine upon selected lysine residues in specific proteins [3]. Once released from protein during proteolysis, trimethyllysine is known to be metabolized via the carnitine biosynthetic pathway [4—6] and to be excreted in the urine [7]. Carnitine [3-hydroxy-4-(N,N,N-trimethylammonio) butanoate] is an essential cofactor in mitochondrial long-chain fatty acid oxidation. The control of the fate of trimethyllysine is not understood, in large part, owing to the lack of sensitive analytical methods. The determination of free trimethyllysine is important not only in

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understanding trimethyllysine metabolism but in the delineation of the regulation of carnitine biosynthesis.

Trimethyllysine has been determined by a number of lengthy procedures of varying sensitivity incorporating ion-exchange chromatography and traditional amino acid analysis [8—14]. We have previously developed a sensitive method for the determination of urinary trimethyllysine concentrations by high-performance liquid chromatography (HPLC) [15]. However, this method does not provide a limit of sensitivity adequate for practical measurement of free trimethyllysine in plasma or tissue specimens.

The reaction of primary amines with *o*-phthalicdicarboxaldehyde and alkanethiols to produce intensely fluorophoric 1-alkylthio-2-alkyl-isoindoles [16] has been applied in the development of very sensitive methods for determination of amino acids at detection limits far lower than possible when ninhydrin or fluorescamine are used as derivatizing agents [17, 18]. We have applied this derivatization process in the development of a method for the determination of free trimethyllysine concentrations in plasma and tissue specimens. In this procedure, trimethyllysine and a chemically analogous internal standard are isolated from plasma and tissue homogenates by protein precipitation and combined ion-exchange—ion-exclusion chromatography of the protein-free supernatant. Determination is accomplished by reversed-phase ion-pair HPLC, postseparation column derivatization by reaction with *o*-phthalicdicarboxaldehyde and 2-mercaptoethanol, and fluorometric detection.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a single Model M6000A pump, a Model U6K syringe loading injection valve, and an RCM-100 radial compression module purchased from Waters Assoc. (Milford, MA, U.S.A.). A precolumn constructed of zero dead volume chromatographic fittings (Crawford Fitting, Solon, OH, U.S.A.) and packed with Co:Pell ODS reversed-phase pellicular chromatographic medium (Whatman, Clifton, NJ, U.S.A.) preceded the column compression unit in the eluent stream. The chromatographic separation was accomplished on a 10 × 0.5 cm cartridge of 10 μm nominal particle diameter Radial-Pack C_{18} (Waters). The postcolumn derivatization reagent solution was delivered by a Milton-Roy minipump (Glenco Scientific, Houston, TX, U.S.A.), and introduced within the eluent stream through a zero dead volume T fitting (Waters). A 50 × 0.023 cm coil of steel tubing provided a short reaction time delay prior to detection. A Kratos-Schoeffel Instruments (Westwood, NJ, U.S.A.) Model SF-970 fluorescence detector was used for eluent fluorescence measurements. The detector output signal was recorded by a Linear Instruments (Irvine, CA, U.S.A.) Model 291 chart recorder. Peak area integration and height measurements were performed by a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354 laboratory automation system. Liquid scintillation counting was performed with a Packard Instruments (Downers Grove, IL, U.S.A.) PRIAS liquid scintillation counter. A Brinkman (Des

Plaines, IL, U.S.A.) Polytron tissue homogenizer, DuPont/Sorvall (Newtown, CT, U.S.A.) table-top centrifuge and a Buchler Instruments Evapomix evaporator (distributed by Fisher Scientific, Cleveland, OH, U.S.A.) were used during sample preparation.

Materials

MCB OmniSolv acetonitrile (non-UV grade) was purchased from Curtin Matheson Scientific (Cleveland, OH, U.S.A.). Sodium dodecyl sulfate (electrophoresis grade) was obtained from Gallard-Schlesinger (Carle Place, NY, U.S.A.). Hydrochloric acid, ammonium hydroxide, sodium hydroxide, and sodium dihydrogen phosphate were obtained from Fisher Scientific. Boric acid, *o*-phthalicdicarboxaldehyde, and the polyether surfactant Brij-35 were obtained from Aldrich (Milwaukee, WI, U.S.A.). The ion-exchange resins Dowex 50W-X8 (200—400 mesh, H^+) and Dowex 1-X8 (200—400 mesh, Cl^-) were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). These resins were washed with water and converted to their ammonium and hydroxide forms, respectively, according to the vendor's instructions. Water was prepared for use as a chromatographic eluent constituent by passage through a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.).

Trimethyllysine was prepared as described previously [4]. 6-N-Triethyllysine was prepared for use as an analytical internal standard by a modification of the same procedure in which iodoethane replaced iodomethane as the alkylating agent. 3-Hydroxy-6-N-trimethyllysine was prepared in our laboratory by a procedure which will be reported elsewhere. [^{14}C-Methyl]trimethyllysine was prepared as reported [4]. Bovine serum albumin, 1-N-methylhistidine and 3-N-methylhistidine were obtained from Sigma (St. Louis, MO, U.S.A.). N^G-Methylarginine and N^G,N'^G-dimethylarginine were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). 6-N-Methyllysine and 6-N-dimethyllysine were purchased from Chemical Dynamics (South Plainfield, NJ, U.S.A.).

Aqueous stock solutions of trimethyllysine and triethyllysine were prepared and standardized spectrophotometrically [19]. Standard solutions of trimethyllysine for use in both plasma and tissue determinations were prepared by serial dilution of the stock solutions with a 2% (w/v) aqueous solution of bovine serum albumin.

Sample preparation

Plasma specimens. Whole blood was collected in heparinized tubes, chilled on ice and centrifuged at 1500 *g* for 10 min. A 2-ml volume of chilled plasma or standard solution was combined with 0.5 ml 12% perchloric acid and 350 μl of triethyllysine internal standard working solution in a 16.8 × 95 mm disposable polypropylene test tube. The tubes were allowed to stand on ice for 1 h and then centrifuged at 1500 *g* for 10 min. The supernatant was transferred to another 16.8 × 95 mm polypropylene test tube containing 0.5 ml of cold 2 *M* potassium bicarbonate, vortexed, placed on ice for 1 h, and centrifuged at 1500 *g* for 10 min. Volumes of all solutions used in sample preparation were adjusted in direct proportion to sample size when less than 2 ml of plasma were harvested from the blood specimens.

Columns fashioned from 5-ml disposable (10 × 116 mm) polypropylene pipette tips (Kew Scientific, Columbus, OH, U.S.A.) were filled in sequence with 2.5 ml Dowex 1-X8 (OH^-) and 2.5 ml Dowex 50W-X8 (NH_4^+) ion-exchange resins; the Dowex 1-X8 layer was allowed to settle completely before addition of the Dowex 50W cation exchanger. The entire sample supernatant was applied to the column. Excluded and weakly retained species were eluted with 6 ml of 1 *M* ammonium hydroxide. Trimethyllysine and the internal standard then were eluted with 3 ml of 1 *M* ammonium hydroxide and 5 ml of deionized water. The combined 8 ml of column effluent were evaporated to dryness under vacuum in a Buchler Evapomix (water bath at 38° C) and reconstituted in 250 μl of 10^{-3} *M* hydrochloric acid before injection into the liquid chromatograph.

Tissue specimens. To 1 g of rat tissue (liver, kidney, heart, or mixed skeletal muscle) were added 3 ml of ice cold 6% perchloric acid. This mixture was homogenized by the Brinkman Polytron homogenizer operated at setting 6 for 40 sec; 2 ml of the suspension were combined with 350 μl of the triethyllysine internal standard working solution in a 16.8 × 95 mm disposable polypropylene test tube. The tubes were placed on ice for 1 h, centrifuged for 10 min at 1500 *g*, and the supernatant was transferred to another 16.8 × 95 mm polypropylene test tube containing 0.5 ml of 2 *M* potassium bicarbonate. This in turn was vortexed, placed on ice for 1 h, and centrifuged as above. The supernatant liquid was decanted from the potassium perchlorate precipitate and subjected to ion-exchange ion-exclusion chromatography exactly as were the plasma specimens. The combined effluent fractions were evaporated to dryness under vacuum in a Buchler Evapomix, with the water bath at 38° C. The sample was reconstituted in 250 μl 10^{-3} *M* hydrochloric acid for injection into the chromatograph.

Chromatography

The chromatographic eluent was 30%, v/v acetonitrile in water containing $5 \cdot 10^{-2}$ *M* sodium dodecyl sulfate and $5 \cdot 10^{-2}$ *M* sodium dihydrogen phosphate. To 700 ml of water were added 6.9 g of sodium dihydrogen phosphate and 14.4 g of sodium dodecyl sulfate. The solution was adjusted to pH 2.75 with concentrated sulfuric acid, filtered through a 0.45-μm nominal pore diameter cellulose acetate membrane, and 300 ml of filtered acetonitrile were added with thorough magnetic stirring. The derivatization reagent solution was prepared by dissolving 300 mg of *o*-phthalicdicarboxaldehyde and 500 μl of 2-mercaptoethanol in 10 ml of a 3%, v/v solution of Brij-35 in 95% ethanol and adding this to 500 ml of a 0.5 *M* sodium borate solution of pH 10.4. The eluent and derivatization reagent solutions were delivered at flow-rates of 3.0 ml/min and 1.2 ml/min, respectively. The fluorometric detector was operated at an excitation wavelength of 229 nm with an emission filter cutoff wavelength of 418 nm.

Quantitation

Standard curves of trimethyllysine:triethyllysine peak height or area ratios versus concentration were established daily over a trimethyllysine standard

solution concentration range of 0.5 to 4.0 nmol/ml. Trimethyllysine concentrations in experimental samples were interpolated from the least-squares regression line through the standard data points. All standard and experimental samples were analyzed in duplicate.

RESULTS AND DISCUSSION

This study was designed for the development of a simple and selective method for the determination of free 6-N-trimethyllysine in plasma and tissue specimens. Sample protein was removed effectively by perchloric acid precipitation. Excess perchlorate was precipitated as its potassium salt during the subsequent sample neutralization with potassium bicarbonate. In a previous study [15], we removed large quantities of interfering acidic and neutral amino acids from urine samples by ion-exclusion chromatography over small columns of Dowex 1-X8 (OH^-) anion-exchange resin prior to derivatization of quaternary ammonio acids and unretained basic amino acids. However, the greater sensitivity of the fluorometric detector revealed that small but troublesome quantities of other primary amines were isolated with trimethyllysine and the internal standard.

The necessary degree of sample simplification was achieved with a larger column containing cation-exchange and anion-exchange resins in serial combination. Elution of this column with 1 *M* ammonium hydroxide resulted in the expected exclusion of basic amino acids and quaternary ammonio acids by the anion-exchange resin, and their partial resolution by cation-exchange chromatography. Recovery of applied [^{14}C-methyl]trimethyllysine from this column was found to be 97% (± 2.2%; $n = 3$).

The reversed-phase, ion-pair separation mechanism can provide excellent chromatographic selectivity for quaternary ammonio acids. In our experience, such separation systems have shown long-term stability and unusual tolerance for sample contaminants. We experimented with several alkylsulfate and alkylsulfonate ion-pairing agents. Sodium dodecyl sulfate of electrophoresis grade was found to provide both greatest chromatographic efficiency for the separation of trimethyllysine from other sample constituents and least mobile phase background fluorescence. Retention of trimethyllysine and triethyllysine was found to vary as expected with changes in the acetonitrile concentration of the mobile phase. Tailing of chromatographic peaks was reduced by inclusion of $5 \cdot 10^{-2}$ *M* sodium dihydrogen phosphate in the aqueous portion of the eluent. The absolute retention of trimethyllysine and the internal standard was found to be sensitive to small alterations in eluent pH from the selected value of 2.75. Recovery of injected [^{14}C-methyl]trimethyllysine through the injector, precolumn, and separation column of the liquid chromatograph was 97% (± 2.7%, $n = 3$). The detection limit corresponded with 25 pmol of trimethyllysine injected into the chromatograph.

The retention of N-methylated amino acids by the ion-exchange—ion-exclusion sample preparation column was investigated. Aqueous solutions of 3-hydroxytrimethyllysine, triethyllysine, 6-N-mono-, di-, and trimethyllysine, 3-N- and 1-N-methylhistidine, and N^G-methyl- and N^G,N'^G-dimethylarginine

TABLE I

RETENTION CHARACTERISTICS OF VARIOUS N-METHYLATED AMINO ACIDS

A 2-nmol amount of each amino acid was applied to and eluted from the sample preparation column. The column effluent was evaporated to dryness and reconstituted in 250 μl of 10^{-3} *M* hydrochloric acid. A second standard portion of 2 nmol of the same amino acid was similarly dried and reconstituted in a second test tube. In succession, a 30-μl aliquot of each sample was injected into the liquid chromatograph. Fractional elution of each amino acid is expressed as the ratio of its chromatographic peak height in the first sample to that in the second, times 100%. These experiments were performed in duplicate.

Amino acid	Percent elution through double resin column	k' (HPLC)
1-N-Methylhistidine	ND*	2.30
3-N-Methylhistidine	ND	2.86
3-Hydroxy-6-N-trimethyllysine	100	3.30
6-N-Methyllysine	ND	4.33
6-N-Dimethyllysine	ND	4.77
6-N-Trimethyllysine	100	5.13
N^G-Methylarginine	30	7.48
N^G,N'^G-Dimethylarginine	58	7.56
6-N-Triethyllysine	100	11.11

*ND = not detected.

were prepared at a 20 nmol/ml concentration. A 2-nmol amount of a single amino acid was applied to the sample preparation column and eluted by the usual series of eluents. Trimethyllsyine, triethyllysine, and 3-hydroxytrimethyllysine were unretained by the sample preparation column, while about 30% of the applied N^G-methylarginine and 60% of the N^G,N'^G-dimethylarginine were eluted. The methylhistidines and the other methyllysines were completely retained by the sample preparation column. These data are summarized in Table I.

The N-methylated amino acids were chromatographed individiually for comparison of their HPLC retention with that of trimethyllysine and triethyllysine. 3-Hydroxytrimethyllysine and the methylhistidines all were retained less strongly than trimethyllysine. The methylarginines eluted between trimethyllysine and triethyllysine, completely resolved from both compounds. 6-N-Methyllysine and 6-N-dimethyllysine were retained less strongly than trimethyllysine. However, 6-N-dimethyllysine was unresolved from trimethyllysine. As seen in Table I, this presented no problem, as 6-N-dimethyllysine was completely retained by the sample preparation column.

Standard curves of trimethyllysine:triethyllysine peak height ratios versus sample trimethyllysine concentration were found to be linear over a sample concentration of 0.5—4.0 nmol/ml trimethyllysine (r^2 = 0.98) with a slightly positive *Y* intercept of 0.07. This intercept value was less than 22% of the peak height ratio obtained for the standard solution containing the smallest trimethyllysine concentration used for standard curve definition. Slopes of five replicate standard curves established on successive days had a relative standard

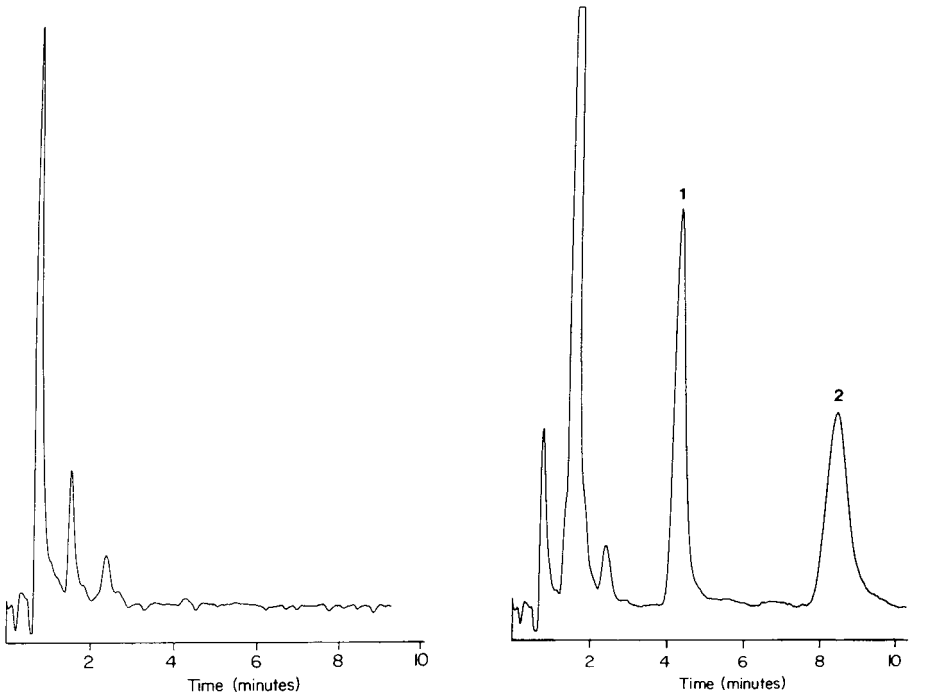

Fig. 1. Chromatogram obtained after complete preparation of an aliquot of 2% (w/v) bovine serum albumin (BSA) according to the described procedure. The column was a 10 × 0.5 cm cartridge of 10-μm nominal particle diameter Radial-Pak C_{18} (Waters). The chromatographic eluent was 30%, v/v acetonitrile in water containing $5 \cdot 10^{-2}$ *M* sodium dodecyl sulfate and $5 \cdot 10^{-2}$ *M* sodium dihydrogen phosphate. The aqueous component was adjusted to pH 2.75 prior to addition of acetonitrile. The chromatographic eluent was pumped at 3.0 ml/min, while the derivatizing reagent solution was pumped at 1.2 ml/min. The fluorometric detector was operated at an excitation wavelength of 229 nm with an emission cutoff filter of 418 nm. The full scale of the ordinate is 1 μA.

Fig. 2. Chromatogram of a derivatized standard solution of trimethyllysine, containing 5.0 nmol/ml trimethyllysine in 2% BSA, which included the internal standard, triethyllysine. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine; 2 = triethyllysine.

deviation (R.S.D.) of 6%. The R.S.D. in trimethyllysine:triethyllysine peak height ratios obtained for five replicate injections of a single prepared standard solution containing 2 nmol/ml trimethyllysine was 5.7%. The R.S.D. observed in three replicate determinations of a single plasma specimen containing 2.0 nmol trimethyllysine per ml plasma was 4.9%. For three determinations of a kidney specimen containing 2.1 nmol trimethyllysine per g kidney, the R.S.D. was 5.5%.

Fig. 1 is a chromatogram obtained upon preparation of a 1-ml aliquot of the 2% (w/v) aqueous solution of bovine serum albumin employed for preparation of the standards. Fig. 2 is a chromatogram of a standard solution containing 5 nmol/ml trimethyllysine, and triethyllysine. A typical chromatogram obtained upon preparation of a rat plasma specimen is shown in Fig. 3, while a chromatogram obtained after preparation of a rat muscle specimen is reproduced in Fig. 4.

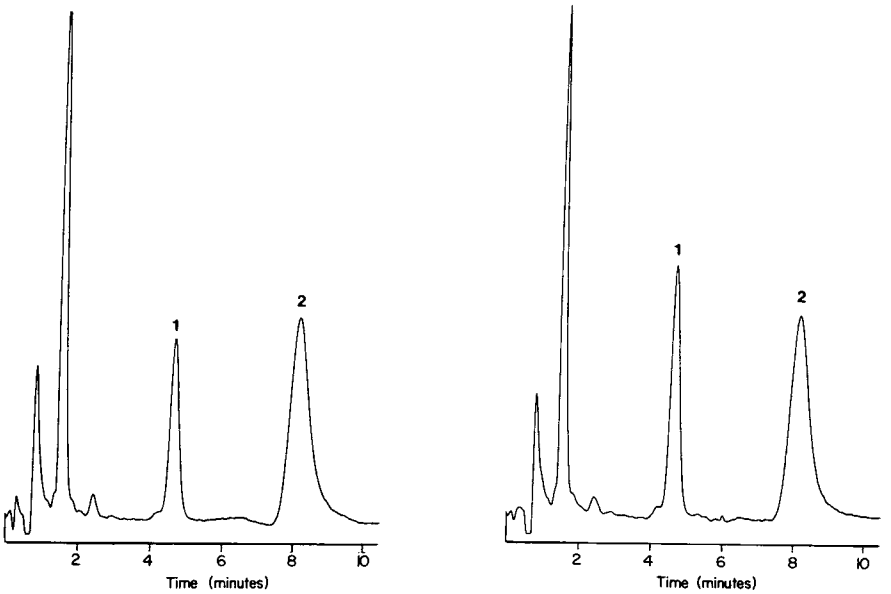

Fig. 3. Chromatogram of a prepared derivatized rat plasma specimen containing 2.0 nmol/ml free trimethyllysine. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine; 2 = triethyllysine.

Fig. 4. Chromatogram of a prepared derivatized rat muscle specimen calculated to contain 16.3 nmol/g free trimethyllysine. The chromatographic conditions were as described in Fig. 1. Peaks: 1 = trimethyllysine; 2 = triethyllysine.

TABLE II

FREE TRIMETHYLLYSINE IN VARIOUS TISSUES AND PLASMA OF RAT

Five rats were killed by decapitation. Whole blood was collected immediately in heparinized tubes and chilled on ice. Tissues to be removed (liver, kidney, skeletal muscle) were frozen between aluminum blocks which had been cooled in a dry ice—acetone bath. Samples were processed and analyzed as described in the text. Values are expressed as the mean ± the standard error of the mean for four replicate determinations upon skeletal muscle and five replicate determinations upon each of the other tissues.

Tissue	Free trimethyllysine
Plasma (nmol/ml)	1.9 ± 0.1
Liver (nmol/g)	3.2 ± 0.2
Kidney (nmol/g)	2.7 ± 0.1
Muscle (nmol/g)	19.3 ± 1.2

Rats fed a trimethyllysine limiting diet (7.9 nmol trimethyllysine per g diet) for seven days were stunned and decapitated. Blood was collected, and specific organs were removed for determination of free trimethyllysine. The plasma concentration of trimethyllysine in the rat is similar to that found in the chicken [20] and about twice that found in the dog [21]. Concentrations of free trimethyllysine in rat liver, kidney, and skeletal muscle are low, amounting to approximately 1% of free lysines in these tissues [22]. These data are

summarized in Table II. Results of our study of the changes in free trimethyllysine concentrations in the rat during starvation will be reported elsewhere [23].

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SIMPLE AND MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS DETERMINATION OF *p*-AMINOHIPPURIC ACID AND IOTHALAMATE IN BIOLOGICAL FLUIDS

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SUMMARY

A simple, rapid and micro high-performance liquid chromatographic method was developed for separate or simultaneous determination of *p*-aminohippuric acid and iothalamate in plasma and urine using *p*-aminobenzoic acid as an internal standard. The method involved deproteinizing samples with two volumes of acetonitrile followed by injection of 5 μl of deproteinized supernatant onto a C_{18} reversed-phase column. The mobile phase contained 3.5% acetonitrile in 0.04% phosphoric acid and flowed at a rate of 1.5 ml/min. The column effluent was monitored by an ultraviolet detector at 254 nm. Retention times for *p*-aminohippuric acid, iothalamate and *p*-aminobenzoic acid were approximately 4.5, 6 and 8 min, respectively. This method requires as little as 5 μl of sample and can be used to measure accurately down to 1 μg/ml *p*-aminohippuric acid and 0.5 μg/ml iothalamate in plasma samples. The coefficients of variation of the assay with or without the use of internal standard were generally low (below 7%). No interferences from endogenous substances or any drugs tested were found.

INTRODUCTION

Determination of glomerular filtration rate and/or renal plasma flow is often useful in pharmacokinetic and pharmacodynamic studies as well as in the diagnosis and treatment of disease [1]. To date, inulin and creatinine appear to have been most commonly employed for estimation of glomerular filtration rate. Potential disadvantages of using the inulin method are the laborious sample preparation, the non-specificity of the colorimetric assay and the need for a sensitive spectrophotometer. Also, in order to achieve steady-state plasma concentrations which can be accurately measured, large doses of inulin are

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usually required for intravenous infusion. In this regard, it seems important to note that the single-injection method with venous sampling might be unsatisfactory, due to potential arterial—venous plasma concentration differences of inulin [2]. Although no administration of exogenous creatinine is required, the use of creatinine clearance for accurate measurement of the glomerular filtration rate has been recently questioned [3]. Contrary to the common notion, creatinine may be extensively secreted and reabsorbed by renal tubules in humans and animals with normal or impaired renal function [4]. Reduction in urine flow or the presence of certain disease states could decrease the creatinine clearance due to enhanced tubular reabsorption, and coadministration of drugs might compete with its tubular secretion. It was also shown that creatinine could be significantly eliminated by non-renal routes [5]. In addition, some commonly used colorimetric assays of creatinine may not be specific, often resulting in a considerable overestimation of plasma levels [6, 7].

The introduction of labeled iothalamate as a substitute for inulin appears very attractive [8, 9]. Its major drawbacks are those associated normally with the use of radioactive compounds, including risks to patients, special storage and handling, and the need for a sensitive detecting instrument. Recently, a highly sensitive high-performance liquid chromatographic (HPLC) method has been reported for the quantitation of low concentrations of non-radioactive iothalamate in plasma and urine [10]. This method is, however, quite tedious, involving four ethyl acetate extractions, and two evaporations of about 10 ml of extract. It also requires 0.5—1.0 ml of sample, and a variable-wavelength ultraviolet (UV) detector (set at 235 nm).

For determination of renal plasma flow, *p*-aminohippuric acid (PAH) has been the most frequently used agent to date [1, 11]. Earlier colorimetric assay methods based on the color reaction between PAH and a reagent have been known to be non-specific and relatively time-consuming [12]. Several HPLC methods have been published in recent years. The method reported by Brown et al. [13] employed direct injection of biological samples which might be detrimental to the column. Their HPLC system had an unusually unstable baseline, necessitating the preparation of buffer solution 12—24 h in advance and storage of mobile phase in the pump. The sensitive method of Shoup and Kissinger [12] employed direct injection of biological samples diluted with distilled water, and a less commonly available and less easy-to-operate electrochemical detector. The method of Gloff et al. described in an abstract [14] appeared adequate for most routine analyses. They employed a high percentage (50%) of acetonitrile in the mobile phase, and the method had a coefficient of variation up to about 14%. A capillary gas chromatographic method reported recently [15] used a special nitrogen—phosphorus detector. The assay also required 1 ml of plasma, ether extraction and derivatization.

The purpose of this paper is to report a simple, rapid and micro HPLC method for separate or simultaneous quantitation of iothalamate and PAH in biological fluids using a fixed-wavelength UV detector.

EXPERIMENTAL

Reagents and standards

PAH (as a sodium salt) was purchased from Sigma (St. Louis, MO, U.S.A.), iothalamate (as an acid form) was purchased from Mallinkrodt (St. Louis, MO, U.S.A.) and *p*-aminobenzoic acid (PABA) from Mann Research Lab. (New York, NY, U.S.A.). Phosphoric acid (85%) and HPLC grade acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Standard stock solutions of PAH, iothalamate and PABA were prepared in distilled water at concentrations of 0.5—2 mg/ml and stored at 4°C in a refrigerator. All the solutions were used within a few weeks because a slight decomposition of PAH was found after 5—6 weeks of storage.

HPLC instrumentation

The HPLC system consisted of a solvent delivery pump (Model 110A), a fixed-wavelength detector with a 254-nm filter (Model 160) from Beckman Instruments (Berkeley, CA, U.S.A.), a syringe loading sample injector (Model 7215, Rheodyne, Cotati, CA, U.S.A.) and a 30-cm μBondapak C_{18} reversed-phase column (particle size 10 μm, I.D. 4.1 mm) from Alltech Associates (Deerfield, IL, U.S.A.). The output from the detector was connected to a 10-mV potentiometric 25-cm recorder (Linear Instruments, Irvine, CA, U.S.A.). A chart speed of 10 cm/h was employed for routine analysis [16]. The detector was set at 0.002—0.01 and 0.005—0.05 a.u.f.s. for plasma and urine analyses, respectively.

Mobile-phase preparation

The mobile phase was prepared by mixing 3.5 parts of acetonitrile with 96.5 parts of 0.04% phosphoric acid solution (pH 2.5 ± 0.05). A flow-rate of 1.5 ml/min resulted in a pump pressure of approximately 84 bar. The optimal percentage of acetonitrile might vary slightly with the column.

Standard curves

Aliquots of 100 μl of pooled human plasma spiked with various known quantities of stock solutions of PAH, iothalamate, and PABA were pipetted into 100 × 13 mm screw-capped culture tubes followed by the addition of 200 μl of acetonitrile. After capping, each tube was vortexed for a few seconds and centrifuged at 800 *g* for 5 min. The clear supernatant, 5 μl, was then injected onto the column. Urine samples were prepared in the same manner. The concentrations of PAH and iothalamate ranged from 2.5 to 50 μg/ml in plasma, and 10 to 100 μg/ml in urine. The internal standard concentrations were 5 or 25 μg/ml and 25 or 50 μg/ml in plasma and urine, respectively.

The peak heights, measured with the assistance of a micrometer (Dial Caliper from Manostat, NY, U.S.A.), were used for quantitation and the ratios of peak heights of PAH or iothalamate to those of the internal standard were used to construct the standard curves.

Reproducibility study

Six replicate analyses of pooled human plasma containing 15 or 30 μg/ml

PAH and iothalamate were analyzed according to the method described earlier.

Recovery study

The recovery was assessed by comparing the absolute peak height of PAH or iothalamate from plasma and urine samples to those obtained by direct injection of aqueous solutions at concentrations of 15 and 30 μg/ml for each compound.

Drug interference study

Many drugs and a potential metabolite of PAH, N-acetyl-*p*-aminohippuric acid (N-acetyl-PAH) [17, 18], were tested for interference with the assay by injecting stock solutions of these compounds onto the column. The drugs tested included those which may produce nephrotoxicity [19] (e.g. *p*-aminosalicylic acid, isoniazid, kanamycin, gentamycin, tobramycin, amikacin, trimethadione) and those which can interfere with PAH determination using colorimetric methods [12] (e.g. methyldopa, sulfathiazole, etc.). Acetaminophen, aspirin, propranolol, acetazolamide, tolazamide, hydralazine and methotrexate were also tested. The N-acetyl-PAH was prepared according to the method of Newman et al. [18].

Preliminary study in dog.

A 5% sterile iothalamate solution was prepared by dissolving an equimolar ratio of iothalamate and sodium hydroxide in water followed by autoclaving. The sterile PAH solution (1% w/v in 0.9% sodium chloride) was prepared by filtering through a 0.2-μm filter unit (Millex-FG from Millipore, Bedford, MA, U.S.A.). Aliquots of each solution in 0.9% sodium chloride (equivalent to 180.5 mg of PAH and 219.2 mg of iothalamate) were then simultaneously infused into the cephalic vein of a male mongrel dog (10.1 kg) over 40 min. Venous blood samples were collected at appropriate times and were immediately centrifuged at 800 *g* for 5 min to separate the plasma. Urine samples were collected at 30-min intervals. All the samples were stored frozen until analyzed (within one week). Blank samples of plasma or urine were obtained before drug administration and processed in a similar manner. Standard curves were constructed using blank samples from the same dog.

RESULTS AND DISCUSSION

Chromatograms from typical blank human plasma and urine, and from those containing known concentrations of PAH, iothalamate, and PABA are shown in Fig. 1, while those from plasma and urine samples obtained from the dog given 180.5 mg of PAH and 219.2 mg of iothalamate are depicted in Fig. 2. The retention times of PAH, iothalamate and PABA were approximately 4.5, 6 and 8 min, respectively. No interfering peaks were found in various plasma and urine blanks examined. Although there was an endogenous peak found between the iothalamate and PABA peaks in dog plasma, it did not affect the present assay. Another endogenous peak appeared from both human and dog plasma with a retention time of 18—19 min. In order to save time between analyses, one can inject the second sample right after the appearance of the PABA peak from the first sample.

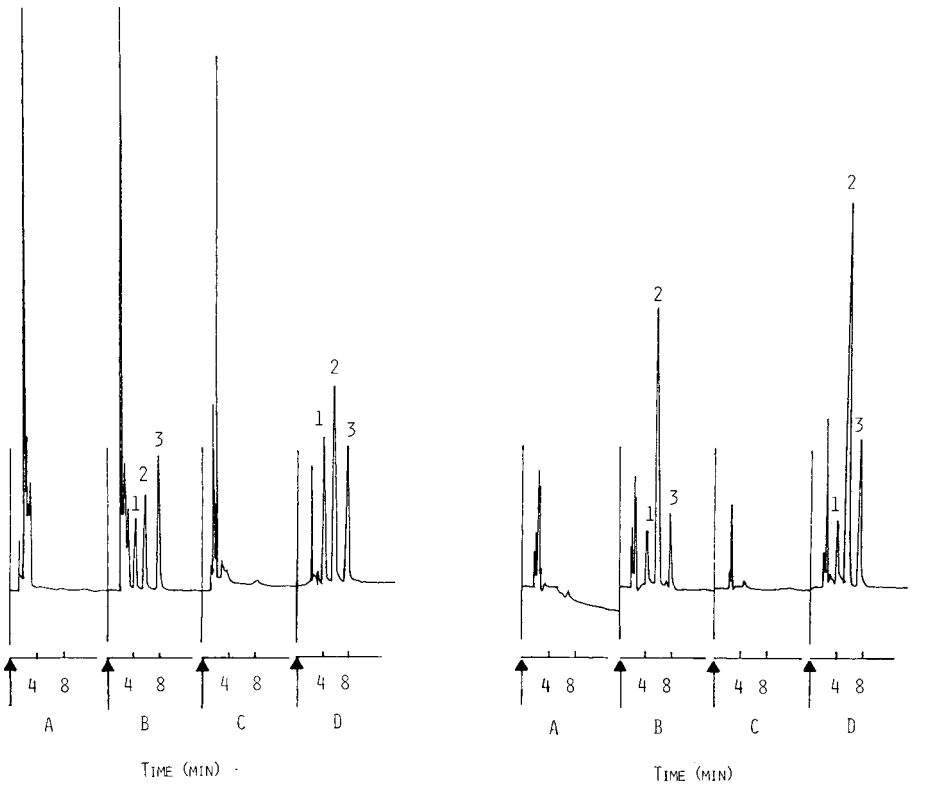

Fig. 1. Chromatograms of: (A) blank human plasma; (B) human plasma spiked with 15 μg/ml PAH, iothalamate and 30 μg/ml PABA; (C) blank human urine; (D) human urine spiked with 30 μg/ml PAH, iothalamate and PABA. Peaks: 1 = PAH, 2 = iothalamate, 3 = PABA. Sensitivity setting: 0.01 a.u.f.s. The arrows mark the points of injections.

Fig. 2. Chromatograms of: (A) blank dog plasma; (B) dog plasma obtained after 1 h infusion of 180.5 mg of PAH and 219.2 mg of iothalamate; (C) blank dog urine; (D) dog urine collected after 3 h of infusion. Peaks: 1 = PAH, 2 = iothalamate, 3 = PABA. Sensitivity setting: 0.01 a.u.f.s. for A and B, 0.025 a.u.f.s. for C and D. The arrows mark the points of injections.

The pH of the mobile phase was found to play an important role in peak resolution. At pH values higher than 2.6, iothalamate was eluted faster and interfered with PAH, whereas at lower pH values (less than 2.4), iothalamate eluted later and interfered with PABA.

The μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm particle size) from Waters Assoc. also gave adequate peak separation and similar retention times for the three compounds under the current HPLC conditions. However, the C_{18} column (Partisil PXS 10/25 ODS, Cat. No. IF 2494) from Whatman (Clifton, NJ, U.S.A.) did not give a satisfactory result under these conditions.

The standard curves for human plasma and urine samples were found to be linear over the concentration range studied. This is illustrated by the low coefficients of variation of response factors as shown in Tables I and II. Without the use of internal standard, the coefficients of variation for plasma and urine were also low (Tables I and II). Based on a signal-to-noise ratio of 3,

TABLE I

RESPONSE FACTORS OF PAH AND IOTHALAMATE IN HUMAN PLASMA

Spiked plasma concentration (μg/ml)	With internal standard (based on concentration of 5 μg/ml)				Without internal standard (based on sensitivity setting of 0.002 a.u.f.s.)	
	Peak height ratio*		Response factor I**		Response factor II***	
	PAH	Iothalamate	PAH	Iothalamate	PAH	Iothalamate
2.5	0.52	0.80	0.20	0.32	0.54	0.83
5.0	0.96	1.47	0.19	0.29	0.56	0.86
10.0	2.09	3.19	0.21	0.32	0.54	0.83
25.0	5.43	7.50	0.22	0.30	0.56	0.77
50.0	10.95	15.20	0.22	0.30	0.55	0.77
Mean ± S.D.			0.21 ± 0.012	0.31 ± 0.012	0.55 ± 0.01	0.81 ± 0.039
C.V. (%)			5.62	3.74	1.84	4.80

*Peak height ratio = ratio of peak height of the compound to that of the internal standard.
**Response factor I = peak height ratio divided by the concentration (μg/ml) of the compound.
***Response factor II = peak height (cm) of the compound divided by its concentration (μg/ml).

TABLE II

RESPONSE FACTORS OF PAH AND IOTHALAMATE IN HUMAN URINE

Spiked urine concentration (μg/ml)	With internal standard (based on concentration of 20 μg/ml)				Without internal standard (based on sensitivity setting of 0.005 a.u.f.s.)	
	Peak height ratio		Response factor I (ml/μg)		Response factor II (cm^2/μg/ml)	
	PAH	Iothalamate	PAH	Iothalamate	PAH	Iothalamate
10.0	0.62	0.84	0.062	0.084	0.19	0.26
20.0	1.24	1.61	0.062	0.080	0.18	0.24
40.0	2.50	3.27	0.063	0.082	0.18	0.23
60.0	3.71	4.81	0.062	0.080	0.17	0.22
100.0	6.20	8.12	0.062	0.081	0.17	0.23
Mean ± S.D.			0.062 ± 0.0003	0.081 ± 0.001	0.18 ± 0.009	0.24 ± 0.015
C.V. (%)			1.74	0.47	4.97	6.53

the detection limits of the present assay for plasma samples were found to be 1 μg/ml for PAH and 0.5 μg/ml for iothalamate.

The intra-day coefficients of variation were 1.60% and 3.79% for PAH, and 1.59% and 0.74% for iothalamate at the concentrations of 15 μg/ml and 30 μg/ml, respectively. The inter-day coefficients of variation for the analysis of the same plasma samples on four days over a period of one week were 2.75% and 7.20% for PAH, while for iothalamate the values were 5.5% and 5.95% at the concentrations of 5 and 25 μg/ml, respectively.

At concentrations of 15 and 30 μg/ml, the percentages of recovery of iothalamate were 94% for plasma and 95—100% for urine, while those of PAH were virtually 100% for both plasma and urine. These high recoveries resulted from the simple one-step deproteinization with two volumes of acetonitrile. The uniqueness of acetonitrile as a simple and effective deproteinizing agent has been extensively studied earlier [20]. Use of 20% perchloric acid to precipitate proteins in plasma samples was not found to give better results

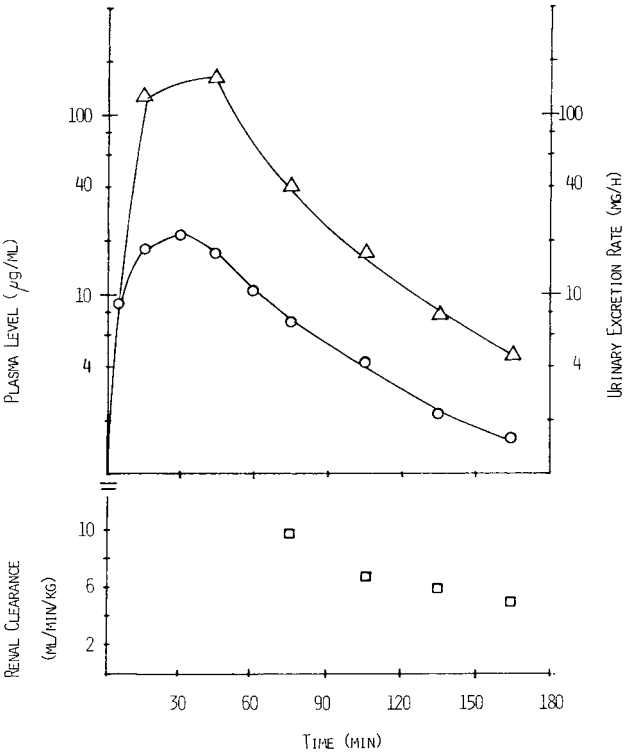

Fig. 3. Plasma concentration profile (○) and urinary excretion rate profile (△) of PAH in the dog following 40 min infusion of 180.5 mg of PAH and 219.2 mg of iothalamate. Renal clearance of PAH from the dog (□) was obtained by dividing urinary excretion rate by plasma concentration at the midpoint of urine collection interval.

than using acetonitrile. With the same volume of injection (5 μl), it resulted in a higher response and more interference. It is obvious that, when necessary, sample sizes smaller than 0.1 ml can be used for quantitation.

Peak splitting was found when the volume of injection was more than 5 μl under the present HPLC conditions.

The results of the drug interference study showed that none of the drugs tested would affect the assay, with the exception of hydralazine which might coelute with the internal standard. N-Acetyl-PAH, a metabolite of PAH in man [17, 18], did not interfere with the assay. Its retention time was about 30 min.

The plasma and urine profiles of both compounds from the preliminary dog study together with their renal clearances (determined by midpoint method) are shown in Figs. 3 and 4. In spite of the venous data employed in the present study [2], the gradual decrease in PAH renal clearance during the apparent terminal phase was evident; this appeared to be different from the result in dogs [18] but consistent with that in man reported earlier [18]. The total urinary recoveries estimated were 94.3% and 87.5% for PAH and iothalamate, respectively. No N-acetyl-PAH was found in either plasma or urine samples, which is in agreement with the previous studies [17, 18]. PABA, which was also reported to be a possible metabolite of PAH in man [21], was not

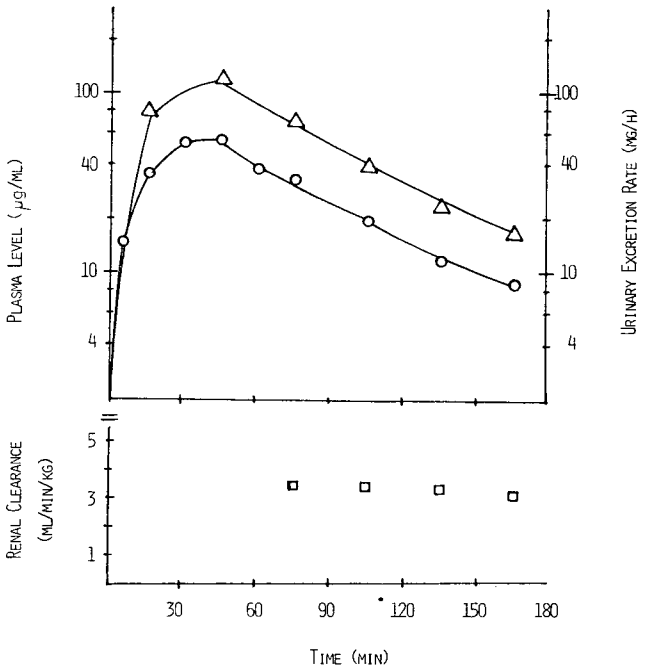

Fig. 4. Plasma concentration profile (○) and urinary excretion rate profile (△) of iothalamate in the dog following 40 min infusion of 180.5 mg of PAH and 219.2 mg of iothalamate. Renal clearance of iothalamate from the dog (□) was obtained by dividing urinary excretion rate by plasma concentration at the midpoint of urine collection interval.

detected. Even if PABA cannot be used as an internal standard due to its presence as a metabolite, the present HPLC method may still be satisfactory. When necessary, an alternative internal standard such as hydralazine might be employed.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NEUROPEPTIDES USING RADIALLY COMPRESSED POLYTHENE CARTRIDGES

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SUMMARY

This study was designed to assess practically the suitability of different C_{18} reversed-phase radially compressed polythene cartridges (Radial-Pak, Waters Assoc.) in two types of radial-compression systems, for the separation and analysis of various neuropeptides at both high (< 5 μg) and low (> 100 pg) levels in biological extracts and to compare them with well established techniques using stainless-steel columns.

A solvent system fully compatible with both radially compressed and steel columns is described. The completely volatile mobile phase (acetonitrile gradient containing trifluoroacetic acid) allows ultraviolet detection below 215 nm, gives good resolution and is readily compatible with the further radioimmunoassay and bioassay of collected fractions.

The efficiency of radially compressed 5 and 10 μm "capped" and "non-capped" C_{18} silica supports and slurry-packed steel columns has been assessed by: (1) separation and recovery of a complex standard mixture of neuropeptides; (2) separation and subsequent identification of degradation products formed during the incubation of neurotensin with rat cortical synaptosomes; (3) analysis of α-melanotropin and corticotropin-(18—39) in tissue culture media containing varying amounts of foetal calf serum; and (4) characterization of corticotropin-like immunoreactivity in human cerebrospinal fluid.

The Z-module fitted with the capped 10-μm irregular C_{18} silica cartridge gave better resolution than with the μBondapak steel column but the selective retention was similar. The back-pressures in the Z-module are much reduced (approximately 13 bar at 1 ml/min); therefore, flow-rates may be increased and analysis times greatly reduced. In order to obtain good resolution with the RCM-100 module which uses a non-capped stationary phase, a salt must be added (e.g. 15 m*M* sodium chloride) to the mobile phase to reduce polar interactions between the peptide and the free silanol groups on the stationary phase. This makes the solvent non-volatile and therefore less useful.

INTRODUCTION

The potential of reversed-phase high-performance liquid chromatography

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(RP-HPLC) in the separation and characterization of neuropeptides has been demonstrated by numerous investigators (see, for example, refs. 1—5). In the vast majority of cases separations are achieved using C_{18} microparticulate (3—10 μm) silica supports slurry packed in stainless-steel columns. After introduction to the column the peptides are eluted selectively according to their hydrophobicity by an aqueous-to-organic solvent gradient. Apart from hydrophobic interactions there are various ion-pairing effects [6—8] and column modifications [9] which will selectively affect retention.

An important criterion in HPLC column efficiency is packed-bed uniformity [10]. This is difficult to achieve in conventional slurry-packed steel columns. However, by applying radial compression to a dry-packed polythene cartridge, heterogeneity of packing in the stationary phase is minimized, especially around the column wall and end fittings. The potential advantages of this chromatographic procedure are: increased sample capacity, shorter analysis times, improved resolution, reduced back-pressures and greater economy. Peptides are not ideal candidates for the assessment of column kinetics as they display both polar and non-polar interactions with the stationary phase in RP-HPLC [11]. However, other studies have shown using non-polar solutes with good thermodynamic properties that radially compressed beds display greater uniformity than slurry-packed columns (assessed by log height versus log volume plots) [11].

Three chromatographic systems have been compared: the μBondapak stainless-steel column slurry packed with 10-μm capped irregular C_{18} silica; the RCM-100 radial-compression module using 5 and 10-μm spherical non-capped C_{18} silica cartridges radially compressed at approximately 170 bar; and, finally, the Z-module using 10-μm irregular C_{18} capped silica cartridges radially compressed at approximately 13 bar.

The efficiency of these systems has been assessed by their ability to separate and resolve a standard mixture of neuropeptides ranging in both size and hydrophobicity. The Z-module has been further evaluated by its use in the separation and identification of products formed during the incubation of neurotensin with nerve endings isolated from rat brain, by monitoring the stability of α-melanotropin (α-MSH) and corticotropin-(18—39) (CLIP) in tissue culture media containing varying concentrations of foetal calf serum and characterizing the corticotropin-like immunoreactivity (ACTH-IR) present in human cerebrospinal fluid.

MATERIALS AND METHODS

Equipment

A Waters HPLC system was used comprising two 6000A pumps, a Model 720 system controller, a WISP automatic sample injector, a 450 variable-wavelength detector, RCM-100 and Z-module radial-compression systems, 5- and 10-μm spherical C_{18} Radial-Pak cartridges, μBondapak 10-μm C_{18} Radial-Pak cartridges (all 10 cm $\times$ 8 mm I.D.) and a μBondapak 10-μm C_{18} stainless-steel column (Waters Assoc., Northwich, U.K.). Fractions were collected using an LKB Ultrorac 2070 collector controlled by a microprocessor, allowing collection of any part of the column effluent defined by its elution time [12].

Chemicals

Acetonitrile (HPLC S-Grade), was obtained from Rathburn Chemicals (Peebles, U.K.). Sodium chloride (AnalaR), trifluoroacetic acid (TFA, spectroscopic grade), acetic acid (chromatographic grade), phosphoric acid (AnalaR) were obtained from BDH (Poole, U.K.). Water was deionized, double glass-distilled and passed through a Porapak Q column. Tissue culture media and foetal calf serum (non-heat-inactivated) were obtained from Gibco (Paisley, U.K.).

Peptides

Neurotensin, thyrotropin-releasing hormone (TRH), TRH-OH, luteinizing hormone–releasing hormone (LH–RH), α-MSH, substance P and somatostatin were obtained from Peninsula Labs. (San Carlos, CA, U.S.A.). ACTH-(18–39) (CLIP) was obtained from Universal Biologicals (Cambridge, U.K.).

Methods

A 20-μl volume of the standard neuropeptide mixture containing TRH, TRH-OH, LH–RH, neurotensin, somatostatin and substance P (each 5 μg) was introduced to each system. The separations were achieved using linear gradients of 3% to 70% of solvent B over 10 and 20 min at flow-rates of 1 and 2 ml/min, respectively. Solvent A = 11 m*M* TFA–2.6 m*M* acetic acid. Solvent B = 70% acetonitrile containing 11 m*M* TFA. In the case of the RCM-100 system, sodium chloride (15 m*M*) was added to the aqueous phase (to counter free silanol groups on the stationary phase).

Metabolically active synaptosomes from rat cortex were prepared using the rapid method of Dodd et al. [13]. The synaptosome pellet (one cortex equivalent) was resuspended in 2.5 ml of Krebs phosphate buffer and incubated at 37°C with 50 μg of synthetic neurotensin. At varying time intervals 100-μl aliquots were removed, acidified with 10 μl of 10% TFA and centrifuged prior to analysis by HPLC.

Amounts of 25 μg of CLIP and 25 μg of α-MSH were incubated in 600 μl of tissue culture media containing 0, 1, 5 and 30% foetal calf serum at 37°C for 24 and 48 h. The incubations were terminated by adding 60 μl of 10% TFA followed by centrifugation prior to HPLC. The incubations containing CLIP were also run on HPLC using heptafluorobutyric acid (HFBA) in place of TFA as the acid modifier [14].

For analysis of cerebrospinal fluid, 7 ml were first concentrated on Sep-Pak cartridges (Waters Assoc.); the fraction containing the ACTH-IR was dried under vacuum and reconstituted in 200 μl of 0.1% TFA prior to injection.

The Radial-Pak cartridges were washed with acetonitrile (25 ml) prior to use. All the chromatography was carried out at ambient temperature, the solvents being filtered and degassed immediately before use.

RESULTS

Good resolution of a standard mixture was obtained on the μBondapak steel column (30 × 0.39 cm) (Fig. 1a) using a solvent system previously described [1]. A gradient of 3% to 70% of solvent B (i.e. 2.1% to 49% aceto-

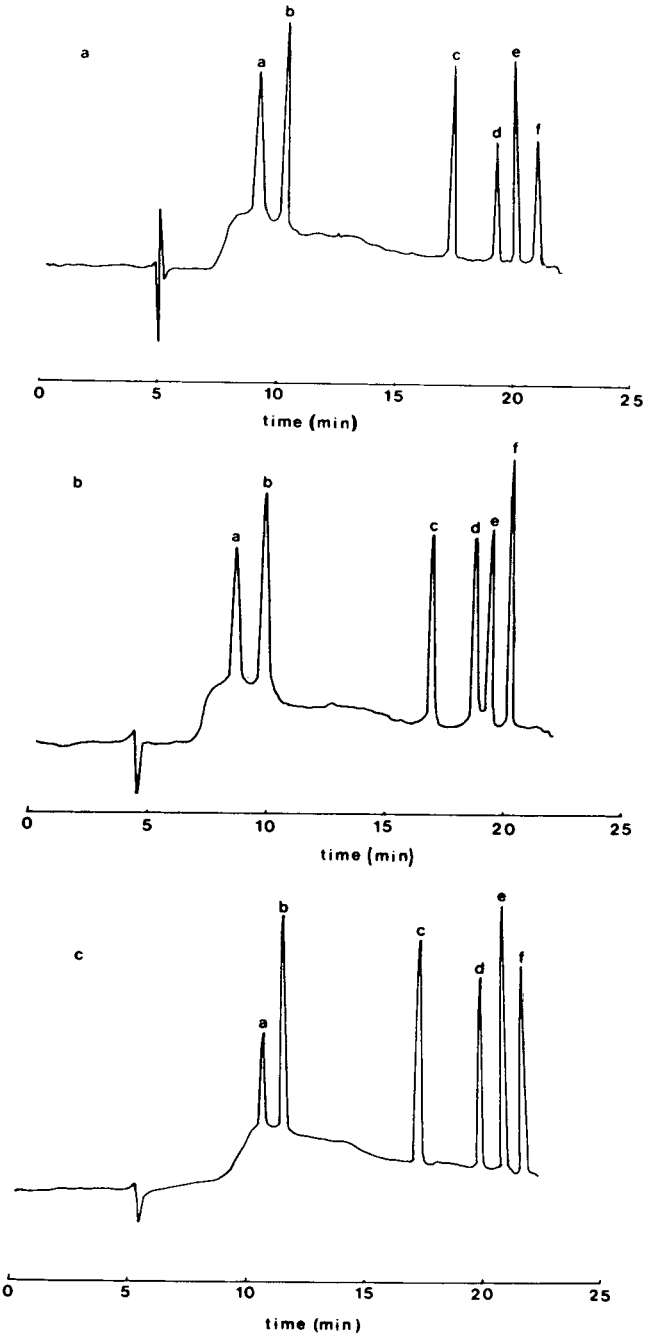

Fig. 1. (a) Separation of neuropeptide standards on the μBondapak steel column using an acetonitrile gradient. Solvent A = 11 m*M* TFA—2.6 m*M* acetic acid; solvent B = 70% acetonitrile containing 11 m*M* TFA; 20-min linear gradient, 3% to 70% solvent B at 1 ml/min. Peaks: a = TRH, b = TRH-OH, c = LH—RH, d = neurotensin, e = substance P, f = somatostatin (all 5 μg); 0.4 a.u.f.s. at 206 nm. (b) Elution conditions and standard as in Fig. 1a except the aqueous phase also contains 15 m*M* sodium chloride; the column used is the RCM-100 containing the 5-μm non-capped C_{18} Radial-Pak cartridge. Peaks as in (a). (c) Elution conditions and standard as in Fig. 1a. The column used is the Z-module containing the 10-μm capped C_{18} Radial-Pak cartridge. Peaks as in (a).

nitrile) in the presence of 11 m*M* TFA (pH 2.1) over 20 min at 1 ml/min is sufficient to resolve TRH, TRH-OH, LH—RH, neurotensin, somatostatin and substance P. Using this gradient a wide range of neuropeptides varying both in hydrophobicity and molecular weight can be separated. Small peptides such as the tripeptide TRH and its metabolites His-Pro diketopiperazine and TRH-free acid (TRH-OH) are well resolved in the early part of the chromatogram. The larger and more hydrophobic peptides (e.g. LH—RH, substance P and somatostatin) are retained longer but are equally well resolved.

The same standard was injected into the RCM-100 system using both 5- and 10-μm non-capped cartridges and the same solvent gradient. Only TRH and its metabolites were eluted and then as broad tailing peaks. However, by introducing a salt (in this case 15 m*M* sodium chloride) into the aqueous solvent A to act as a counter-ion to suppress the ionic interactions between the

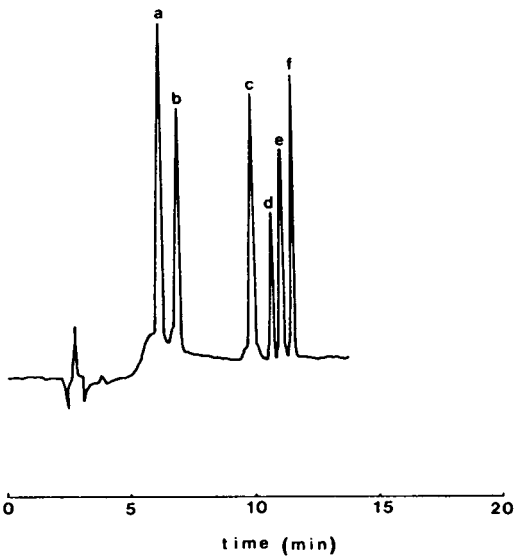

Fig. 2. Separation of the neuropeptide standards using the Z-module. Solvents as in Fig. 1a; 10-min linear gradient of 3% to 70% solvent B at 2 ml/min; peaks a—f as in Fig. 1a (a, 10 μg; b—f, 5 μg); 0.4 a.u.f.s. at 206 nm.

TABLE I

PEPTIDE RETENTION TIMES (min)

Elution conditions as in Fig. 1a.

Peptide	Z-module			μBondapak steel		
	$\bar{x}$	S.D.	*n*	$\bar{x}$	S.D.	*n*
TRH	9.17	0.067	10	5.56	0.041	10
LH—RH	19.81	0.058	10	18.51	0.052	10
Neurotensin	20.29	0.059	10	20.26	0.059	10
Substance P	22.06	0.053	10	21.60	0.050	10

free silanol groups on the stationary phase and the ionic amino acid side-chains in the peptide, all the peptides eluted as discrete sharp peaks (Fig. 1b).

The Z-module containing the 10-μm irregular capped Radial-Pak cartridge separated all the peptides in the standard under the same elution conditions used on the μBondapak steel column (Fig. 1c). Because of the low back-pressure with the Z-module (13 bar at 1 ml/min, 20% of that in the steel column), it was possible to reduce the analysis time by both increasing the flow-rate to 2 ml/min and reducing the gradient time to 10 min. The components of the standard mixture were again well resolved (Fig. 2).

Repeated injections of the standard were made onto both the Z-module and the μBondapak steel column in order to assess the reproducibility of each column type by comparing the variation in the peptide elution times in each system (Table I).

PRACTICAL APPLICATIONS

The products formed from the incubation of synthetic neurotensin with rat cortical synaptosomes were well separated using the Z-module (Fig. 3). The peaks (1—6) were collected and hydrolysed (6 *M* hydrochloric acid, 20 h, 110°C) prior to amino acid analysis. The amino acid ratios for each peak show that peak 1 is neurotensin-(12—13), peak 2 is neurotensin-(1—8), peak 3 is neurotensin-(1—10), peak 4 is neurotensin-(1—11), peak 5 contains both neurotensin-(1—12) and -(9—13) eluting together and, finally, peak 6 corresponds to synthetic neurotensin.

As part of a study on the characterization of α-MSH and CLIP-like peptides released by cultured pituitary cells, we examined the stability of the two peptides in tissue culture medium using the Z-module. The HPLC traces (Fig. 4) demonstrate the rates at which both α-MSH and CLIP are degraded in medium containing varying amounts of foetal calf serum (0—30%). α-MSH was shown to be degraded fairly rapidly without forming any visible stable products. The 18—39 fragment of ACTH, however, formed a stable product

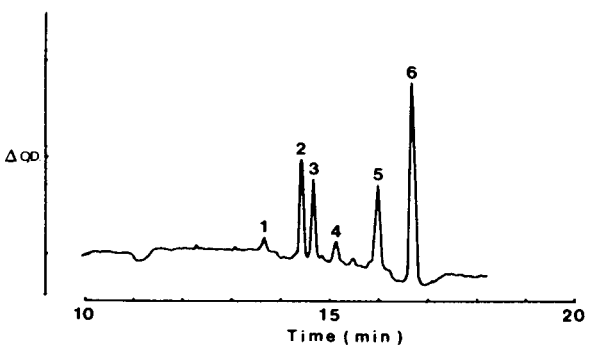

Fig. 3. HPLC profile of neurotensin degradation products formed on incubation with rat cortical synaptosomes. Elution conditions as in Fig. 1a. Peaks: 1 = neurotensin-(12—13), 2 = neurotensin-(1—8), 3 = neurotensin-(1—10), 4 = neurotensin-(1—11), 5 = both neurotensin-(1—12) and neurotensin-(19—13) eluting together, and 6 = synthetic neurotensin. The equivalent of 10 g of peptide was injected onto the Z-module.

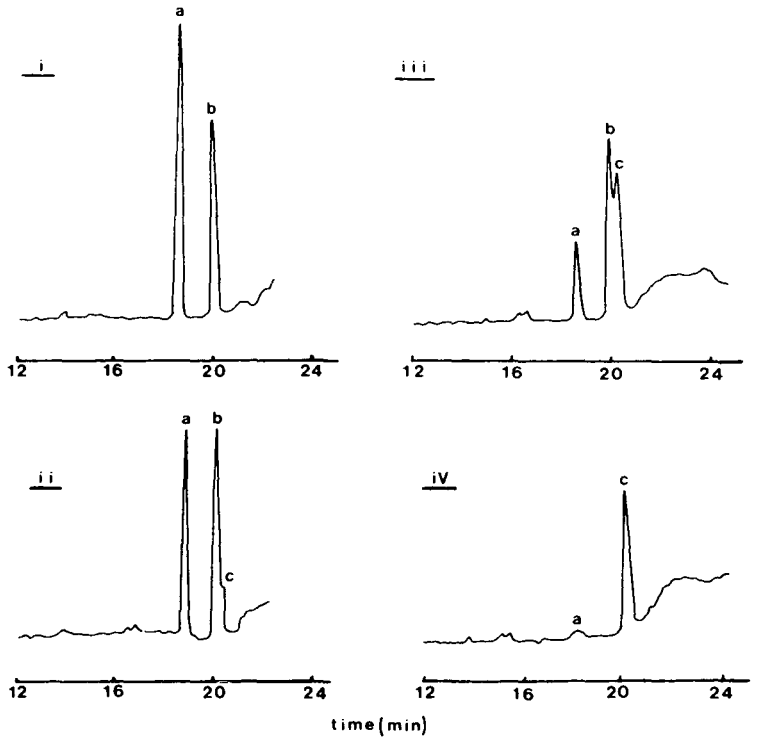

Fig. 4. HPLC profiles, using the Z-module, of α-MSH and CLIP incubated for 48 h at 37°C in tissue culture media containing 0% (i), 1% (ii), 5% (iii) and 30% (iv) foetal calf serum. Elution conditions and solvents are as in Fig. 3. Peaks: a = α-MSH, b = CLIP [ACTH-(18—39)], c = ACTH-(19—39). The equivalent of 20 μg of peptide was injected onto the column.

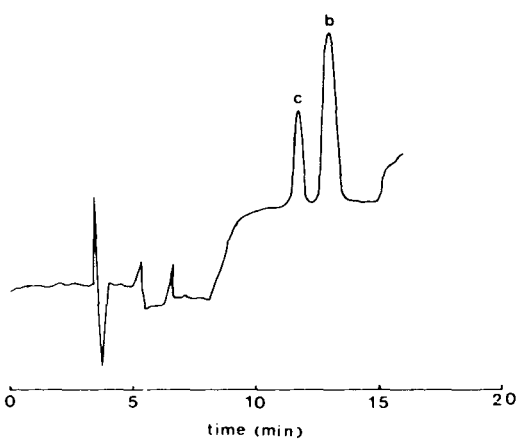

Fig. 5. The separation, using the Z-module, of peaks b and c (see Fig. 4) enhanced by substituting HFBA for TFA in the mobile phase. Linear gradient 45% to 60% solvent over 10 min at 1 ml/min; solvent A = 10 m*M* HFBA, solvent B = 70% acetonitrile in 10 m*M* HFBA; monitored at 220 nm, 0.4 a.u.f.s. The equivalent of 10 μg of peptide was injected onto the column.

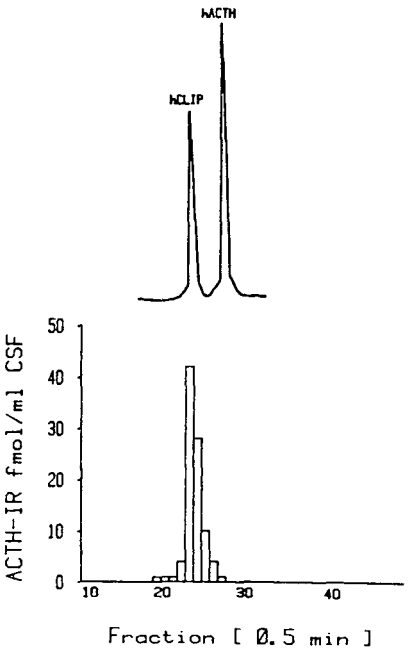

Fig. 6. HPLC trace of 5 μg of standard human ACTH and human CLIP over radioimmunoassay profile or human cerebrospinal fluid extract. Elution conditions are as in Fig. 5.

eluting very close to the parent peptide (peak c). In order to identify this product the separation was enhanced by substituting HFBA (10 m*M*) for TFA as the acid modifier in the mobile phase (Fig. 5), which will increase peptide retention depending on the number of basic amino acid residues present in the structure [6, 14]. The degradation product was then isolated and shown by amino acid analysis to be ACTH-(19—39), i.e. loss of the N-terminal arginine had occurred.

Human cerebrospinal fluid contains a significant amount of ACTH-IR [15]. To determine the molecular nature of this immunoreactivity we subjected a concentrated fraction of cerebrospinal fluid to HPLC again using HFBA in the mobile phase. The radioimmunoassay (using an antiserum directed against the C-terminus of ACTH) profile of the collected fractions (Fig. 6) shows that the ACTH-IR is not due to ACTH itself but is more likely to be CLIP, the 18—39 fragment of ACTH.

DISCUSSION

In slurry-packed steel columns the inside diameter of the column is limited in order to minimize the wall effect (i.e. when the dispersion of the mobile phase around the column casing is greater than that in the core). It has been claimed that in a column of 5 mm I.D. containing 20-μm particles, the peripheral area affected by the wall effect may be as great as 40% [16]. In a radially compressed flexible cartridge this effect may be much reduced as the column wall is able to mould around the column packing giving a more homo-

geneously packed bed thereby potentially increasing column efficiency. It is therefore possible to use a shorter column with a wider bore, the advantages being reduced back-pressures, improved resolution and greater sample capacity. The reduction in back-pressure will allow increased flow-rates resulting in shorter analysis times. As sample capacity is much increased (up to 20 mg) the system may be used in a preparative mode for the purification and isolation of neuropeptides.

In this report we have tried to assess the suitability of radial compression for the separation and isolation of neuropeptides and for the first time compared several C_{18} packing materials in different compression systems. Previous reports have successfully demonstrated the separation of proteins and peptides derived from the partial hydrolysis of human lipoproteins using a non-capped C_{18} 10-μm spherical packing radially compressed at 170 bar, the mobile phase being an acetonitrile or isopropanol gradient against a triethylammonium phosphate buffered (pH 3.2) aqueous phase [10, 17]. The neuropeptides angiotensin, and α-, β- and γ-endorphin have also been separated using the 10-μm spherical packing (Radial-Pak A) with triethylamine formate and ammonium bicarbonate as counter-ions [18]. The Z-module having a lower compression rating (13 bar) fitted with the 10-μm irregular C_{18} packing which has undergone a secondary derivatization or end-capping process (to ensure maximal coverage of the silica core) may be used successfully to separate a variety of peptides without the addition of any salts to the mobile phase (water—TFA—acetonitrile; Fig. 1c). This mobile phase is particularly useful by being ultraviolet-transparent below 220 nm to allow the sensitive detection of peptides containing non-aromatic residues, and by being totally volatile, collected fractions may be dried for further analysis without the problems associated with interference from residual salts [1]. Other solvents systems containing triethylamine formate or ammonium bicarbonate with acetonitrile are also volatile and ultraviolet-transparent but are much less convenient to use.

Hearn et al. [18] have demonstrated differences in selectivity for several peptides between radially compressed packings (Radial-Pak A) and conventional steel columns. However, using the Z-module fitted with the capped 10-μm irregular C_{18} packing we found that the selectivities for all the peptides we tried were similar to those obtained with the μBondapak steel column. It is also our experience that these columns have a much longer life than steel columns, especially when used for the analysis of large numbers of relatively crude biological extracts.

Three examples of the practical applications of radial compression using the Z-module are described briefly and full details will appear elsewhere. The putative neurotransmitter neurotensin has been shown to be degraded by specific peptidases present in soluble and particulate fractions prepared from rat brain [19]. Using HPLC we have identified a potential inactivation pathway for neurotensin at the nerve terminal using metabolically active rat cortical synaptosomes incubated with synthetic neurotensin (Fig. 4), the collected peaks being positively identified by amino acid analysis following acid hydrolysis.

One of the problems associated with the monitoring of peptide release from cultured cells is the peptidase activity present in tissue culture media containing foetal calf serum (normally 20—30%). In order to optimize the conditions

necessary for maximal cell growth and peptide stability we have used HPLC to look at the rate at which α-MSH and CLIP are degraded in tissue culture media containing varying amounts of foetal calf serum (Fig. 5). By substituting HFBA for TFA in the mobile phase we were able to isolate and identify a major degradation product formed from CLIP (Fig. 6). We have recently demonstrated that cells isolated from rat intermediate lobe may be successfully cultured in media containing 1% foetal calf serum where peptide breakdown is minimal (Hughes and Smith, unpublished results).

By subjecting a concentrated sample of cerebrospinal fluid to HPLC, we have been able to show that the immunoreactivity in collected fractions does not correspond to ACTH but is more likely to be CLIP, the 18—39 fragment of ACTH.

In conclusion, the 10-μm, irregular, capped C_{18} packing radially compressed in the Z-module compares favourably with steel columns slurry packed with similar material. The major advantages of this system are speedier analysis and increased sample capacity, longer life and therefore greater economy.

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DETECTION AND QUANTITATION OF FLUORESCAMINE-LABELED BRADYKININ, ITS ANALOGUES AND METABOLITES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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(First received August 23rd, 1983; revised manuscript received October 14th, 1983)

SUMMARY

A sensitive technique is described for detecting and quantitating fluorescamine-labeled kinins and their usual metabolic products using reversed-phase high-performance liquid chromatography (HPLC) linked with a fluorescence detector. Kinins and their enzymatic products were labeled with fluorescamine, subjected to HPLC, and scanned for the fluorescence signal with excitation at 390 nm and emission at 476 nm. The fluorescence signal was linear with bradykinin, Lys-bradykinin and Met-Lys-bradykinin in amounts upward from 2.5 ng. Separation of the fluorescamine-labeled kinins using HPLC was carried out with a solvent system of methanol—triethylammonium formate buffer. Labeled kinins were eluted in the following order: bradykinin, Lys-bradykinin, and Met-Lys-bradykinin.

When native (unlabeled) kinins were subjected to HPLC using a solvent system of acetonitrile—triethylammonium formate buffer, the minimum amount of native kinin detected at 210 nm was 1 μg. All three kinins showed linearity at 210 nm in amounts upward from 1 μg. Kinins were eluted in the following order: Lys-bradykinin, bradykinin and Met-Lys-bradykinin.

The different elution patterns of kinins by means of these two separation techniques provide a useful method for identification of purified kinins. The fluorescamine label provides a 400-fold more sensitive detection technique than ultraviolet absorbance of the native kinins and may be used to identify the metabolic products of kinins.

INTRODUCTION

Different analytical procedures such as paper chromatography, paper and gel electrophoresis, gel chromatography and ion-exchange chromatography have

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been used in conjuction with bioassay on isolated tissues to detect and differentiate bradykinin and its analogues Lys-bradykinin and Met-Lys-bradykinin [1—9]. Stewart and Morris [10] used reversed-phase high-performance liquid chromatography (HPLC) to separate and identify kallikrein products. The absorbance was monitored by UV at 210 nm and elution was carried out using a methanol—ammonium formate mixture. Kinins were detected in the microgram range. Geiger et al. [11], Powers and Nasjletti [12] and Dizdaroglu et al. [13] also separated kinins by HPLC and used detection by UV absorbance at 215—225 nm or by radioimmunoassay or by fluorescamine.

The purpose of the present paper is to report a method of direct separation, identification and quantitation of fluorescamine-labeled kinins and their usual metabolic products at nanogram levels using reversed-phase HPLC. This method, in conjunction with reported separation techniques of native kinins on HPLC provides an excellent tool for identification and quantitation of kinins.

MATERIALS AND METHODS

Ile-Ser-Bradykinin, synthetic bradykinin, Lys-bradykinin and Met-Lys-bradykinin were from Peninsula Labs. (San Carlos, CA, U.S.A.). Rat plasma T-kinin (Ile-Ser-bradykinin) was a gift of H. Okamoto, Medical College of Georgia, Department of Pharmacology.

Triethylamine was from Aldrich (Milwaukee, WI, U.S.A.). Converting enzyme (E.C. 3.4.15.1) and carboxypeptidase-B (E.C. 3.4.17.1) were from Calbiochem (La Jolla, CA, U.S.A.). α-Chymotrypsin (E.C. 3.4.21.1) was from Worthington (Freehold, NJ, U.S.A.). Carboxypeptidase-A (E.C. 3.4.17.1), carboxypeptidase-M (E.C. 3.4.11.2) and fluorescamine were from Sigma (St. Louis, MO, U.S.A.). Disposable extraction columns (Bond-Pak column) packed with reversed-phase octadecylsilane (C_{18}) bonded to silica gel (40 μm average particle diameter) were from J.T. Baker (Phillipsburg, NJ, U.S.A.). The Ultrasphere ODS (C_{18}) (75 mm $\times$ 4.6 mm I.D.; with 5 μm mean particle diameter) was a prepared column from Beckman (Concord, CA, U.S.A.). HPLC grade solvents were from J.T. Baker. Deionized glass-distilled water passed through a Bond-Pak C_{18} 3-ml column was used for the preparation of all HPLC buffers. The buffer was degassed for 20 min before applying to the HPLC column.

A Beckman Model 110A dual pump HPLC instrument linked to a Perkin-Elmer 650-10S fluorescence spectrophotometer with a 20-μl flow cell was used for this study.

Labeling of kinins with fluorescamine

This was carried out by the method of Prakash et al. [14]. From a stock solution of 1 mg/ml kinin in 0.03 *M* hydrochloric acid, concentrations of standard kinin solutions ranging from 1.25 to 40 μg/ml were prepared by serial dilution with 0.05 *M* sodium borate buffer, pH 8.3. To 100 μl of each concentration, 5 μl of freshly prepared fluorescamine solution (3 mg/ml in anhydrous acetone) were added, mixed vigorously using a Vortex mixer, and kept at room temperature for 10 min. A 0.05 *M* borate buffer blank was similarly treated. Although the pyrollinone kinin derivatives were generally

found to be stable, chromatography was carried out within 2 h after fluorescamine treatment.

HPLC of fluorescamine-labeled peptides and kinins

The reversed-phase column was equilibrated with a minimum of 20 ml of 0.04 *M* triethylammonium formate, pH 4.4—methanol (80:20). For kinin separation, a 2-μl sample (2.5—80 ng) of the fluorescamine-labeled kinin was injected and a linear gradient of methanol from 20% to 100% over 30 min was programmed. The sample was eluted at a flow-rate of 0.5 ml/min at ambient temperature. In the case of separation of kinins and their metabolic products, elution was carried out with a linear gradient of acetonitrile from 20 to 40% over 60 min at a flow-rate of 0.4 ml/min at ambient temperature. Detection was carried out using an excitation wavelength of 390 nm and an emission wavelength of 476 nm.

HPLC of native kinins

The reversed-phase column was equilibrated with a minimum of 20 ml of 0.04 *M* triethylammonium formate, pH 4.4—acetonitrile (88:12). Concentrations of native kinins ranging from 1 to 8 μg were injected and eluted with the same solvent system at a flow-rate of 0.5 ml/min at ambient temperature.

Detection and quantitation were carried out at 210 nm using an on-line Altex Model 155-10/40 UV—VIS spectrophotometer. Between each sample, the sample port was cleaned with 50 μl of acetonitrile followed by 50 μl of buffer.

HPLC of enzymatic products of bradykinin

Incubation of bradykinin with different enzymes was carried out in 1-ml plastic centrifuge cups with a substrate concentration of 10 μg/ml in a final volume of 500 μl of buffer at appropriate pH at 37°C for the time indicated. Following incubation, a 20-μl aliquot of sample was drawn and mixed with 80 μl of 0.1 *M* sodium borate buffer, pH 8.3 and immediately labeled with 5 μl of freshly prepared fluorescamine solution and subjected to HPLC. The conditions of the incubations were as follows:

Converting enzyme: 0.1 *M* sodium phosphate buffer containing 0.3 *M* sodium chloride at pH 7; enzyme concentration 100 μg/ml; incubated for 48 h.

α-Chymotrypsin: 0.1 *M* sodium borate buffer containing 0.2 *M* calcium chloride at pH 7.8; enzyme concentration 10 μg/ml; incubated for 10 min.

Carboxypeptidase-B: 0.025 *M* sodium phosphate buffer containing 0.1 *M* sodium chloride at pH 7.55; enzyme concentration 1 μg/ml; incubated for 10 min.

Carboxypeptidase-B followed by -A: after 10 min of incubation of bradykinin (10 μg/ml) with carboxypeptidase-B (1 μg/ml) in 0.25 *M* sodium phosphate buffer containing 0.5 *M* sodium chloride at pH 7.55, carboxypeptidase-A (10 μg/ml) was added and incubated for an additional 5 h.

Digestion of T-kinin by aminopeptidase-M

T-Kinin (0.8 μg of bradykinin equivalent by the rat uterus assay) was incubated with 2 ng of aminopeptidase-M in 0.05 *M* sodium phosphate buffer

pH 7.0 containing 3 m*M* disodium EDTA in a total volume of 60 μl. After the incubation at 37°C, the enzyme reaction was terminated by addition of 20 μl of methanol. A 50-μl aliquot of the sample was removed and labeled with fluorescamine and chromatographed.

RESULTS

The retention times of the fluorescamine-labeled kinins chromatographed in nanogram quantities were found to be for bradykinin 10.1 ± 0 min (n = 15), Lys-bradykinin 23.3 ± 0 min (n = 22) and Met-Lys-bradykinin 25.1 ± 0 min (n = 15) (Fig. 1B). The retention times of native kinins chromatographed in microgram quantities were found to be for Lys-bradykinin 11.0 ± 0.2 min (n = 16), bradykinin 21.7 ± 0.2 min (n = 16) and Met-Lys-bradykinin 25.1 ± 1 min = 0.1 (n = 16) (Fig. 1A).

The fluorescent peaks of the fluorescamine-labeled kinins were highly symmetrical. Quantitation was carried out measuring the peak height of the fluorescence signal and plotting it against the amount of kinin injected into the column. The buffer blank did not give any fluorescence signal. As can be seen in Fig. 2, the kinins exhibited different fluorescent sensitivities following labeling with fluorescamine but all showed linearity in terms of fluorescence versus concentration. Fluorescamine-labeled bradykinin could be detected at minimal amounts of 2.5 ng applied to the column. Lys-bradykinin was detected at a minimum of 5-ng quantitites and Met-Lys-bradykinin at 15-ng quantities. Linearity was observed for concentrations at least 10-fold higher than the minimum concentrations without altering the sensitivity of the fluorescence detector. Quantitation of native kinins was carried out by measuring the

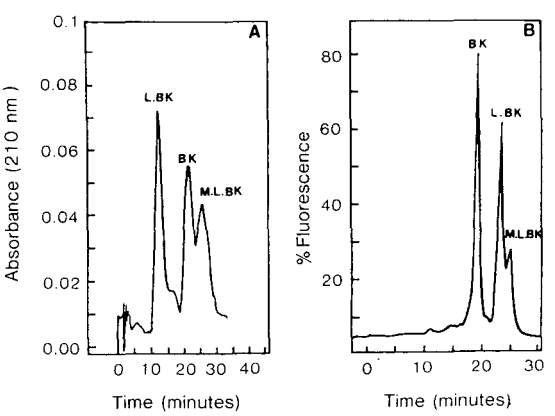


Fig. 1. (A) Elution pattern of a native kinin mixture (2 μg each) on a reversed-phase HPLC column. Kinins were eluted with a solvent system of 0.04 *M* triethylammonium formate buffer, pH 4.4—acetonitrile (88:12) at a flow-rate of 0.5 ml/min at ambient temperature. Kinins were detected at 210 nm. (B) Elution pattern of a fluorescamine-labeled kinin mixture (10 ng each) on a reversed-phase HPLC column. Labeled kinins were eluted with a linear gradient of methanol from 20% to 100% over 30 min at a flow-rate of 0.5 ml/min at ambient temperature. The fluorescence signal was detected at an excitation wavelength of 390 nm and an emission wavelength of 476 nm. Peaks: BK = bradykinin; L.BK = Lys-bradykinin; M.L.BK = Met-Lys-bradykinin.

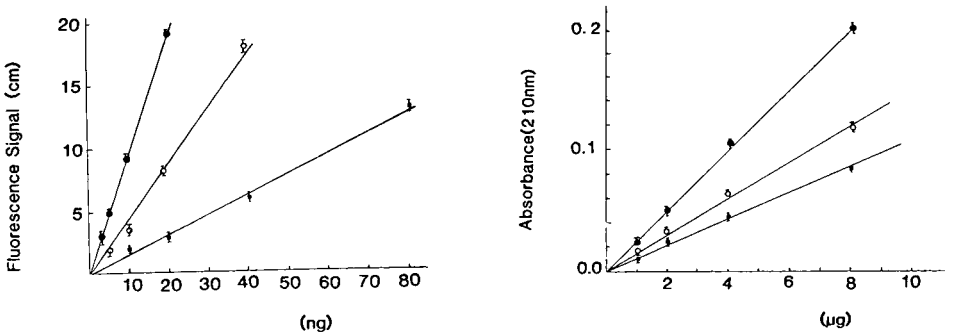


Fig. 2. Fluroescence signal of eluted fluorescamine-labeled kinins from the HPLC column versus concentration of kinin labeled with fluorescamine. Elution was with a linear gradient of methanol from 20% to 100% over 30 min at a flow-rate of 0.5 ml/min at ambient temperature. Fluorescence was determined as in Fig. 1. Each point represents mean ± standard error of quadruplicate experiments: (•) bradykinin (correlation coefficient 0.998; slope 0.954; S.E., 0.018); (○) Lys-bradykinin (correlation coefficient 1.000; slope, 0.370; S.E., 0.003); (▪) Met-Lys-bradykinin (correlation coefficient, 0.998; slope, 0.157; S.E. 0.003).

Fig. 3. Absorbance at 210 nm of eluted native kinins versus concentration injected into the HPLC column. Kinins were eluted with 0.04 *M* triethylammonium formate, pH 4.4,—acetonitrile (88:12). Each point represents the mean ± standard error of quadruplicate experiments: (○) bradykinin (correlation coefficient, 0.998; slope, 0.0145; S.E., 0.0002); (•) Lys-bradykinin (correlation coefficient, 0.997; slope, 0.0253; S.E., 0.0005); (▪) Met-Lys-bradykinin (correlation coefficient, 0.998; slope, 0.0099; S.E., 0.0001).

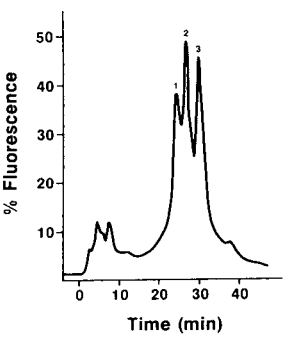
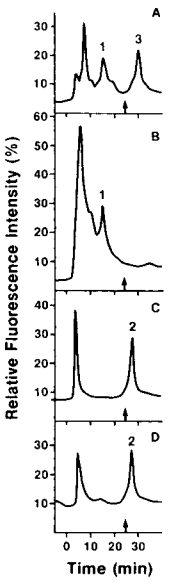


Fig. 4. Elution patterns of a mixture of fluorescamine-labeled bradykinin, des-Arg^9 -bradykinin and Phe-Arg dipeptide. A mixture of 20 ng of each peptide was injected into the HPLC column. Elution was with a linear gradient of acetonitrile from 20% to 40% over 60 min at a flow-rate of 0.4 ml/min at ambient temperature. Fluorescence was determined as in Fig. 1. Peaks: 1 = bradykinin; 2 = des-Arg^9-bradykinin; 3 = Phe-Arg-dipeptide.

absorbancy at 210 nm and plotting it against the amount of kinins injected into the column. Native kinins could be detected at a minimum of 1 µg. Linearity was observed for concentrations at least 8-fold higher than the minimum concentrations without altering the sensitivity of the UV detector (Fig. 3).

The elution patterns of fluorescamine-labeled bradykin and its common metabolic products, des-Arg^9-bradykinin and the dipeptide Phe-Arg, using a linear gradient of acetonitrile from 20% to 40% over 60 min are seen in Fig. 4.



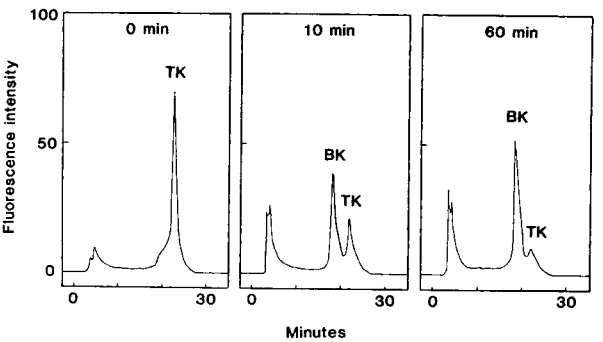


Fig. 5. Elution pattern of fluorescamine-labeled enzymatic products of bradykinin (see text for details of incubations and labeling). The elution time for bradykinin is indicated by an arrow. (A) Converting enzyme products: des-Phe^8-Arg^9-bradykinin (1) and Phe-Arg dipeptide (3); (B) carboxypeptidase-B followed by carboxypeptidase-A: des-Phe^8-Arg^9-bradykinin (1); (C) α-chymotrypsin: des-Arg^9-bradykinin (2); (D) carboxypeptidase-B: des-Arg^9-bradykinin (2).

Fig. 6. T-Kinin was incubated with aminopeptidase-M as described in the text. At 0, 10, and 60 min aliquots were removed, treated with fluorescamine and chromatographed. Labeled kinins were eluted with a linear gradient of methanol from 30% to 100% over 35 min at a flow-rate of 0.5 ml/min at ambient temperature. Peaks: BK = bradykinin, (appears with increasing time of incubation); TK = T-kinin.

The retention times were found to be for bradykinin 26.3 ± 0.3 min (n = 5), des-Arg^9-bradykinin 28.7 ± 0.3 min (n = 5) and Phe-Arg dipeptide 31.8 ± 0.3 min (n = 5). Using this same separation system, the elution patterns of fluorescamine-labeled products of bradykinin incubated respectively with converting enzyme, α-chymotrypsin, carboxypeptidase-B and carboxypeptidase-B followed by carboxypeptidase-A are shown in Fig. 5.

The retention time of fluorescamine-labeled des-Arg^9-bradykinin formed after the hydrolysis of bradykinin by carboxypeptidase-B (Fig. 5D) and by α-chymotrypsin (Fig. 5C) was found to be the same as the standard (see above). Labeled des-Phe^8-Arg^9-bradykinin, generated by the action of converting enzyme (Fig. 5A) and by carboxypeptidase-B followed by -A (Fig. 5B) on bradykinin, eluted with a retention time of 15 min. Labeled Phe-Arg dipeptide, formed by the action of converting enzyme on bradykinin was eluted as above.

The elution patterns of labeled T-kinin and its hydrolytic product, bradykinin, formed by the action of aminopeptidase-M with time are shown in Fig. 6. T-kinin was eluted with a retention time of 22.5 min.

DISCUSSION

As described here, fluorescamine labeling of kinins followed by HPLC provides for rapid identification and quantitation at nanogram levels of bradykinin and its analogues Met-Lys-bradykinin, Lys-bradykinin and Ile-Ser-bradykinin (T-kinin). The magnitude of the sensitivity of detection is the same as the bioassay on the rat uterus with the added advantages of distinct identification and the reproducibility and speed of a chemical procedure. In addition to kinin identification, slight alterations of the chromatographic procedure allow for detection of the enzymatic products of bradykinin when incubated with converting enyzme (des-Phe^8-Arg^9-bradykinin and Phe-Arg dipeptide), carboxypeptidase-B, (des-Arg^9-bradykinin), α-chymotrypsin (des-Arg^8-bradykinin and carboxypeptidase-B followed by carboxypeptidase-A (des-Phe^8-Arg^9-bradykinin). The conversion of T-kinin to bradykinin by aminopeptidase-M is also clearly demonstrable.

The fluorescamine method is much more sensitive (400-fold) than the UV detection of native kinins. Both methods taken together, however, are very useful in providing clear identification of bradykinin and its analogues since the sequence of elution of the kinins differs in the two systems. In the native kinin separation using acetonitrile and triethylammonium formate buffer, the order of elution is Lys-bradykinin, bradykinin and Met-Lys-bradykinin. The separation may be improved by using longer columns (e.g. 25 cm) and increasing the concentration of acetonitrile (e.g. 18%). When fluorescamine-labeled kinins (pyrollinone derivatives [14]) are chromatographed, using methanol and triethylammonium formate, the order of elution is bradykinin, Lys-bradykinin and Met-Lys-bradykinin.

It should be pointed out that attempts to identify a kinin by only one of these two HPLC procedures can be misleading since the hydrophobic nature of the molecule is an important criterion for separation. For example, T-kinin is clearly separated from bradykinin and its known analogues on one system of HPLC but chromatographs the same as Lys-bradykinin in the second system [15, 16]. Gabriel et al. [17] have made similar observations with other peptides. It should also be noted that these procedures are useful for detecting kinins only following purification, since, as pointed out by Geiger et al. [11], proteins may interfere with HPLC.

The fluorescamine—HPLC procedure for studies on kinetics of enzymatic degradation or conversions of purified kinins is simple, quantitative and rapid and needs no bioassay. However, during purification of a kinin from natural sources, the use of chromatography of the native kinin in conjunction with bioassay or radioimmunoassay is necessary since fluorescamine labeling will produce a plethora of peaks which are difficult to identify. However, once purification is moderately achieved, the identification by HPLC of both labeled and non-labeled kinins can be carried out without bioassay.

Radioimmunoassay is still the most sensitive method for quantitation of kinins at picogram levels [18]. A drawback of this method is that it cannot distinguish kinin analogues. Also, the antibody may react with biologically inactive peptides (des-Arg^9-bradykinin) and other degradative products of kinins. Though bioassay is a very useful method for quantitating kinins during

purification procedures, the method also cannot conveniently distinguish kinin analogues.

Finally, by using the fluorescamine—HPLC procedure the amounts necessary are minimal for determining purity as well as for identification, and peptide purification may proceed with a minimal loss of product.

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PURIFICATION OF SOLUBLE SPECIFIC ANTIGENS OF SYSTEMIC CANDIDIASIS BY ANTIBODY AFFINITY CHROMATOGRAPHY

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SUMMARY

Serological methods can be applied to the diagnosis of systemic candidiasis, but cell wall mannans can detect antibodies occurring in several cases unrelated to candidiasis. The present study proposes a procedure for the preparation of specific antigens obtained from an experimental infectious model. The specific immunoglobulins were obtained from rabbits with chronic systemic candidiasis. After precipitation by ammonium sulfate and purification by ion-exchange chromatography on DEAE-Sephadex A-50, these immunoglobulins were fixed on cyanogen bromide-activated Sepharose 4B and used for the preparation of an affinity chromatography column. This column allowed isolation of specifically bound fractions of *Candida albicans* soluble antigens. When analyzed by quantitative immunoelectrophoresis with a polyvalent hyperimmune antiserum, these fractions showed eight precipitation lines, whereas the complete soluble antigen exhibited 48 lines. Possible applications of these antigens to specific serodiagnosis of systemic candidiasis are being evaluated.

INTRODUCTION

Systemic candidiasis remains a major infectious problem for high-risk patients [1]. Direct diagnosis is extremely difficult; clinical signs are poorly evocative and hemocultures may be negative. The serological diagnosis can

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be helpful, particularly detection of precipitins to *Candida albicans* soluble cytoplasmic antigen. However, antigenic extracts used to this purpose contain contaminants of cell wall origin [2—6] which give rise to false-positive reactions for sera of apparently normal subjects [1, 6—9]. As a consequence, it seems necessary to utilize antigenic fractions specific for systemic candidiasis. Elimination of cell wall mannans and characterization of specific mycelial antigens have been tried [3, 4, 10—17].

The present paper reports the preparation of specific antigens of deep candidiasis using an original chromatographic procedure: antibodies obtained from rabbits with experimentally induced chronic systemic candidiasis were fixed on cyanogen bromide (CNBr) activated Sepharose 4B [18]. Antigens thus prepared were then analyzed by quantitative immunoelectrophoresis.

MATERIALS AND METHODS

Antibody preparation

Experimentally induced infection. Twelve hybrid albino rabbits in the weight range 3.5—4 kg were used. Three rabbits were inoculated with saline by injection into the external auricular vein, nine were inoculated with *Candida albicans*, six received 1.38×10^7 colony-forming units (CFU) per kg ($\equiv 0.1$ LD_{50}), and the remaining three received 2.8×10^7 CFU/kg ($\equiv 0.2$ LD_{50}).

From day 20 after inoculation, a 10-ml blood sampled was drawn from each rabbit every seven days for a three-month period. The course of infection was controlled by line immunoelectrophoresis of antibodies [19]. At the end of the three-month period, the rabbits were sacrificed and autopsies were done. Organs exhibiting macroscopic alterations were removed under sterile conditions and were used for post-mortem cultures after homogenization and for histopathologic tests performed in the Department of Anatomo-Pathology of the Institut Pasteur (Lyon, France).

Specific immunoglobulin G (IgG) purification. Only rabbit sera rich in antibodies were selected, mixed, precipitated by 25% ammonium sulfate and purified by ion-exchange chromatography on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals), as described by Harboe and Ingild [20], in pH 5 acetate buffer, flow-rate 25 ml/h. Purification was checked by cellulose acetate electrophoresis, immunoelectrophoresis, line immunoelectrophoresis and fused rockets, using anti-rabbit whole protein antiserum and anti-rabbit IgC antiserum (Institut Pasteur de Lyon).

Soluble antigen preparation

Culture was carried out in Sabouraud medium (Institut Pasteur Production), 30 g/l, enriched with 3 g/l of yeast extract (Difco), for 48 h at 30°C in a shaker incubator (GFL, Touzart et Matignon, Vitry-sur-Seine, France). After centrifugation for 15 min at 15,000 *g* the yeasts were collected and washed twice with sterile distilled water. Then a mixture of 40 g of 0.5 mm glass beads and 10—15 g of yeast was suspended in 15 ml of sterile distilled water in a special 70-ml flask (Braun Melsungen). Disruption of cells was done under liquid carbon dioxide for 2 min at 2800 cycles/min in a Braun Melsungen homogenizer. Breakage was checked under the light microscope and if the

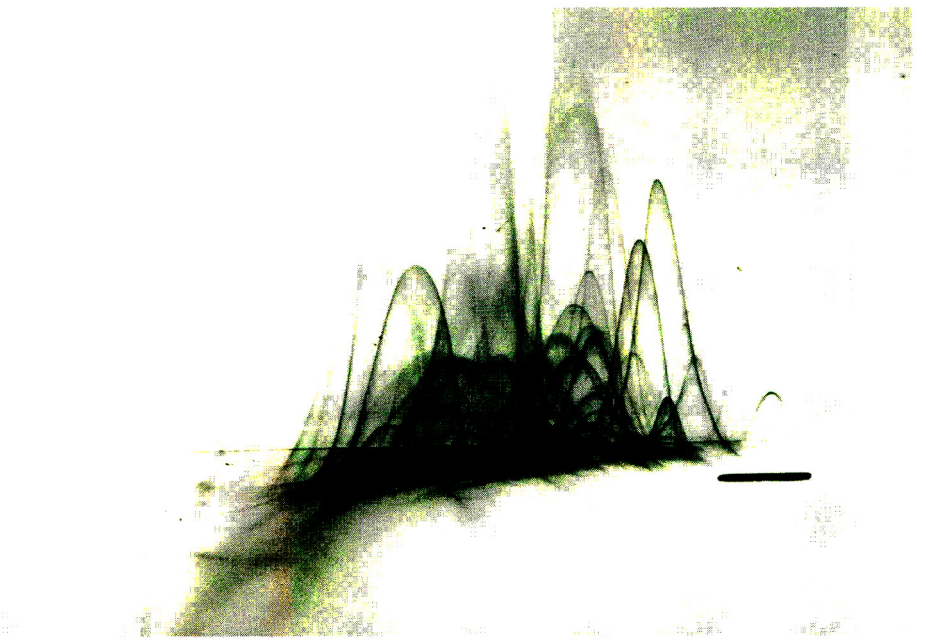

Fig. 1. Soluble *Candida albicans* antigen showing 65 fractions by crossed immunoelectrophoresis with the corresponding polyvalent antiserum.

amount of intact cells exceeded 30%, a second 1-min breakage was carried out.

The supernatant was clarified by a 30-min double centrifugation at 48,000 *g* (Beckman J2-21). The soluble antigen obtained had a protein concentration of 30 g/l (determined by refractometry) and exhibited 65 fractions on crossed immunoelectrophoresis (Fig. 1). The antigen was transferred to flasks and lyophilized.

Specific antigen purification

Immunoabsorbent preparation. A 15-g quantity of CNBr-activated Sepharose 4B (Pharmacia) was made to swell for 15 min in 10^{-3} *M* HCl solution. Then 500 mg of purified IgG were added to the 15 g of activated gel and coupling was achieved in pH 8.3 buffer (0.1 *M* $NaHCO_3$, 0.5 *M* NaCl) within 2 h at room temperature and by gentle rotary shaking. Remaining active groups were blocked by 1 *M* glycine at pH 8. The gel was then washed several times alternately in pH 4 buffer (0.1 *M* acetate, 0.5 *M* NaCl) and in pH 8.3 buffer (0.1 *M* $NaHCO_3$).

Chromatography. Chromatography was performed in a $C_{16/20}$ column (Pharmacia) with a bed volume of 13 ml and at flow-rate of 25 ml/h. The washed gel was equilibrated with 200 ml of pH 8.3 buffer (0.2 *M* Tris—HCl). Then 150 mg of the soluble antigenic preparation were applied at the top of the column. Elution was performed successively with pH 8.3 buffer (0.2 *M* Tris—HCl, 0.5 *M* NaCl) and pH 2.8 buffer (0.2 *M* glycine—HCl). The various fractions were salted out by dialysis and lyophilized. Antigenic analysis of these fractions was made by line immunoelectrophoresis [19] and fused rockets [21, 22] using a polyvalent anti-*Candida albicans* antiserum.

RESULTS

Experimentally induced infection

All rabbits receiving a 0.1 LD_{50} dose survived for three months and exhibited important kidney lesions and, to a lesser degree, liver and spleen lesions. They had lost 15% of their initial weight. Post-mortem cultures were positive for kidneys and negative for the other organs examined. Line immunoelectrophoresis of their sera showed 4—8 precipitation lines in relevant continuity with the reference line system.

The rabbits receiving a 0.2 LD_{50} dose also survived for three months but their pathologic conditions were more severe: tubular and interstitial nephritis, liver and spleen lesions, and lung, brain (paralysis) and eye damages were observed. They had lost 40% of their initial weight. Here again, only post-mortem cultures of kidney were positive. Line immunoelectrophoresis demonstrated 12—13 precipitation lines, also in continuity with the reference line system.

The rabbits receiving saline did not present any signs of infection.

Specific IgG purification

When using ion-exchange chromatography, type G immunoglobulins appeared in the first fraction (pH 5 and 0.06 *M*). The purified IgG were obtained with nearly 99% purity as checked by cellulose acetate electrophoresis. Line immunoelectrophoresis (Fig. 2) and fused rockets (Fig. 3) showed that only IgG were present in this fraction.

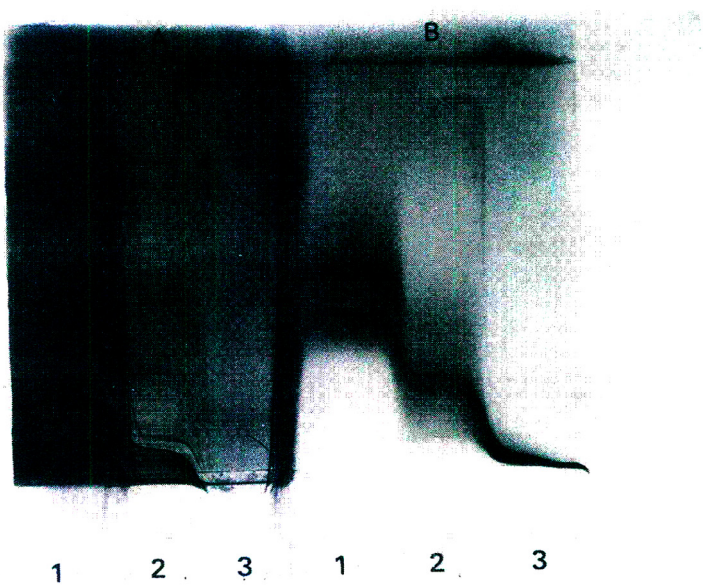

Fig. 2. IgG purification from sera of experimentally infected rabbits: control of separation steps by line immunoelectrophoresis. (A) 450 µl of anti-rabbit whole protein antiserum in 4.7 ml of agarose; (B) 450 µl of anti-rabbit IgG antiserum in 4.7 ml of agarose. 1 = 25 µl of serum from rabbit No. 7 in 250 µl of agarose; 2 = 25 µl of the same serum treated with ammonium sulfate; 3 = 25 µl of IgG purified by ion-exchange chromatography.

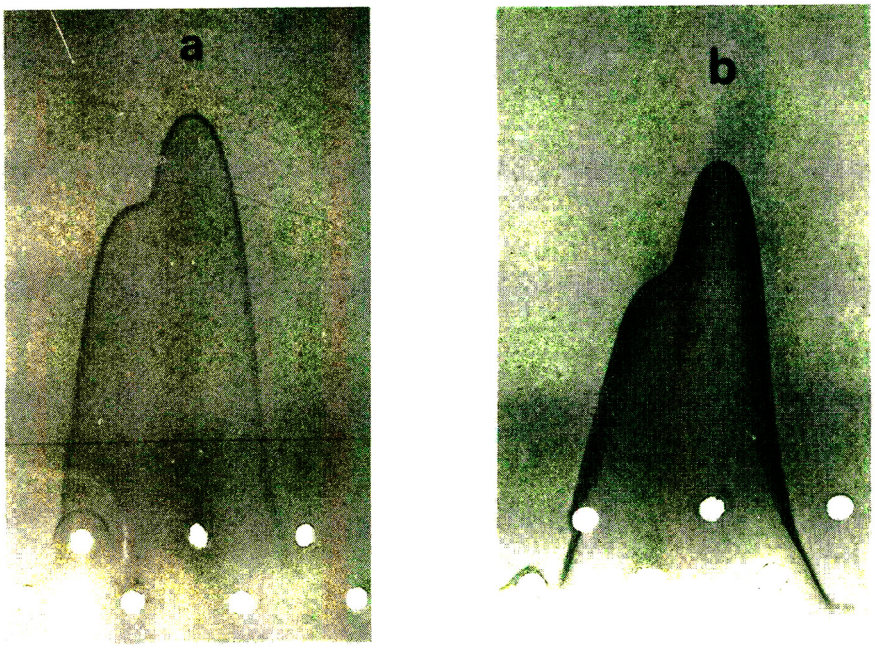

Fig. 3. IgG purification from sera of experimentally infected rabbits: control of separation steps by fused rockets (each well contained 2 μl of antigen). (a) 1 ml of anti-rabbit whole protein antiserum in 10 ml of agarose; (b) 1 ml of rabbit IgG antiserum in 10 ml of agarose.

Specific antigens purification

Using antibody affinity chromatography, unbound antigens were eluted with a total volume of 200 ml of pH 8.3 buffer and the eluate was collected in 28 fractions. The first eight fractions contained the greatest part of these

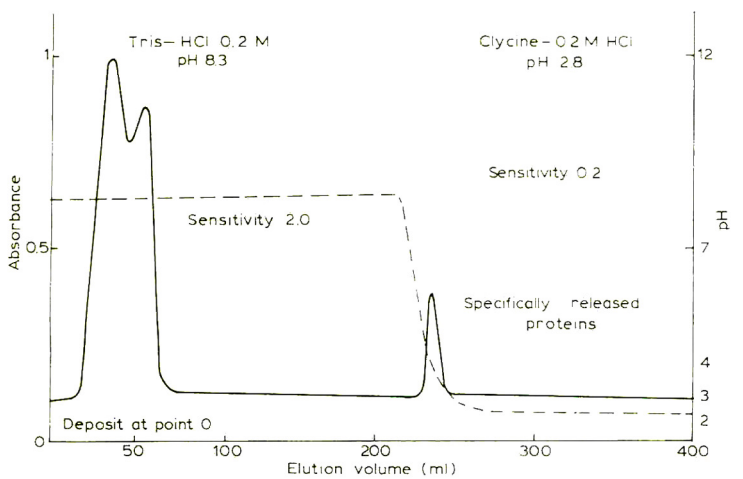

Fig. 4. UV absorbance chromatogram at 278 nm showing *Candida albicans* antigen separation by antibody affinity chromatography. The greater part of the antigens was not retained. The first peak recorded with low sensitivity was more important than the second peak recorded with maximum sensitivity, which implies that only a very small amount of antigen was retained by the affinity column.

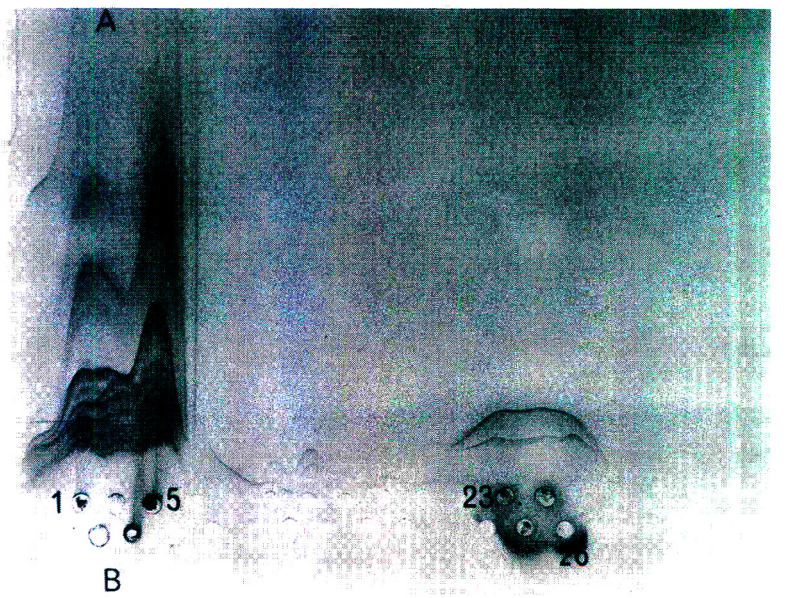

Fig. 5. Fused rockets of fractionated *Candida albicans* antigens by affinity chromatography. (A) 1 ml of anti-*Candida albicans* hyperimmune serum in 10 ml of agarose; (B) 4.2 ml of blind agarose. The distance between the wells was 4 mm. The wells received 2 μl of each fraction. Wells 1—5 corresponded to the first UV absorbance peak, wells 23—26 to the second peak (Fig. 4).

antigens. Then specifically bound antigens were eluted with a total volume of 30 ml of pH 2.8 buffer and the eluate collected in five fractions (Fig. 4).

Immunoelectrophoretic analysis and fused rockets of these fractions (Fig. 5) allowed antigen separation to be controlled during chromatography. In the line immunoelectrophoresis plate (Fig. 6) it was possible to count and identify the antigens that were present only in the second elution fractions. Eight precipitation lines could be identified; they all existed in the initial complete antigen showing 48 lines.

DISCUSSION

The chronic systemic *Candida albicans* infection experimentally induced in rabbit was clinically close to that observed in man, which is in agreement with results previously reported [4, 5, 16]. The infection allowed antibodies detectable by immunoprecipitation to be obtained. These antibodies were obtained with a nearly 99% degree of purity by combining ammonium sulfate precipitation with ion-exchange chromatography, as described elsewhere [20]. The antigens that were specifically adsorbed onto the antibody affinity column were wholly released at pH 2.8 and were recorded in one single, low concentration peak (Fig. 4). The yield was rather low: it was necessary to run the same sample three times in the column in order to retain the greater part of the specific fractions.

The purification of cytoplasmic extracts of *Candida albicans* by eliminat-

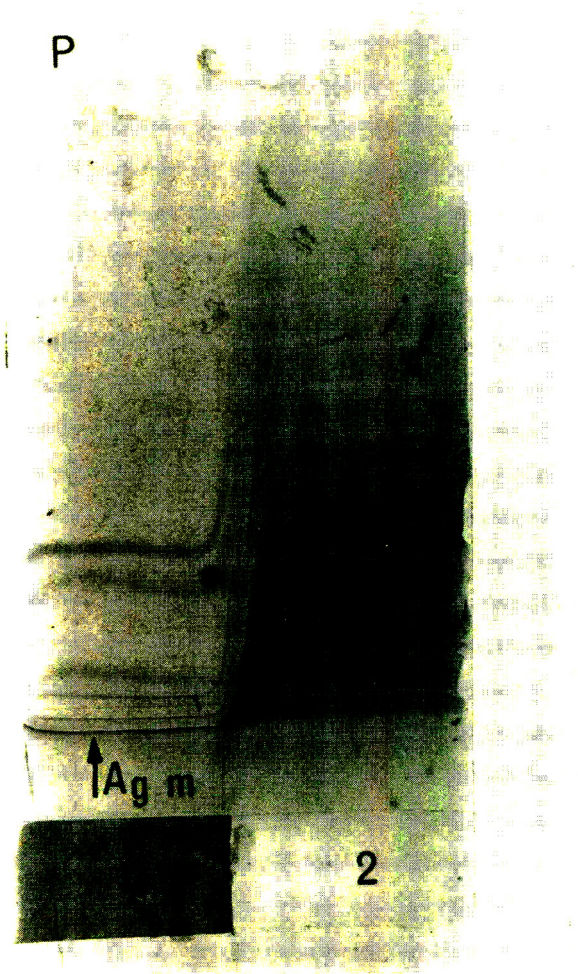

Fig. 6. Line immunoelectrophoretic identification of specific antigens of systemic candidiasis. P = polyvalent hyperimmune serum; 1 = purified specific antigen corresponding to the second peak in Fig. 4. Agm = major antigen forming a markedly intense precipitation line in the purified antigen as well as in the soluble complete antigen; 2 = soluble complete antigen.

ing mannans by affinity chromatography using concanavalin A [3, 14] improves the diagnostic specificity of systemic candidiasis. However, glycoproteins which play a role in candidal pathology are eliminated along with the mannans [4, 5]. Gabriel and Guinet [2, 23], using the immunoaffinoelectrophoresis method with concanavalin A Sepharose as ligand, discovered that 38% of the *Candida albicans* antigens studied showed marked affinity for this ligand. Our method for obtaining specific antigens of systemic candidiasis uses only their immunological properties, retaining exclusively those antigens that proved to develop immune response in animals with deep candidiasis.

The antibodies elaborated in the course of experimentally induced infec-

tion in the rabbit were all found to be present in hyperimmunized rabbits. This is in agreement with the findings of Axelsen et al. [10, 24], who demonstrated that antibodies developing in human systemic candidiasis were in all cases found in the hyperimmunized rabbit.

CONCLUSION

The serodiagnosis of systemic candidiasis depends mainly on the preparation of specific antigens of this pathologic entity. Our method, using antibodies elaborated during experimental systemic infection followed by affinity chromatography (antibody CNBr-activated Sepharose 4B), allows antigens to be obtained whose specificity and efficiency are being evaluated.

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SCREENING PROCEDURE FOR DETECTION OF PHENOTHIAZINE AND ANALOGOUS NEUROLEPTICS AND THEIR METABOLITES IN URINE USING A COMPUTERIZED GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC TECHNIQUE*

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SUMMARY

A method for the identification of phenothiazine and analogous neuroleptics and their metabolites in urine after acid hydrolysis is described. The acetylated extract is analysed by computerized gas chromatography—mass spectrometry. An on-line computer allows rapid detection using mass fragmentography with the masses *m/e* 58, 72, 86, 98, 100, 113, 114, 141 and 132, 148, 154, 191, 198, 199, 243, 267. The identity of positive signals in the reconstructed mass fragmentograms is established by comparison of the stored entire mass spectra with those of standards. The mass fragmentograms, the underlying mass spectra and the gas chromatographic retention indices (OV-101) are documented.

INTRODUCTION

Screening for phenothiazine and analogous neuroleptics is necessary in analytical toxicology to diagnose a probable intoxication. Furthermore, neuroleptics are encountered frequently in analysis when monitoring patients who may have taken addictive drugs and simultaneously take neuroleptics therapeutically. Detection of some of these drugs using ultraviolet spectrophotometry [2], paper chromatography [3], thin-layer chromatography [3—6], gas chromatography [6—8] and mass spectrometry [9] has been described. However, none of these methods allows the rapid and exact identification of all neuroleptics and their metabolites. This is important in clinical

*These results were reported in part at the Jahrestagung der Deutschen Gesellschaft für klinische Chemie in Stuttgart, F.R.G., September 23—24, 1982 [1].

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or forensic estimation of an intoxication because the various neuroleptics have different pharmacological potencies. Furthermore, all chromatographic spots or peaks must be identified because any of these may represent a potential poison. These demands are met by the computerized gas chromatographic—mass spectrometric (GC—MS) technique described below. This method has the further advantage that several other groups of drugs can be detected simultaneously by simply searching for typical fragment masses in the stored spectra.

If necessary, plasma levels of the identified phenothiazines can be determined using a GC or a GC—MS procedure described in the literature [10—13].

EXPERIMENTAL

Apparatus

A Varian Aerograph gas chromatograph series 1400 combined with a Varian mass spectrometer type 311 A, a Varian data system 111 MS and a Tektronix storage display unit type 611 was used. The GC conditions were as follows. Column: nickel-capillary 60 cm × 1 mm I.D., packed with Chromosorb G HP 100—120 mesh coated with 5% OV-101. Column temperature: programmed from 100 to 310°C at 20°C/min, final time 3 min. Injector port temperature: 270°C. Carrier gas: helium, flow-rate 7 ml/min.

The MS conditions were as follows: ionization energy, 90 eV; ion-source temperature, 200°C. The technique of open coupling was used. About 2 ml/min of gas were dosed by an SGE micro-needle-valve and an SGE shut-off valve (Scientific Glass Engineering, Ringwood, Australia) and introduced into the ion source by a nickel capillary (0.15 mm I.D., heated at 270°C).

For the exact measurement of retention indices a Varian gas chromatograph series 3700 was used. The column effluent went to a flame-ionization detector and a nitrogen-sensitive flame-ionization detector after a 1:1 split by a splitter made from nickel tubing. The column was a nickel tube, 60 cm × 2 mm I.D., packed as for GC—MS. The column and injector temperatures were as for GC—MS; the temperature of the detectors was 270°C. Carrier gas was nitrogen at a flow-rate of 30 ml/min.

Urine samples

The investigations were carried out using urine from psychiatric in-patients, who were treated with therapeutic dosages of neuroleptics. If no human samples were available urine was used from rats that were given 20—40 mg/kg body weight of an aqueous suspension of the drug by gastric tube (see column "S" in Table I).

Hydrolysis and extraction procedure

Ten millilitres of urine were refluxed with 3 ml of hydrochloric acid (37%) for 15 min, then made basic with about 3 g of potassium hydroxide pellets and mixed with 10 ml of 30% aqueous ammonium sulphate to obtain a pH between 8 and 9. The samples were extracted twice with 10 ml each of a mixture of two parts of dichloromethane, two parts of isopropanol and six parts of ethyl acetate. After phase separation by centrifugation the combined organic extracts

were evaporated to dryness under vacuum. The residue was redissolved in 0.1 ml of methanol [14].

Acetylation

A 40-μl volume of extract was evaporated and then acetylated for 30 min at 60°C with 40 μl of a mixture of three parts of acetic anhydride in two parts of pyridine. After evaporation of the acetylation mixture the residue was redissolved in 40 μl of ethyl acetate [14]. A 1—4-μl volume of this sample was injected into the gas chromatograph.

Gas chromatographic—mass spectrometric analysis

Mass spectra were recorded at a speed of 6 sec/decade and stored on computer tape during the temperature-programmed GC analysis. Scanning at this relatively slow rate ensures at least two spectra for each GC peak and avoids excessive data accumulation. The identity of positive signals in the reconstructed mass fragmentograms was established by a comparison of the entire mass spectra with those of standards (Fig. 1).

RESULTS AND DISCUSSION

Some of the neuroleptics are excreted in urine completely metabolized and conjugated. Therefore, the conjugates were cleaved by acid hydrolysis, which can be completed more quickly than enzymatic hydrolysis. To improve their GC characteristics hydroxy and amino groups were acetylated.

The results of our investigations are shown in Table I. The two mass fragmentograms with eight masses each allow the detection of 29 neuroleptics and their metabolites. Some of these compounds are acetylated (see formulae in Fig. 1). Flupenthixol, homofenazine, oxypertine, sulforidazine, tetrabenazine and thiopropazate are not detected in urine because they are almost completely excreted as their metabolites or they are not volatile under the applied GC conditions which are approved for toxicological analysis.

The retention indices were determined using a gas chromatograph combined with flame-ionization detection and nitrogen-sensitive flame-ionization detection with a temperature programme. In our experience, retention indices are not necessary when employing a GC—MS technique, but since they give preliminary indications and may be useful to gas chromatographers without the latter facility, they are given here. The molecular ions and the mass spectra numbers of Fig. 1 are included.

The entire mass spectra are shown in Fig. 1 for the precise identification of the compounds. Formulae are proposed for probable structures of metabolites. Only those metabolites which were usually found are given. Because of inter-individual differences in metabolism or the variable time elapsed after administration, not all given metabolites were detected in each sample. Further metabolites can be found. The mass spectra and retention indices of these will be included in a forthcoming handbook [15].

Although some neuroleptics lead to common metabolites (mass spectra Nos. 1, 5, 7, 8, 18, 29, 45, 70, 80, and 84 in Fig. 1), they can be differentiated because the parent compounds or additional unique metabolites are also

TABLE I

MONITORING PROGRAM FOR PHENOTHIAZINE AND ANALOGOUS NEUROLEPTICS AND THEIR METABOLITES

MS No.	M^+	Name*	Species**	*m/e* (relative intensities)***					
				58	72	86	98	100	113
25	326	Acepromazine	R	+++	+	+			
49	370	M (dihydro-)	R	+++	+	+			
60	384	M (HO-)	R	+++	+	+			
40	354	M (nor-)	R	+	+	+		+	
23	326	Acepromethazine	M	+	+++				
48	370	M (dihydro-)	M	+	+++				
39	354	M (nor-)	R	+++	+			+	
59	384	M (HO-)	M	+	+++				
13	298	Alimenazine	M	+++		+		+	
01	199	M (ring)	M						
42	356	M (HO-)	M	+++		+		+	
24	326	M (nor-)	M	+	+	++			
18	312	M (bis-nor-)	M		+				
58	384	M (nor-HO-)	M			+			
74	409	Butaperazine	R	+	+		+		++
81	437	M (nor-)	R	+		+			
22	318	Chlorpromazine	M	+++	+	++			
05	233	M (ring)	M						
29	332	M (bis-nor-)	M		+		+++		
37	346	M (nor-)	M	+	+	+		+	
21	315	Chlorprothixene	M	+++					
08	270	M (N-oxide)	M						
55	375	M (HO-dihydro-)	M	+++					
36	345	M (nor-dihydro-)	M	+					
35	343	M (nor-)	M			+			
71	403	M (nor-HO-dihydro-)	M		+	+			
83	442	Clopenthixol	R				++		
08	270	M (N-oxide)	R						
69	400	M (desalkyl-dihydro-)	R				+		
67	398	M (desalkyl-)	R			+			
26	326	Clozapine	M						
41	396	M (nor-)	M			+			
65	354	M (nor-) monoacetyl-	M						
86	469	Dixyrazine	R	+			+		+
01	199	M (ring)	R						
18	312	M (amino-)	M		+				
77	425	M (O-desalkyl-)	R				+		
57	381	M (N-desalkyl-)	R						
		Flupenthixol							
87	478	M (dihydro-)	R				+		
79	434	M (desalkyl-dihydro-)	R				+		

114	141	132	148	154	191	198	199	243	267	Retention index
						+				2757
										2765
										3041
+++						+	+			3143
						+				2626
										2690
++										2940
+										3026
						+				2313
						+	+++			2080
										2600
						++	+			2709
++						+	+			2767
										2930
	+					+				3188
	+++					++				3800
										2500
						++	+			2099
						+				2990
+++						+				3068
										2510
										2409 FID
										2800
+							+			2930
										2945
+										3194
										3462
										2409 FID
	+++									3450
	+++									3490
					+			+++		2893
					+			+	+	3492
					+			++		3650
						+	+++			3531
						+	+++			2080
++						+	+			2767
						+	+			3350
	+++					+	+			3355
									+	3004
	++									3054

(Continued on p. 130)

TABLE I (continued)

MS No.	M^+	Name*	Species**	*m/e* (relative intensities)***					
				58	72	86	98	100	113
88	479	Fluphenazine	R	+			+		+
07	267	M (ring)	R						
45	366	M (amino-)	R	+	+			+++	
80	435	M (desalkyl-)	R	+		+			
		Homofenazine							
07	267	M (ring)	R						
45	366	M (amino-)	R	+	+			+++	
85	449	M (desalkyl-)	R	+			+		+
27	328	Levomepromazine	M	+++				+	
62	386	M (HO-)	M	+++				+	
43	356	M (nor-)	M	+		+		+	
76	414	M (nor-HO-)	M	+		+			
		Oxypertine							
03	204	M (phenylpiperazine)	R						
06	262	M (HO-phenylpiperazine)	R						
16	310	Pecazine	R	+++			+		
01	199	M (ring)	R						
47	368	M (HO-)	R	+++			+		
30	338	M (nor-)	R				+		
64	396	M (nor-HO-)	R				+		
31	339	Perazine	M	+	+				+
01	199	M (ring)	M						
66	397	M (HO-)	M	++					+
46	367	M (nor-)	M	+		+		+	
73	407	Periciazine	R				+		
04	224	M (ring)	R						
84	445	Perphenazine	R			++	++		+
05	233	M (ring)	R						
29	332	M (amino-)	R		+			+++	
70	401	M (desalkyl-)	R	+					
54	373	Prochlorperazine	R	+	+				++
05	233	M (ring)	R						
29	332	M (amino-)	R		+			+++	
70	401	M (nor-)	R	+					
11	284	Promazine	M	+++	+	+			
01	199	M (ring)	M						
33	342	M (HO-)	M	+++	+	+			
19	312	M (nor-)	M					+	
52	370	M (nor-HO-)	M		+	+		+	
10	284	Promethazine	M		+++				
01	199	M (ring)	M						
17	312	M (nor-)	M	+++	+			+	
32	342	M (HO-)	M	+	+++				
50	370	M (nor-HO-)	M	+	+			+	

114	141	132	148	154	191	198	199	243	267	Retention index
									+	3169
									+++	2190
									+	2765
	+++								++	3150
									+++	2190
									+	2765
	+								+++	3240
										2542
										2747
										2970
										3220
		+++								1872
			+++							2355
						+	+			2546
						+	+++			2080
										2750
						+	+			2985
										3414
		+				+	+			2790
						+	+++			2080
		+								3190
	+	+					+++			3212
	+									3391
					+					2552
							+			3468
						++	+			2099
						+				2990
		+++								3500
		+				+				2983
						++	+			2099
						+				2990
		+++								3500
						+	+			2313
						+	+++			2080
										2709
						+	+			2804
	+++									3196
	+++									
						+	+			2272
						+	+++			2080
										2640
	++									2690
										3017
	+++									

(Continued on p. 132)

TABLE I (continued)

MS No.	M^+	Name*	Species**	*m/e* (relative intensities)***					
				58	72	86	98	100	113
12	285	Prothipendyl	M	+++	+	++			
02	200	M (ring)	M						
34	343	M (HO-)	M	+++	+	+			
14	299	M (bis-nor-)	M		+			+	
20	313	M (nor-)	M	+	+	+		+	
53	371	M (nor-HO-)	M					+	
		Sulforidazine							
9	277	M (ring)	M						
78	430	M (nor-)	R						
		Tetrabenazine							
28	331	M (bis-desmethyl-)	M						
44	361	M (desmethyl-HO-)	M						
63	389	M (bis-desmethyl-HO-)	M						
84	445	Thiopropazate	R			++	++		+
05	233	M (ring)	R						
29	332	M (amino-)	R		+			+++	
70	401	M (desalkyl-)	R	+					
		Thioproperazine							
15	306	M (ring)	R						
51	370	Thioridazine	M	+			+++		
68	398	M (nor-)	M						
61	384	M (oxo-)	M	+					+
72	407	Trifluoperazine	R	+					++
07	267	M (ring)	R						
45	366	M (amino-)	R	+	+			+++	
80	435	M (nor-)	R	+		+			
38	352	Triflupromazine	R	+++	+	+			
07	267	M (ring)	R						
75	410	M (HO-)	R	+++		+			
56	380	M (nor-)	R		+	+		+	
45	366	M (bis-nor-)	R	+	+			+++	
82	438	M (nor-HO-)	R	+	+	+		+	

*M ( ) = metabolite, HO- = hydroxy
**M = man, R = rat.
***+++ = > 95% relative intensity, ++ = 50—95%, + = < 50%.

excreted. Only perphenazine and its acetyl ester thiopropazate cannot be differentiated.

The Cope elimination reaction of the N-oxides of chlorprothixene and clopenthixol is the only kind of artifact which results from the analytical procedure used (mass spectrum No. 8).

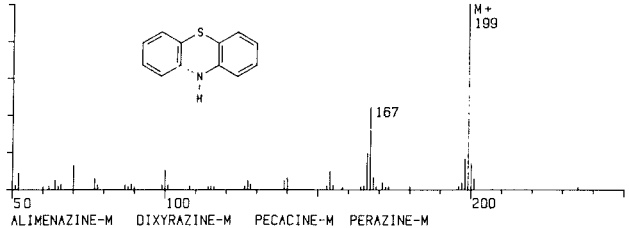
Because all compounds possibly indicated by the mass fragmentograms can be positively identified by comparison of the underlying mass spectra with those of standards (Fig. 1), interferences by other drugs are impossible.

114	141	132	148	154	191	198	199	243	267	Retention index
							+			2348
							+			2049
							+			2781
							+			2830
+++							+			2880
+++										3070
						+				3180
				+++		+				3800
					+++					2510
					+					2584
					++					2666
							+			3468
						++	+			2099
						+				2990
	+++									3500
						++	+			3200
										3125
				+++						3490
						+	+			3500
	+								+	2683
									+++	2190
									+	2765
	+++								++	3150
									+	2239
									+++	2190
										2720
+++									+	2740
									+	2765
+++									+	3120

To illustrate the method two mass fragmentograms from the urine of a psychiatric patient are shown in Fig. 2. Peak 1 indicates thioridazine, peak 2 its N-desmethyl metabolite and peak 3 its oxo metabolite (mass spectra Nos. 51, 68, 61).

The presented screening procedure allows a rapid and exact identification and differentiation of phenothiazine and analogous neuroleptics and their metabolites in urine. It has the advantage that other groups of drugs can be detected simultaneously by simply searching for typical fragment masses in the

1

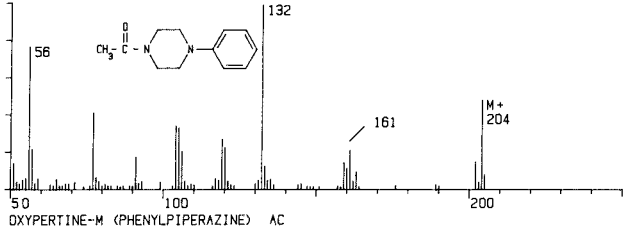


ALIMENAZINE-M DIXYRAZINE-M PECACINE-M PERAZINE-M
PROMAZINE-M PROMETHAZINE-M

2

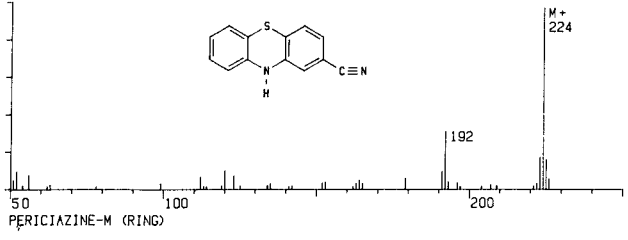
PROTHIPENDYL-M (RING)

3



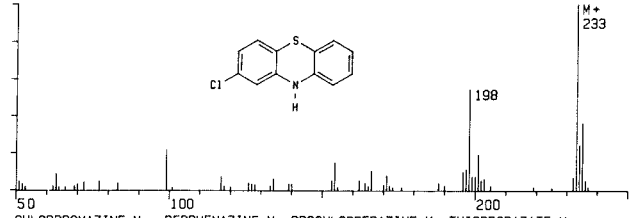
OXYPERTINE-M (PHENYLPIPERAZINE) AC

4



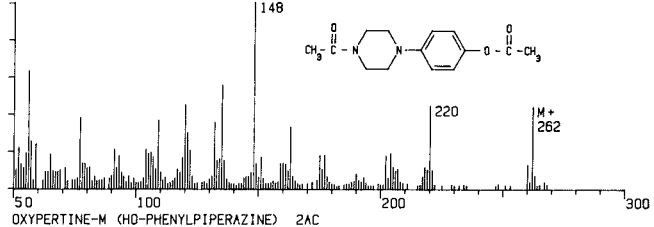
PERICIAZINE-M (RING)

5



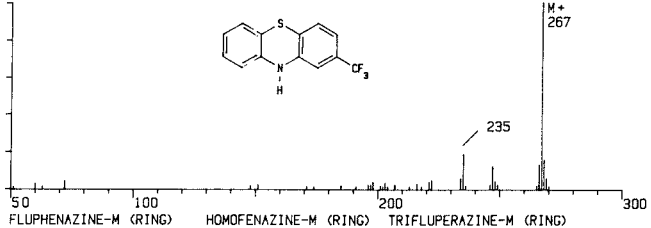
CHLORPROMAZINE-M PERPHENAZINE-M PROCHLORPERAZINE-M THIOPROPAZATE-M

6



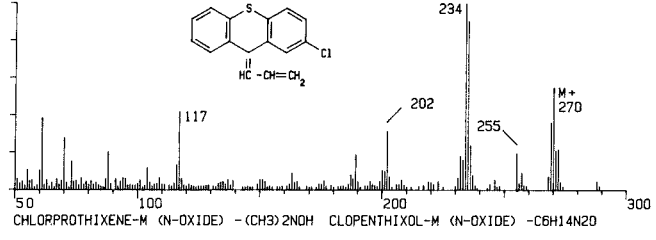
OXYPERTINE-M (HO-PHENYLPIPERAZINE) 2AC

7



FLUPHENAZINE-M (RING) HOMOFENAZINE-M (RING) TRIFLUPERAZINE-M (RING)
TRIFLUPROMAZINE-M (RING)

8



CHLORPROTHIXENE-M (N-OXIDE) -(CH3)2NOH CLOPENTHIXOL-M (N-OXIDE) -C6H14N2O

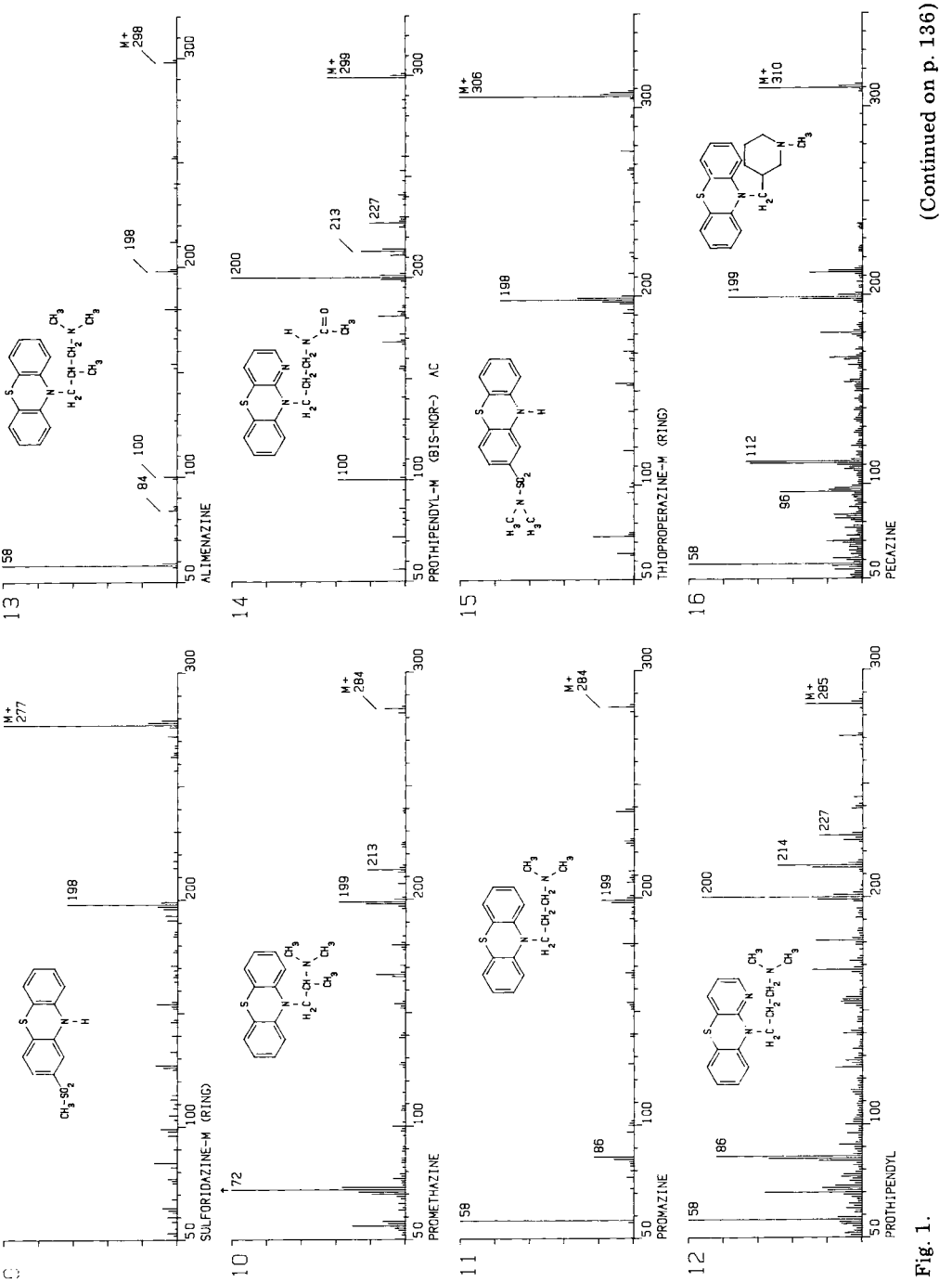
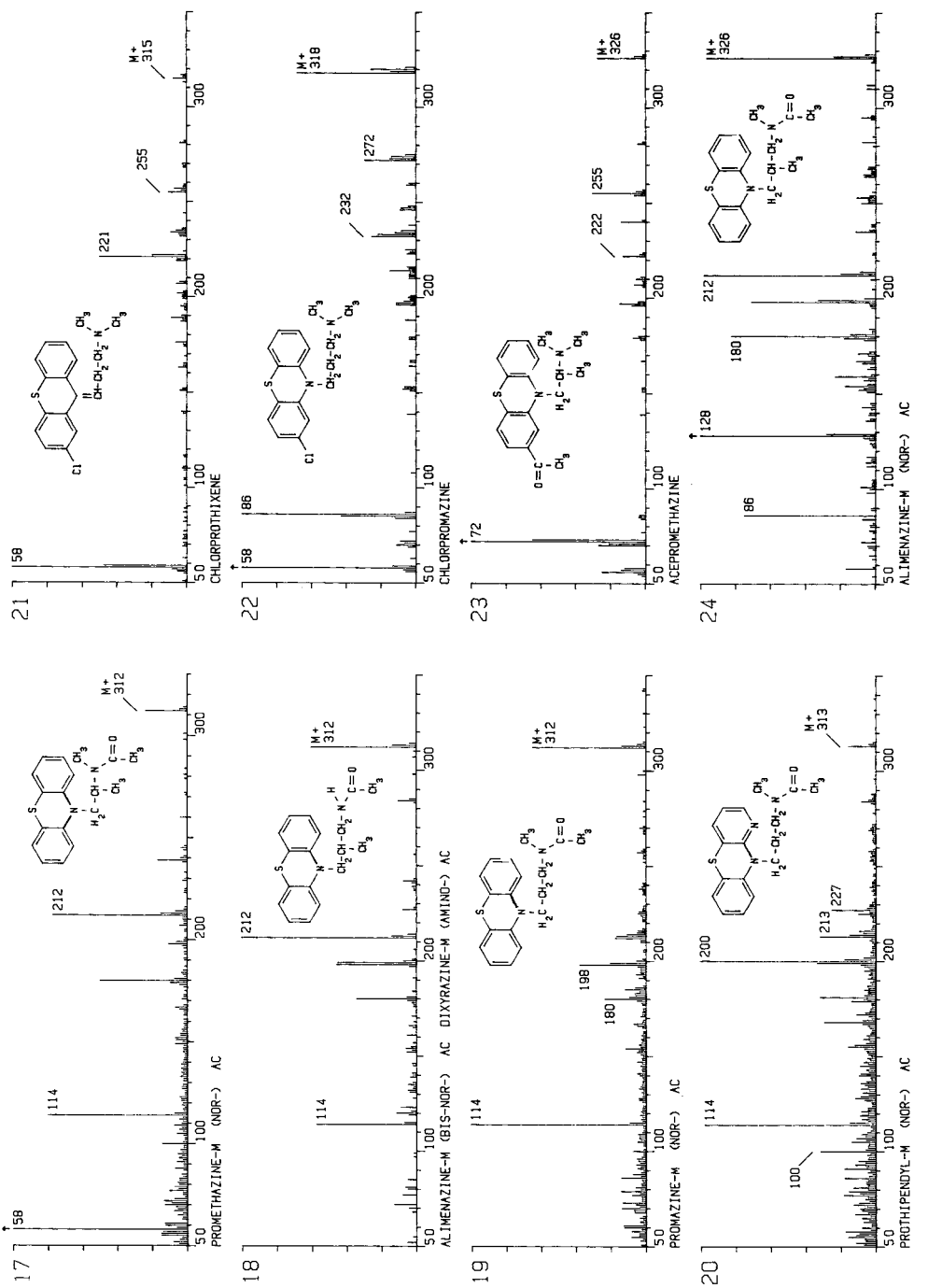


Fig. 1.

(Continued on p. 136)


17
PROMETHAZINE-M (NOR-) AC
18
ALIMENAZINE-M (BIS-NOR-) AC
DIXYRAZINE-M (AMINO-) AC
19
PROMAZINE-M (NOR-) AC
20
PROTHIPENDYL-M (NOR-) AC
21
CHLORPROTHIXENE
22
CHLORPROMAZINE
23
ACEPROMETHAZINE
24
ALIMENAZINE-M (NOR-) AC

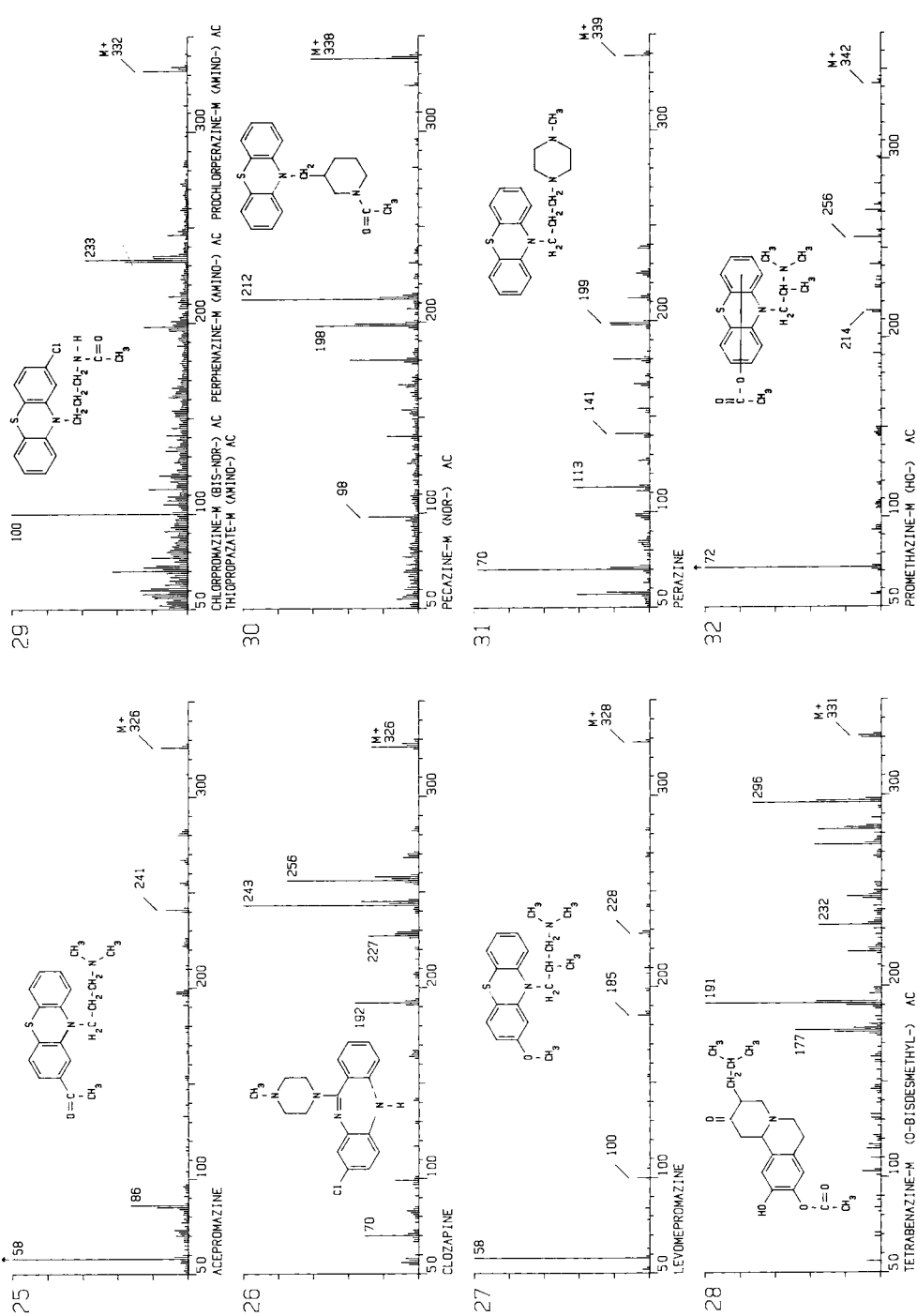
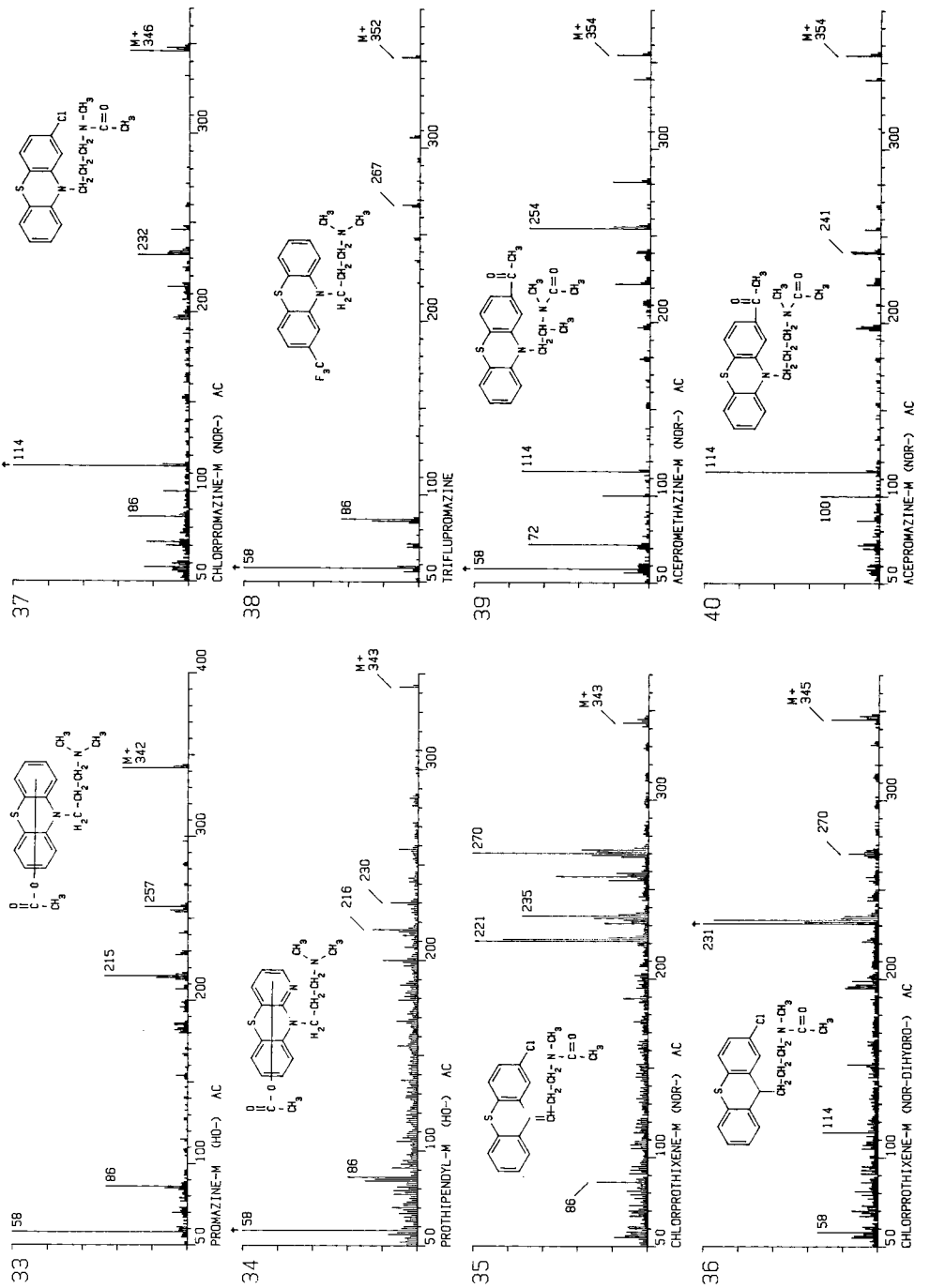


Fig. 1.

(Continued on p. 138)


33
PROMAZINE-M (HO-) AC
34
PROTHIPENDYL-M (HO-) AC
35
CHLORPROTHIXENE-M (NOR-) AC
36
CHLORPROTHIXENE-M (NOR-DIHYDRO-) AC
37
CHLORPROMAZINE-M (NOR-) AC
38
TRIFLUPROMAZINE
39
ACEPROMETHAZINE-M (NOR-) AC
40
ACEPROMAZINE-M (NOR-) AC

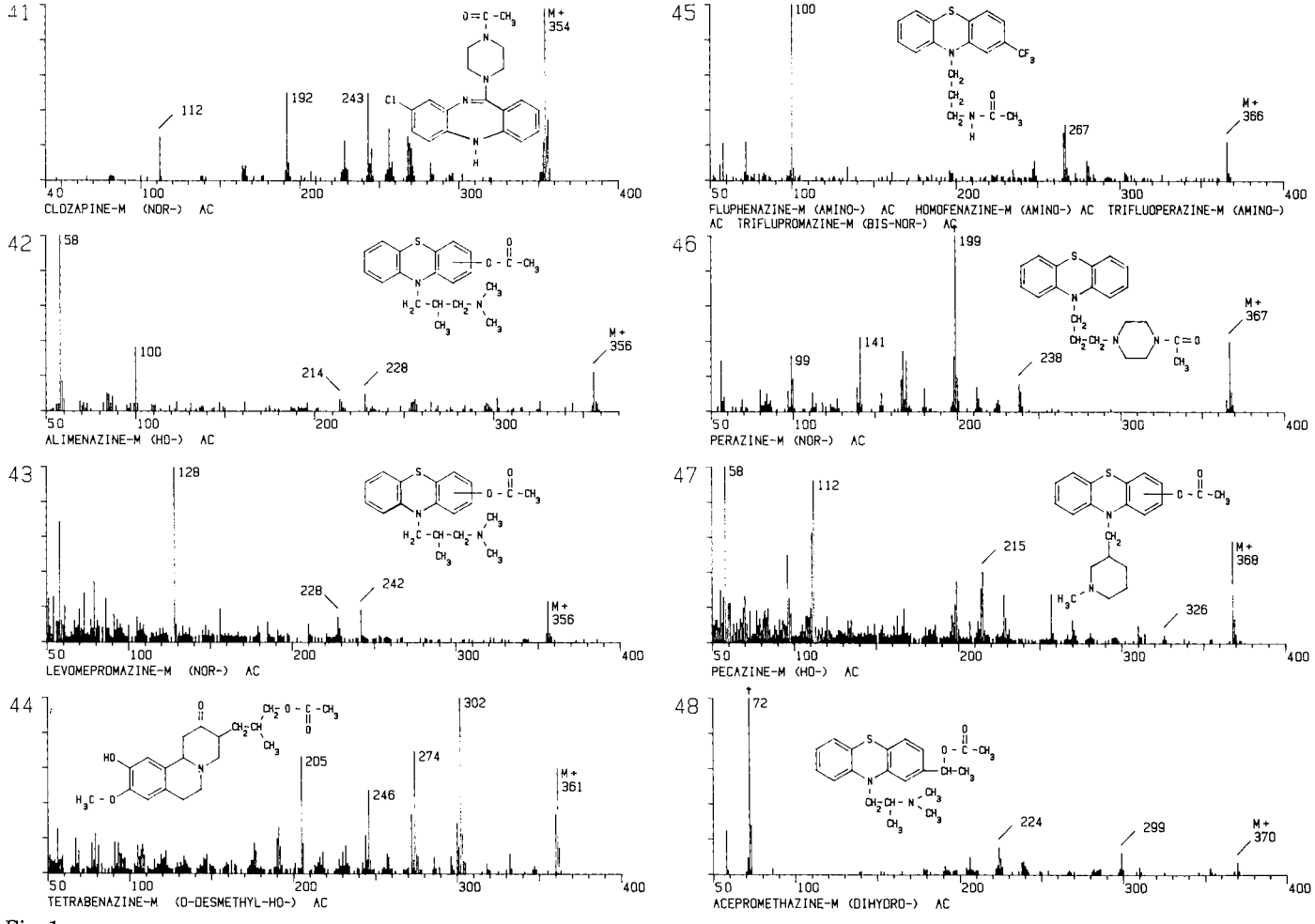
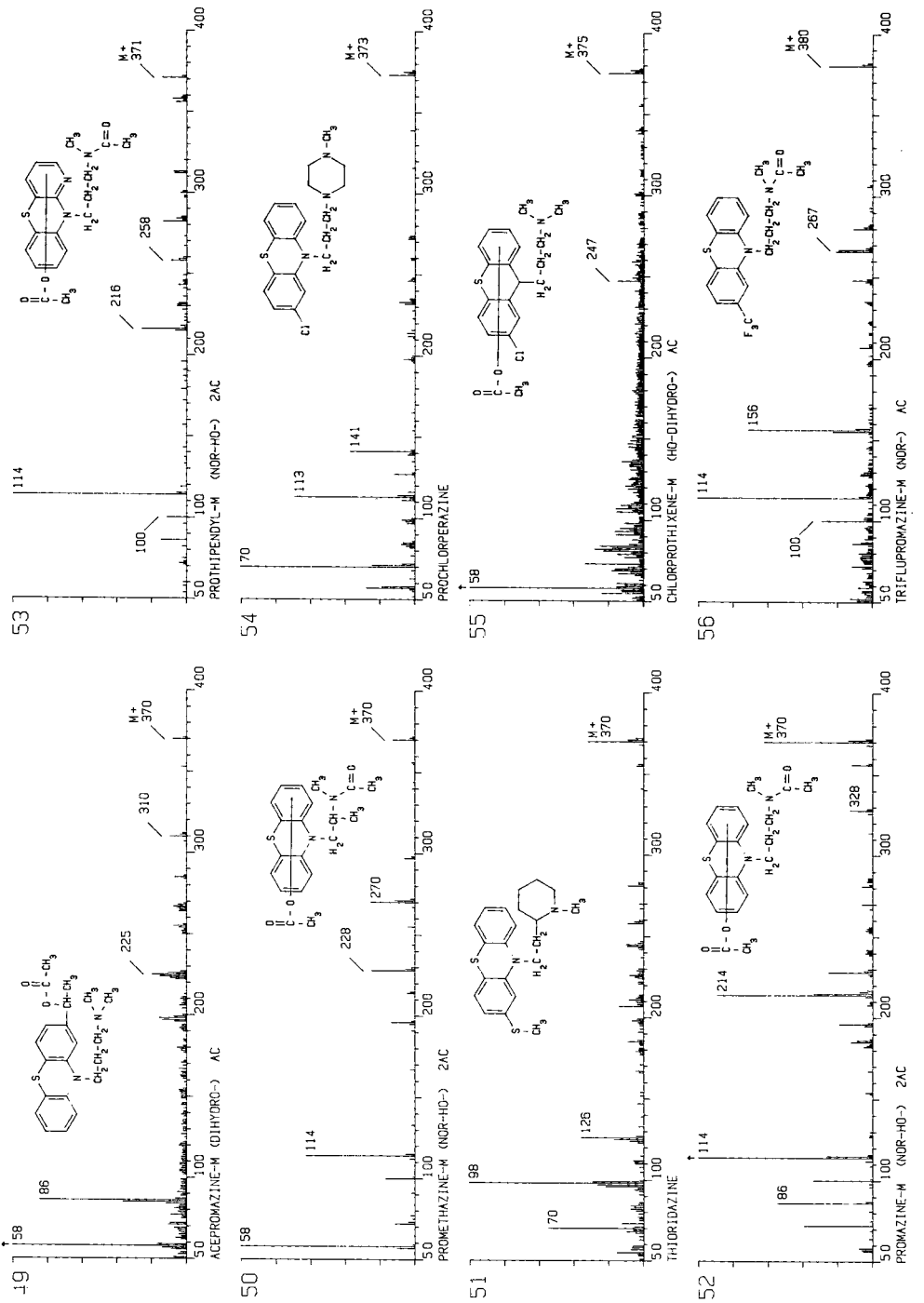


Fig. 1.

(Continued on p. 140)


49
ACEPROMAZINE-M (DIHYDRO-) AC
50
PROMETHAZINE-M (NOR-HO-) 2AC
51
THIORIDAZINE
52
PROMAZINE-M (NOR-HO-) 2AC
53
PROTHIPENDYL-M (NOR-HO-) 2AC
54
PROCHLORPERAZINE
55
CHLORPROTHIXENE-M (HO-DIHYDRO-) AC
56
TRIFLUPROMAZINE-M (NOR-) AC

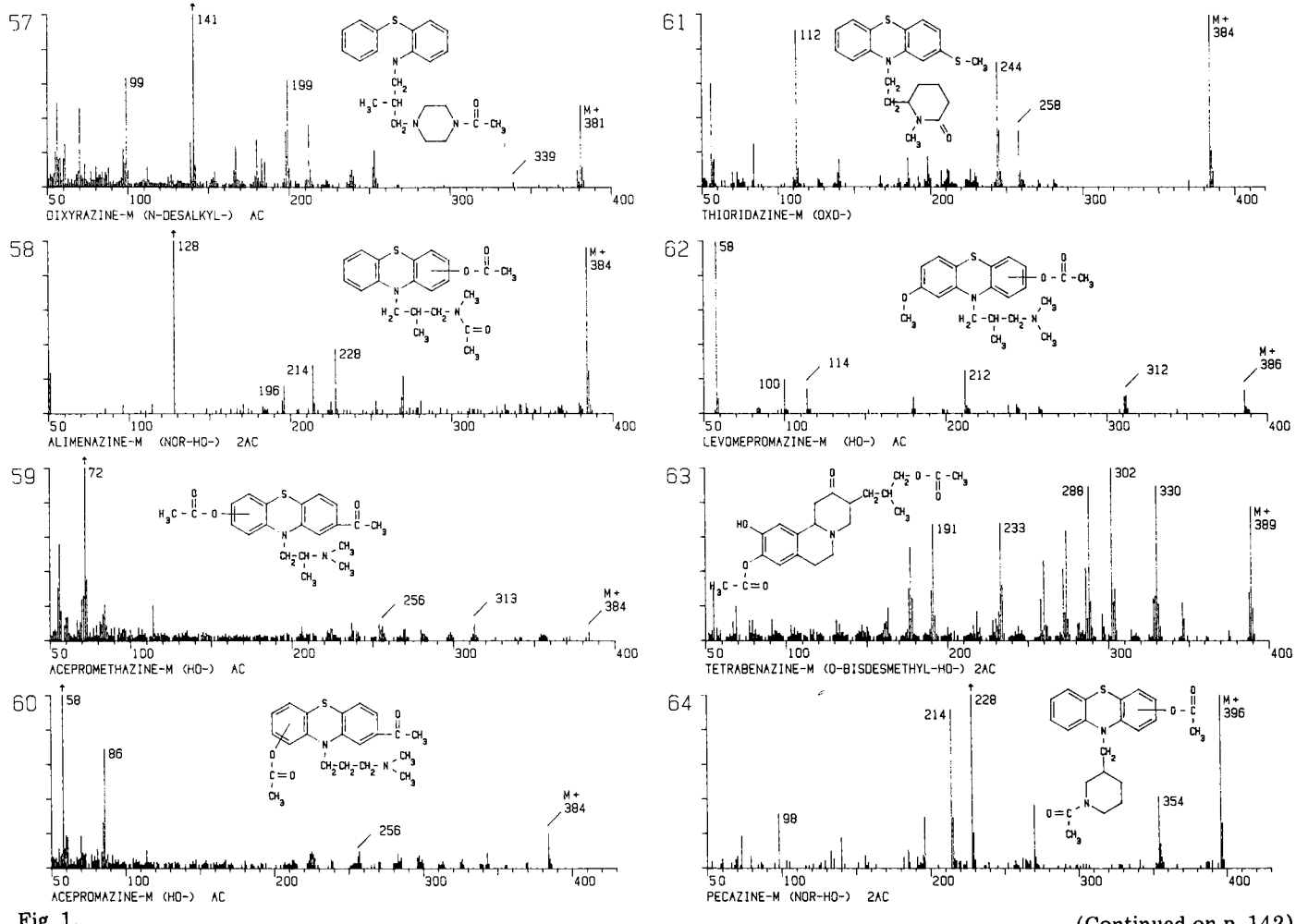
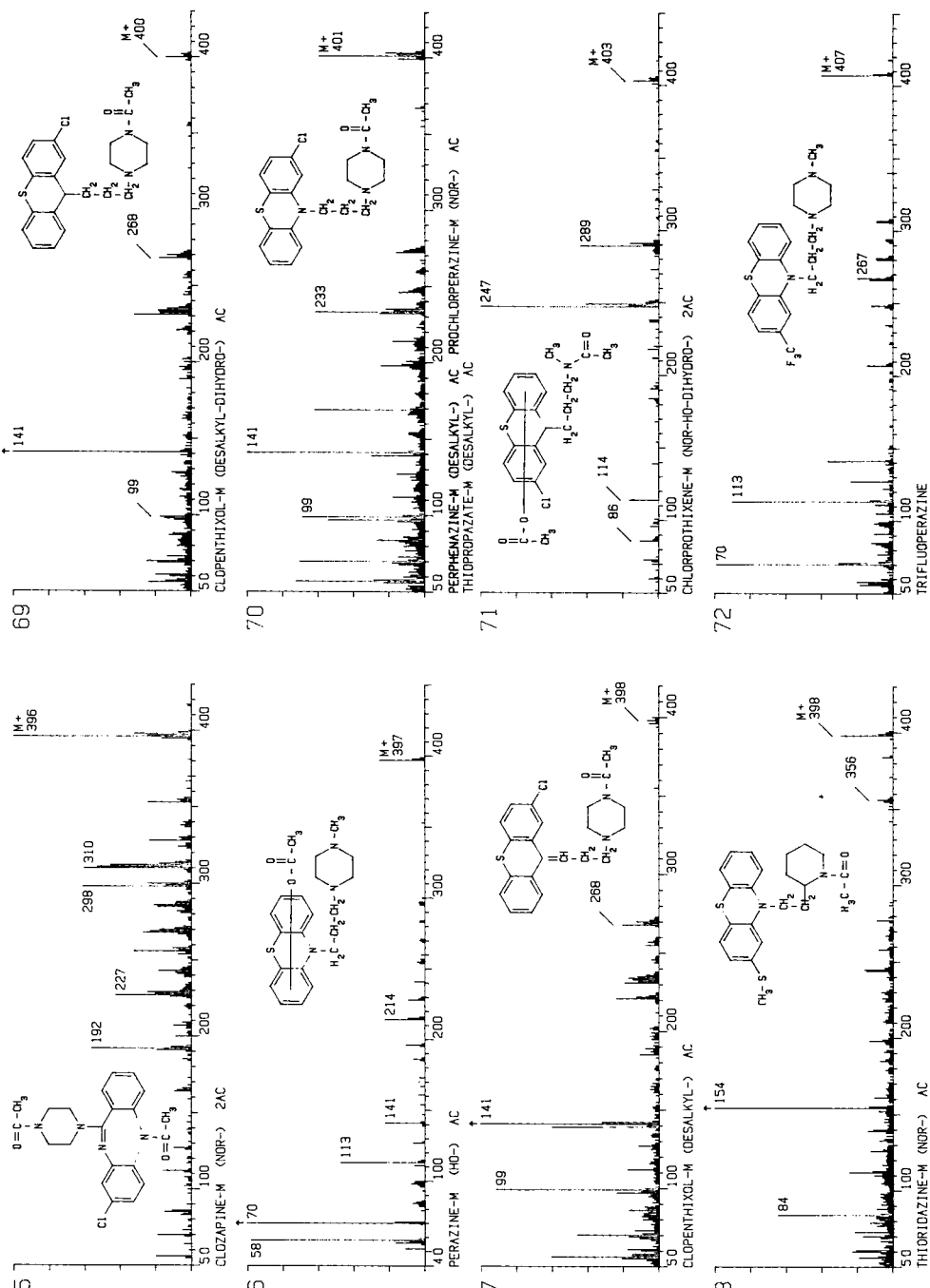


Fig. 1.

(Continued on p. 142)


65
CLOZAPINE-M (NOR-) 2AC
66
PERAZINE-M (HO-) AC
67
CLOPENTHIXOL-M (DESALKYL-) AC
68
THIORIDAZINE-M (NOR-) AC
69
CLOPENTHIXOL-M (DESALKYL-DIHYDRO-) AC
70
PERPHENAZINE-M (DESALKYL-) AC PROCHLORPERAZINE-M (NOR-) AC
THIOPROPAZATE-M (DESALKYL-) AC
71
CHLORPROTHIXENE-M (NOR-HO-DIHYDRO-) 2AC
72
TRIFLUOPERAZINE

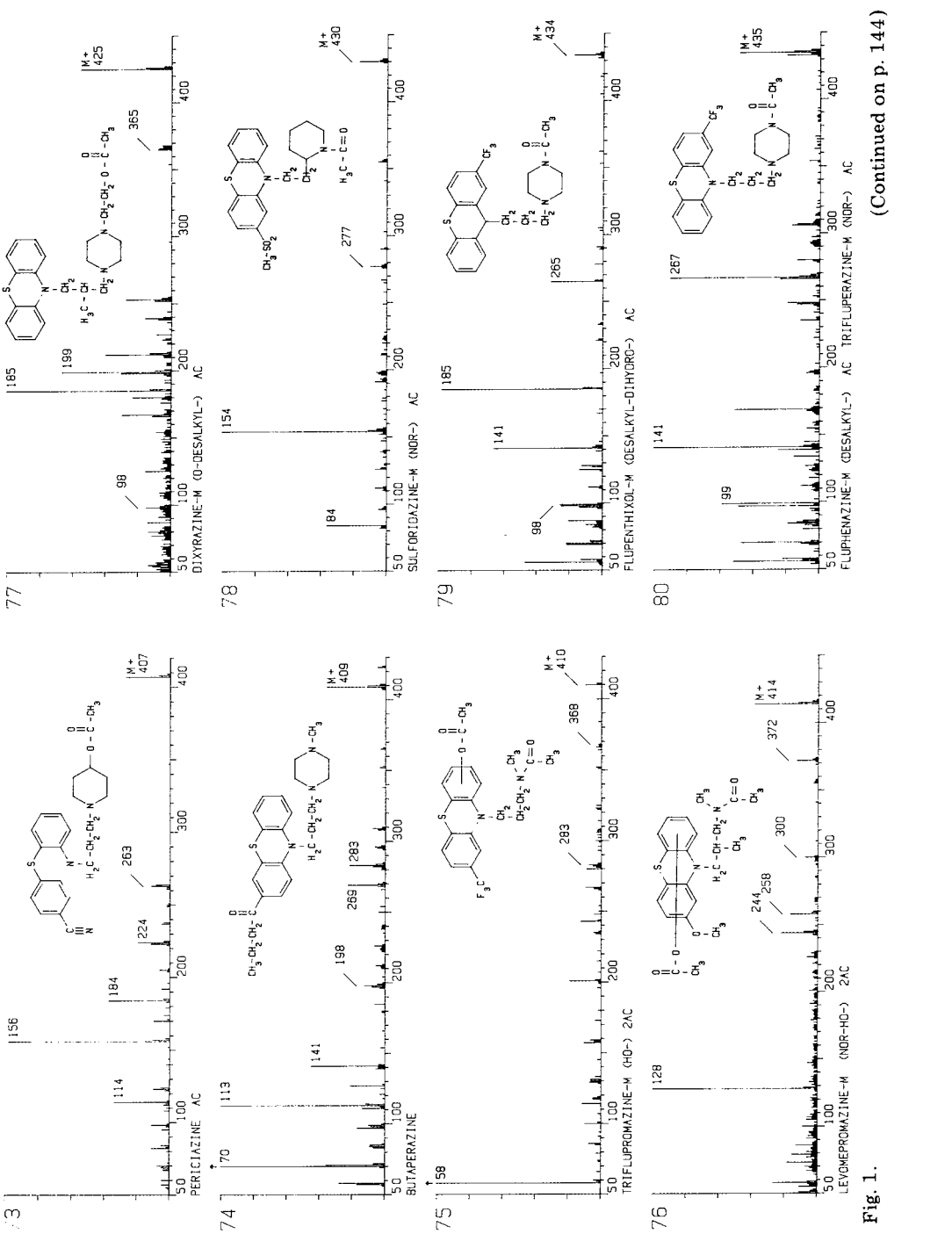


Fig. 1.

(Continued on p. 144)

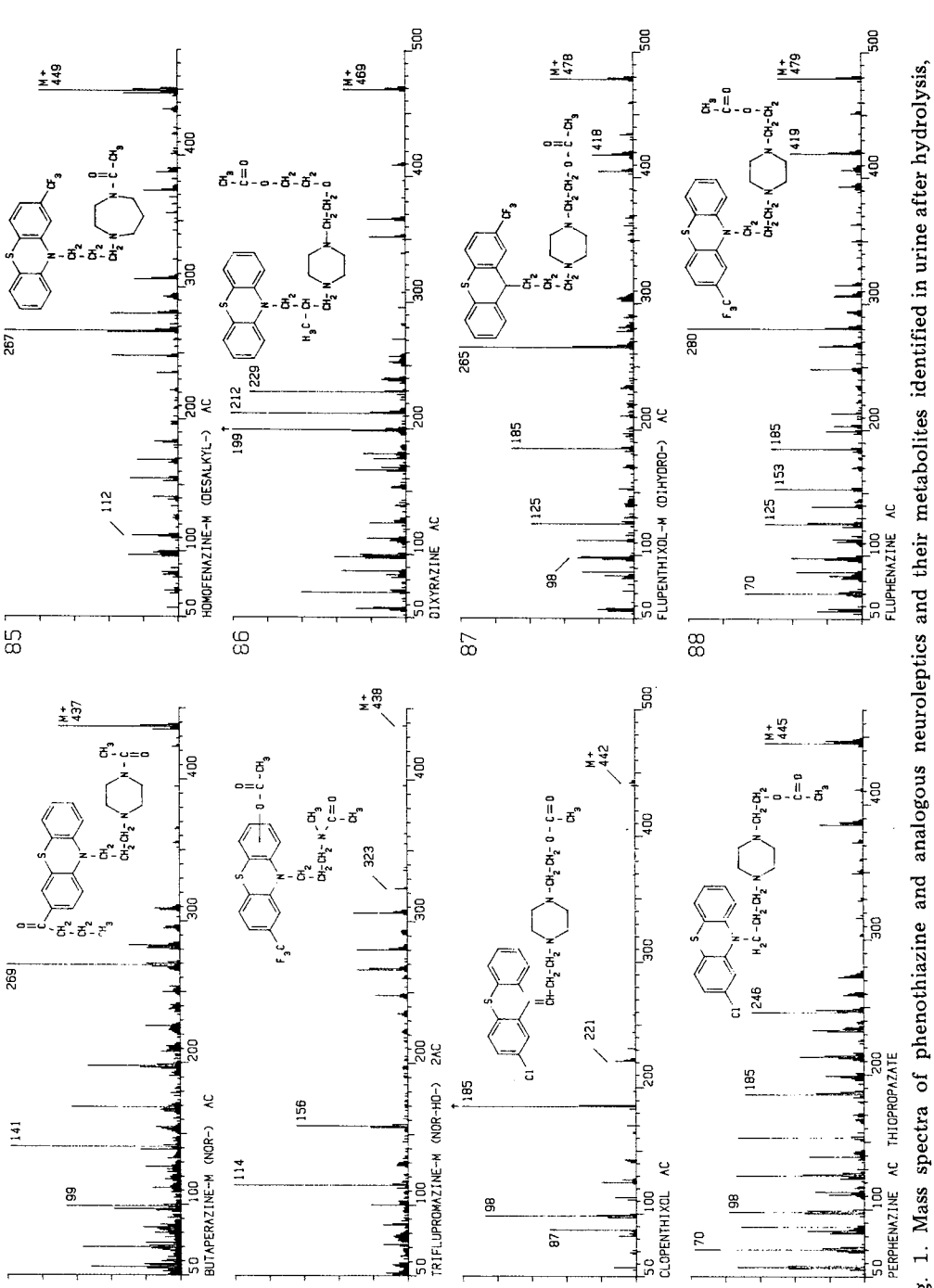


Fig. 1. Mass spectra of phenothiazine and analogous neuroleptics and their metabolites identified in urine after hydrolysis, extraction and acetylation.

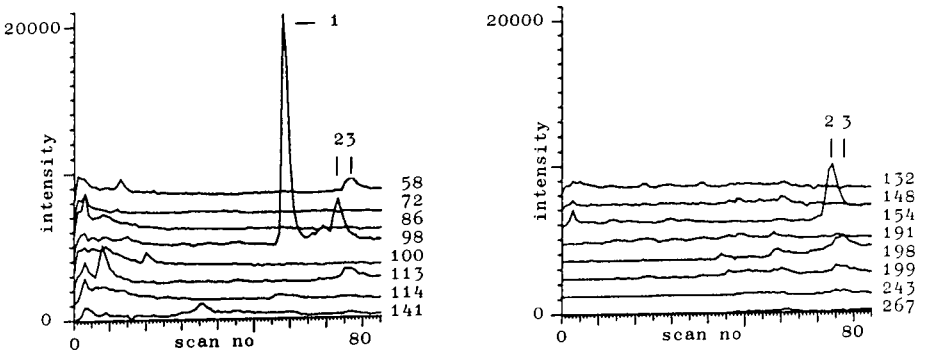


Fig. 2. Mass fragmentograms indicating thioridazine (1), nor-thioridazine (2) and oxo-thioridazine (3).

stored spectra. Such mass fragmentograms typical for benzodiazepines [16], butyrophenone and bisfluorophenyl neuroleptics [17, 18], anti-inflammatory analgesics [19], opioids and other potent analgesics [20] and antidepressants [21] have been published previously. Screening for antiparkinsonian drugs is in preparation [22]. Similar data of other compounds of toxicological interest will be collected so that nearly all relevant drugs will be detectable in urine or other biological materials within 1 h.

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Journal of Chromatography, 306 (1984) 147—153
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CHROMBIO. 1958

AN IMPROVED GAS CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CHLOROQUINE AND TWO METABOLITES USING CAPILLARY COLUMNS

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SUMMARY

A gas chromatographic method for the simultaneous determination of therapeutic levels of chloroquine, and the metabolites deethyl chloroquine and bideethyl chloroquine in human whole blood, plasma and urine has been developed by use of the capillary column technique. The analytes were extracted as bases with *n*-hexane—1-pentanol (90:10) and re-extracted into an acidic aqueous phase. After a further extraction to a small volume of chloroform, the substances were injected by the falling needle technique onto a fused-silica capillary column followed by nitrogen-selective detection. Limits of determination using 2 ml of the sample were found to be 10 nmol/l (3 ng/ml) for chloroquine and deethyl chloroquine and 30—40 nmol/l (10 ng/ml) for bideethyl chloroquine with a coefficient of variation of < 15%. The precision of the method at the 100 nmol/l level was about 4% (n = 8) for chloroquine.

INTRODUCTION

Chloroquine (CQ) is an antimalarial drug which is also used in the treatment of rheumatoid arthritis. Methods based on liquid chromatography (LC) for determination of CQ and its main metabolite deethyl chloroquine (CQM) in human plasma, whole blood and urine were recently reported [1, 2]. An alternative method with gas chromatography (GC) for the simultaneous determination of CQ and CQM, with acylation of CQM to obtain a separation between CQ and CQM on a packed column with OV-17 has been developed in our laboratories [3]. With this method CQ and CQM could be determined down to 100—200 nmol/l.

In this paper we describe a modification of the GC method which uses the capillary column technique. CQ and CQM as well as the bideethyl metabolite (CQMM) could be separated without derivatization. With split or splitless

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injection techniques problems such as "ghosting peak" and adsorption of the substances occur. The falling needle technique [4] seems to be the superior injection technique for CQ and its main metabolites. The evaporation step in the extraction procedure could be omitted, and the injection technique is simple and easy to handle. By isothermal chromatography 4—5 min give a sufficient separation of CQ, the two metabolites and the internal standard.

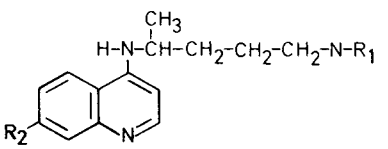
EXPERIMENTAL

Gas chromatography

A Varian VISTA 6000 gas chromatograph with a standard injector for split or splitless injection, equipped with a nitrogen-selective thermoionic detector was used. Fused-silica capillary columns, type J & W DB-5 (12 m × 0.32 μm, 0.25 μm film) and SGE BP-5 (7 m × 0.32—0.50 μm, 0.50 μm film) were used. The sample was injected by an all-glass solid injector with a falling needle from Chrompack and chromatographed isothermally at 255° C. The carrier gas (nitrogen) flow-rate was approximately 1.0 ml/min. The hydrogen and air flow-rates were 4.5 ml/min and 175 ml/min, respectively. In order to obtain optimized detector performance, auxiliary carrier gas was added at the end of the column at 30 ml/min. The nitrogen was purified from oxygen by OXY-TRAP (Alltech, U.S.A.).

Chemicals and reagents

Chloroquine, deethyl chloroquine and 7-bromo-4-(1-methyl-1-diethylamino-butylamino)quinoline, used as an internal standard, were kindly supplied by Sterling-Winthrop (Skärholmen, Sweden). Bideethyl chloroquine (CQMM) was synthesized at the Department of Organic Chemistry, Uppsala University, Uppsala, Sweden. The molecular structures are shown in Fig. 1. All the reagents were of analytical quality from Merck (Darmstadt, F.R.G.). Polypropylene



Compound	R_1	R_2
Chloroquine, CQ	$-N(C_2H_5)_2$	-Cl
Deethyl chloroquine, CQM	$-NH(C_2H_5)$	-Cl
Bideethyl chloroquine, CQMM	$-NH_2$	-Cl
Internal standard, IS	$-N(C_2H_5)_2$	-Br

Fig. 1. Molecular structures of the compounds of interest.

tubes from Sarstedt (Malmö, Sweden) and glass tubes used in the extraction procedure were cleaned by washing in a non-phosphate detergent Decon-90R (Decon Laboratories, Hove, U.K.), followed by cleaning in nitric acid (5 mol/l) in an ultrasonic bath, and by a rinse with deionized Milli Q filtered water (Millipore, Bedford, MA, U.S.A.).

Preparation of standards

Equal amounts of CQ, CQM and CQMM were dissolved in hydrochloric acid (0.01 mol/l) to give a concentration of 100 μmol/l. Aliquots of this solution were diluted with drug-free plasma. The standards were handled and analysed simultaneously with the samples. An example of a calibration curve in the concentration range 100—1000 nmol/l, passing through the origin, is shown in Fig. 2.

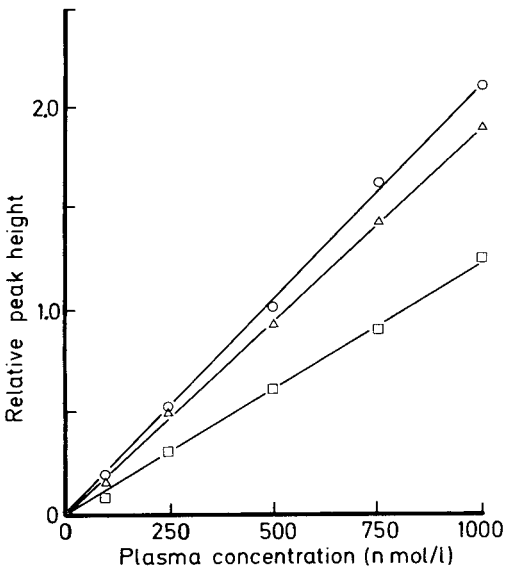


Fig. 2. Calibration curves for chloroquine (○), deethyl chloroquine (△) and bideethyl chloroquine (□).

Injection techniques

Different injection techniques were tested by means of repeated injections of the same extract of CQ, CQM, CQMM and internal standard at a concentration of 10 μmol/l with the same syringe (SGE SK-101) and on the same column. The tested injection techniques were as follows: split injection: split ratio 1:10, isothermal 255°C. Splitless injection: 170°C for 2 min, temperature-programmed 20°C/min up to 260°C. Falling needle injection: isothermal 255°C.

Injector and detector temperatures were 280°C. For split and splitless injection the solvent was ethyl acetate with and without *n*-decylamine (1000 μmol/l). For falling needle injection the solvent was chloroform with and without *n*-decylamine.

Evaporation experiment

One millilitre of an alkalinized aqueous solution of CQ, CQM and CQMM at a concentration of 500 nmol/l in polypropylene tubes was extracted with 3 ml of chloroform. For comparison of recovery, 2.5 ml of the chloroform extract were either re-extracted with 500 μl of 0.1 mol/l hydrochloric acid or evaporated to dryness at 50°C in a glass tube (evaporation interrupted just when completed) and the residue reconstituted in 500 μl of 0.1 mol/l hydrochloric acid. A 100 μl aliquot of each hydrochloric acid phase was injected into the LC system described in ref. 1 and peak heights for CQ, CQM and CQMM were compared.

Extraction procedure

A volume of 0.5—2.0 ml of sample and 100 μl of internal standard solution were made alkaline (pH > 11) with 1.0 ml of sodium hydroxide (1 mol/l) and extracted for 15 min with 4.0 ml of *n*-hexane—1-pentanol (90:10) in polypropylene tubes. Whole blood was hemolysed by diluting (1:2, v/v) with water before extraction. After centrifugation, 3.0 ml of the organic upper phase were transferred to a new polypropylene tube containing 3.0 ml of hydrochloric acid (0.2 mol/l). After extraction for 15 min and centrifugation, the aqueous phase was transferred to a conical glass test tube containing 200 μl of sodium hydroxide (5 mol/l), and 200 μl of chloroform were added. After mixing for 1 min on a Vortex mixer and centrifugation, 2 μl of the chloroform phase were injected into the gas chromatograph using the falling needle technique.

RESULTS AND DISCUSSION

Evaluation of injection techniques

Initial experiments testing the different injection techniques — split, splitless and falling needle — by repeated injection of the same extract of CQ, CQM, CQMM and internal standard, showed that the highest sensitivity and best precision were achieved with falling needle injection. An example of these experiments is shown in Table I. Addition of *n*-decylamine (1000 μmol/l)

TABLE I

REPRODUCIBILITY OF DIFFERENT INJECTION TECHNIQUES

Injection of 2 μl of the same extract (n = 10) of CQ, CQM and CQMM at a concentration of 10 μmol/l.

Injection technique	Solvent	Mean peak height (mm) (attenuation 128 · 10^{-12})			Peak height relative to internal standard (R.S.D.*, %)		
		CQ	CQM	CQMM	CQ	CQM	CQMM
Split	Ethyl acetate	13	11	—	1.35(9.5)	1.35(7.1)	—
	Ethyl acetate + DA**	14	13	5	1.37(5.4)	1.33(7.6)	0.46(8.6)
Splitless	Ethyl acetate	123	98	10	1.29(15.2)	1.04(15.3)	0.09(11.0)
	Ethyl acetate + DA	178	145	52	1.33(9.5)	1.07(7.9)	0.38(10.6)
Falling needle	Chloroform	402	385	228	1.39(3.5)	1.35(3.8)	0.79(8.9)
	Chloroform + DA	430	410	232	1.36(3.5)	1.35(2.4)	0.81(5.8)

*R.S.D. = relative standard deviation.
**DA = *n*-decylamine, 1000 μmol/l.

to the solvent as an adsorption suppressor improved the sensitivity, especially for the primary amine CQMM with split and splitless injection.

With falling needle injection no significant improvement of sensitivity by addition of *n*-decylamine could be observed. However, when column performance had been degraded due to injection of a large number of samples, non-linear standard curves were obtained for CQMM in the concentration range 25—1000 nmol/l unless *n*-decylamine was added to the solvent before injection.

With falling needle injection no "ghosting peak" (< 5%) was observed, which was a problem reported by Churchill et al. [5] in their method using on-column injection and which we also observed with split and splitless injection.

Evaporation

Adsorptive losses of CQ and metabolites must be considered whenever the drug is in contact with glass surfaces [6], especially in the low (ng/ml) concentration range. The results in Fig. 3 indicate that 18% of CQ, 42% of CQM and 43% of CQMM were lost during the chloroform evaporation in comparison with re-extraction to an aqueous phase. The precision was also decreased with evaporation.

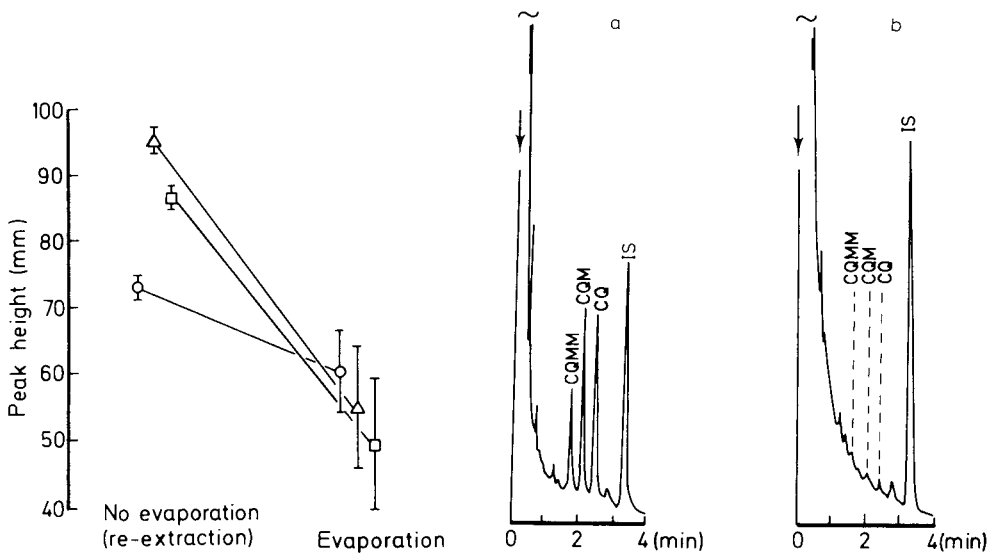


Fig. 3. Comparison of re-extraction and evaporation as a concentration step of 500 nmol/l chloroquine (○), deethyl chloroquine (△) and bideethyl chloroquine (□). The length of the bar corresponds to the standard deviation (n = 5).

Fig. 4. Capillary gas chromatograms from the analysis of (a) chloroquine in plasma with "spiked plasma sample" at a concentration of 100 nmol/l of each substance, and (b) drug-free plasma sample, with internal standard (IS). Column: 7 m × 0.32 μm I.D., BP-5. Injection: 2 μl by falling needle. Column temperature: 255°C. Detector temperature: 280°C.

Extraction conditions

The earlier work [7] had shown that hexane—1-pentanol (90:10) and chloroform could quantitatively extract CQ and CQM from an alkalinized sample. When this extraction was used with biological samples, there were no interfering endogenous compounds with the same retention time as CQ, CQM and CQMM left after the double-extraction procedure. Fig. 4 shows a chromatogram from a plasma spiked with 100 nmol/l of each substance. This figure also shows a chromatogram from a drug-free plasma sample. It can be seen that the plasma does not contain endogenous substances that interfere with CQ and its metabolites. The substances were adequately separated within 4 min.

Recovery

Adding known quantities (100, 500 and 1000 nmol/l) of CQ, CQM and CQMM to plasma gave 100 ± 5% recovery for CQ, 97 ± 8% for CQM and 85 ± 7% for CQMM, by the above extraction procedure.

The limit of determination

The limits of determination from spiked plasma samples are presented in Table II together with the relative standard deviation. These limits of determination are for CQ and CQM about 10 nmol/l and for CQMM 30—40 nmol/l, and are well below the expected drug concentration in biological specimens from patients given therapeutic doses of CQ both in treatment against rheumatoid arthritis as well as for prophylaxis against malaria [8].

TABLE II

PRECISION OF THE DETERMINATION OF CHLOROQUINE AND ITS METABOLITES BY CAPILLARY GAS CHROMATOGRAPHY

Within-day precision of spiked samples; $n = 8$.

	CQ	CQM	CQMM
Mean (nmol/l)	478	460	500
C.V. (%)	5.1	4.8	12
Mean (nmol/l)	113	107	75
C.V. (%)	3.7	1.8	11
Mean (nmol/l)	65	59	34
C.V. (%)	7.8	7.7	14
Mean (nmol/l)	20	17	16
C.V. (%)	4.3	8.7	24
Mean (nmol/l)	14	10	—*
C.V. (%)	10	11	

*Relative standard deviation > 30%.

Selectivity of the present method

A comparison of the present method and the LC method [1] for the assay of CQ, CQM and CQMM is shown in Fig. 5. Samples were taken from patients undergoing chloroquine dihydrogen phosphate treatment (250 mg per day). The results indicate that the two methods are equivalent.

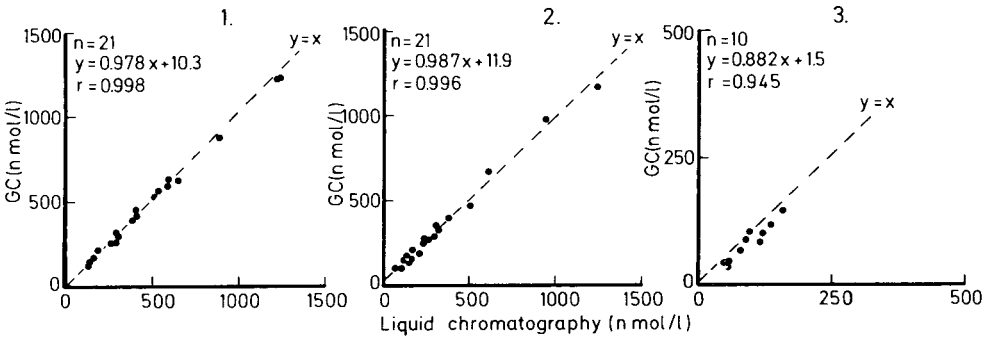


Fig. 5. Comparison of results for chloroquine (1), deethyl chloroquine (2) and bideethyl chloroquine (3) in plasma obtained by liquid chromatography (X) and gas chromatography (GC) with capillary column (Y).

With the present extraction procedure and the chromatographic system no interference with other commonly used drugs in combination therapy for rheumatoid diseases and malaria prophylaxis with CQ was seen. The tested drugs were primaquine, pyrimetamine, quinine, chlorproguanil, and phenylbutazone.

CONCLUSION

In the present work we have shown that sample preparation and injection technique are critical steps for analysis of amines like CQ and metabolites in the ng/ml range. By using the falling needle injection technique the method would be useful for simultaneous determination of CQ, CQM and CQMM down to 10 nmol/l (3 ng/ml) for both CQ, and CQM and for CQMM down to 30—40 nmol/l (10 ng/ml) with a precision of $< 15\%$. Recently other methods have been published usign GC [5, 9, 10] for the determination of CQ in biological specimens, but none of them dealt with the separation of the bideethyl metabolite (CQMM) of CQ.

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SIMULTANEOUS DETERMINATION OF THE ENANTIOMERS OF TOCAINIDE IN BLOOD PLASMA USING GAS—LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

Tocainide is a new experimental antiarrhythmic agent used clinically as the racemic mixture of two enantiomers. Since the optical isomers may differ in their efficacy and toxicity, we have initiated studies on the stereoselective disposition of tocainide. For this purpose, an assay was developed for the simultaneous determination of the enantiomers of tocainide in blood plasma. Alkalinized 1-ml plasma samples containing tocainide and an internal standard, 2-amino-2′,6′-acetoxylidide, are extracted with ethyl acetate. The organic extract is treated with the chiral reagent (*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride, and the resulting derivatives are resolved and quantified by gas—liquid chromatography with electron-capture detection. Calibration data were fitted by least-squares power curves of the form: drug enantiomer/internal standard peak area ratio = $A \times C^B$ where A and B were constants and C was the concentration of tocainide enantiomer. The lower limit of sensitivity of the assay was 10 ng/ml of each enantiomer. Intra-assay coefficients of variation were 3.3 and 2.1% for (*R*)-(—)-tocainide at concentrations of 0.125 and 1.25 μg/ml, respectively, and 3.4 and 2.4% for the (*S*)-(+) enantiomer at the same concentrations. Diazepam may interfere with the determination of (*R*)-(—)-tocainide if concentrations smaller than 1 μg/ml of this enantiomer are measured in the presence of higher-than-therapeutic (> 1.5 μg/ml) concentrations of diazepam.

INTRODUCTION

Tocainide (2-amino-2′,6′-propionoxylidide, TOC, Fig. 1), a primary-amine analogue of lidocaine, is a new antiarrhythmic agent often useful in the treatment of life-threatening arrhythmias unresponsive to conventional therapy [1]. Unlike lidocaine, however, TOC is effective after oral administration. This difference between the two drugs is the result of structural modification of

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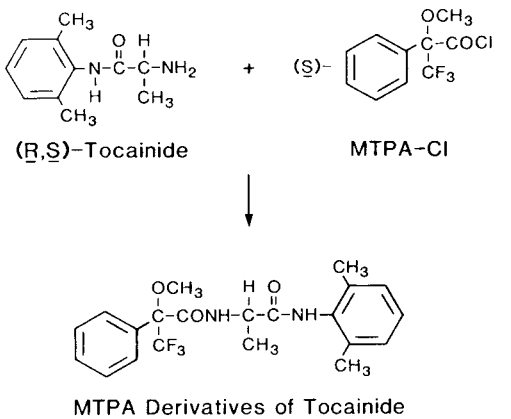

Fig. 1. Reaction of tocainide with (*S*)-MTPA-Cl.

TOC which makes it less susceptible to hepatic metabolism. The chemical structure of TOC (Fig. 1) includes an asymmetric center, and the drug is administered clinically as the racemic mixture. Studies in animals [2] have demonstrated that the enantiomers may differ in their antiarrhythmic efficacy and toxicity. This finding prompted us to undertake studies on the stereoselective disposition of tocainide [3, 4]. Planned pharmacokinetic studies require that we measure each enantiomer in serum, in the presence of the other, at concentrations as low as 50 ng/ml. In this communication, we describe the method developed for this purpose.

EXPERIMENTAL

Chemicals

Racemic and (*R*)-(−)-TOC hydrochloride were provided by Astra Pharmaceutical Products (Framingham, MA, U.S.A.). (*S*)-(+)-TOC hydrochloride was synthesized via a published procedure [2]. 2-Chloro-2′,6′-acetoxylidide and (*S*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid ((*S*)-MTPA) were purchased from Aldrich (Milwaukee, WI, U.S.A.). (*S*)-MTPA was converted to the corresponding acid chloride, (*S*)-MPTPA-Cl, as described previously [5]. 2-Amino-6′-chloro-*o*-acetotoluidide (ACAT) hydrochloride was purchased from the ABC Library of Rare Chemicals, Aldrich. Ethyl acetate, distilled-in-glass grade, was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other reagents were of analytical grade quality.

2-Amino-2′,6′-acetoxylidide (GX) was synthesized as follows: 2-chloro-2′,6′-acetoxylidide (1.0 g) was added to concentrated ammonium hydroxide (10 ml), and the mixture was stirred at room temperature for 24 h. This solution was extracted with diethyl ether (20 ml, three times) and the combined ether extracts were extracted with 1 *M* hydrochloric acid (20 ml, twice). The acidic aqueous extracts were combined, cooled in ice, and saturated with sodium hydroxide. The resulting alkaline aqueous solution was extracted with dichloromethane (25 ml, three times), the combined organic extracts were washed with water (25 ml, twice) and dried (potassium carbonate). The solvent was

evaporated at reduced pressure, and the solid obtained was dissolved in 50 ml dry diethyl ether. Hydrogen chloride gas was bubbled through the solution until the precipitation of GX hydrochloride ceased. The precipitate was filtered and washed with diethyl ether. The yield was 53%. GX free base was prepared by extracting an alkalinized (pH 14) aqueous solution of the hydrochloride with dichloromethane. Evaporation of the organic solvent gave a colorless solid, m.p. 77—80°C (literature value [6] m.p. 78—80°C).

(*R,S*)-TOC free base was similarly obtained from the hydrochloride and had a m.p. of 55—56°C.

Working solutions

Human plasma samples containing racemic TOC at several concentrations were prepared by appropriate dilutions of an aqueous stock standard solution containing TOC hydrochloride at 1.0 mg/ml free base concentration. GX hydrochloride was dissolved in distilled water to give a free base concentration of 16.6 μg/ml. (*S*)-MTPA-Cl was dissolved in ethyl acetate to give a concentration of 1 mg/ml.

Chromatography

A Hewlett-Packard (Avondale, PA, U.S.A.) Model 5736A gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The stationary phase was 3% SP 2250-DA on 100-120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) contained in a 2 m × 2 mm I.D. glass column. Oven temperature was 255°C, injector temperature 300°C, and detector temperature 350°C. The carrier gas was nitrogen at a flow-rate of 30 ml/min.

Assay procedure

The sample, 1.0 ml, was placed in a 12-ml conical glass centrifuge tube and 50 μl of the internal standard GX solution, 1.5 ml ethyl acetate, and ca. 200 mg of a 4:1 mixture of sodium chloride and sodium carbonate were added. The mixture was swirl-mixed (vortex) for 60 sec, followed by centrifugation at 500 *g* for 5 min. The upper organic layer was transferred to a 12-ml screw-cap glass centrifuge tube and 10 μl of pyridine and 50 μl of the (*S*)-MTPA-Cl solution were added. The tube was tightly capped, swirl-mixed for 5 sec, and placed in a water bath at 70°C for 30 min. The tube was then cooled in an ice bath for 5 min, and 1 ml of 1 *M* hydrochloric acid was added. The mixture was swirl-mixed for 30 sec, followed by centrifugation for 30 sec. The organic layer was transferred to a 5-ml centrifuge tube, and 1 ml of 15% sodium carbonate solution was added. The mixture was swirl-mixed for 30 sec, and centrifuged for 30 sec. The organic layer was transferred to a 5-ml conical glass tube and evaporated to dryness at 50—60°C under a stream of nitrogen. The residue was stored at —20°C, and reconstituted with 100 μl of ethyl acetate immediately before gas—liquid chromatographic (GLC) analysis. Injection volumes were 0.5—6 μl.

Stability of derivatives

A 1-ml sample of water containing 50 μg of TOC was added to 100 μl of an aqueous solution containing 166 μg of GX and the mixture was processed

according to the assay procedure described above. The residue obtained upon evaporation of the solvent was reconstituted in 2 ml of ethyl acetate. A series of fourteen 100-μl aliquots of the resulting solution were evaporated to dryness in separate tubes. Two samples were reconstituted with 100 μl of ethyl acetate and analyzed by GLC immediately, while the remaining dried samples were stored for 6, 24, or 48 h at 4°C or —20°C. Each set of storage conditions was studied in duplicate. Absolute peak areas and TOC/GX area ratios were determined.

Recovery studies

A 1-ml sample of plasma, 1.5 ml of a stock ethyl acetate solution containing TOC free base at 0.667 μg/ml and GX free base at 0.553 μg/ml, and ca. 200 mg of a 4:1 mixture of sodium chloride and sodium carbonate were added to a centrifuge tube. The mixture was swirl-mixed for 60 sec, followed by centrifugation for 5 min. A 750-μl aliquot of the organic layer was treated with (*S*)-MTPA-Cl as described under Assay procedure. The derivatized mixture was analyzed by GLC, and the peak areas obtained were compared to the peak areas obtained when 750-μl aliquots of the stock ethyl acetate solution were derivatized with (*S*)-MTPA-Cl without prior extraction.

Calibration curves and detector response

Standard curves were constructed by analyzing a series of plasma samples of known racemic TOC concentration in the range 20 ng/ml—5.0 μg/ml.

Detector response linearity was studied as follows: the (*S*)-MTPA derivatives of TOC and GX were separately prepared. The derivative of TOC was serially diluted with an ethyl acetate solution of the derivative of GX in such a manner that the resulting samples contained the same fixed concentration of the GX derivative, and the concentration of the TOC derivative varied in the range which would be obtained in the analysis of 52 ng/ml—5.0 μg/ml plasma TOC samples.

Precision

Within-day precision was determined by analyzing sets of ten replicate plasma samples containing racemic TOC at 250 ng/ml and at 2.5 μg/ml concentrations. Day-to-day variability was determined by analyzing replicate samples containing TOC at a concentration of 2.5 μg/ml on ten separate days.

Quantitative evaluation of assay data

The TOC isomer: GX peak area ratios were used to construct standard curves. Calibration data were fitted by least-squares power curves through the origin of the form: peak area ratio = $A \times C^B$, where A and B were constants and C was the concentration of the TOC enantiomer.

Interference studies

Aliquots of ethyl acetate solutions of diazepam and nordiazepam at a concentration of 1.0 μg/ml were injected into the gas—liquid chromatograph and the peak areas obtained were compared to peak areas obtained from injections of the same solutions after extraction with 1 *M* hydrochloric acid. Triplicate injections of each solution were performed.

RESULTS

Fig. 2 shows the chromatogram of the MTPA derivatives of TOC and GX, and results of the analysis of a patient sample. When plasma from subjects not receiving TOC was analyzed, no interfering peaks were observed. In preliminary studies OV 17 GLC columns were used, but it was found that better chromatography was achieved on SP-2250 DA stationary phase. Retention times of the MTPA derivatives were: (*R*)-(−)-TOC, 8.9 min; (*S*)-(+)-TOC, 10.2 min; GX, 12.6 min.

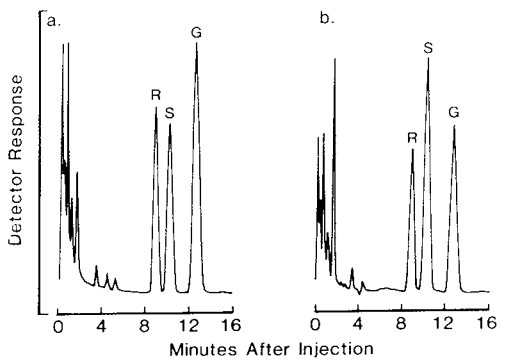

Fig. 2. Chromatograms of (a) the MTPA derivatives of standards of (*R*)-(−)-TOC (R), (*S*)-(+)-TOC (S), and GX (G); (b) extract of serum from patient receiving chronic TOC therapy assayed as described in Experimental. Concentration of (*R*)-(−)-TOC 1.54 μg/ml; (*S*)-(+)-TOC 3.73 μg/ml.

Recoveries of (*R*)-(−)- and (*S*)-(+)-TOC and of GX were 90.4 ± 6.8 (S.D.), 89.2 ± 6.7, and 89.6 ± 8.2, respectively (n = 5). In the derivatization procedure, the use of larger amounts of MTPA-Cl, e.g., 10—250 times that given in the assay procedure, was evaluated. No increase in the yield of derivatives was found, and interfering peaks appeared in the chromatograms.

TABLE I

STABILITY OF TOCAINIDE-MTPA DIASTEREOMERS AS A FUNCTION OF STORAGE TIME AND TEMPERATURE

Time (h)	Enantiomer response*			
	4°C		−20°C	
	(*R*)-(−)	(*S*)-(+)	(*R*)-(−)	(*S*)-(+)
0	797**	710**	797**	710**
6	813	724	812	725
24	813	727	823	735
48	821	736	823	736

*Ratio of tocainide enantiomer peak area to GX peak area × 1000. Tocainide enantiomer concentration was 1.25 μg/ml; mean of duplicate samples.
**Baseline samples were analyzed immediately after preparation.

TABLE II

TYPICAL CALIBRATION DATA

Least-squares power curves: tocainide (*R*)-(—) enantiomer: GX peak area ratio = 1704 (tocainide (*R*)-(—) enantiomer)$^{0.9325}$, r^2 = 0.999; tocainide (*S*)-(+) enantiomer: GX peak area ratio = 1720 (tocainide (*S*)-(+) enantiomer)$^{0.9435}$, r^2 = 0.999.

Tocainide concentration (μg/ml)				Relative errors (%)	
(*R*)-(—)		(*S*)-(+)		(*R*)-(—)	(*S*)-(+)
Calculated*	Observed**	Calculated*	Observed**		
0.0100	0.0103	0.0100	0.00945	+3.0	—5.5
0.0250	0.0248	0.0250	0.0238	—0.8	—4.6
0.0500	0.0510	0.0500	0.0575	+2.0	+15.0
0.125	0.116	0.125	0.122	—7.2	+2.0
0.250	0.253	0.250	0.257	+1.2	+3.0
0.500	0.505	0.500	0.495	+1.0	—1.0
1.25	1.24	1.25	1.24	—0.4	—0.4
2.50	2.56	2.50	2.43	+2.2	—2.6
Spiked samples					
0.250	0.267	0.000	0.000	+6.8	0.0
0.000	0.000	0.250	0.252	0.0	+0.8
0.250	0.253	0.125	0.127	+1.2	+1.6

*By dilution, in plasma.
**By chromatography using the least-squares power curves; mean of triplicate samples.

The MTPA derivatives appeared to be stable when stored at 4°C or —20°C for at least 48 h as there was no significant change in the absolute peak areas or TOC/GX peak area ratios (Table I). The effect of longer storage times was not investigated systematically, but reassay of several samples after a two-week storage period gave results essentially identical to those obtained after 48 h of storage.

Calibration data are given in Table II for the determination of the enantiomers of TOC. Over the wide concentration range the calibration data were curvilinear, and attempts to describe the data with linear least-squares regression resulted in regression lines with marked systematic deviation at low TOC concentrations. Concentration-dependent extraction efficiency, limiting derivatization reaction, and non-linear electron-capture detector response were all investigated as possible sources of non-linearity. Non-linear detector response was found to be the cause of the curvilinearity of the calibration data, as demonstrated in Fig. 3. Calibration data for each enantiomer were well fitted by least-squares power curves (r^2 = 0.999) and are presented in Table II. These curves were used to calculate the concentration of each enantiomer in unknown samples, as illustrated for spiked plasma samples in Table II.

Intra-assay coefficients of variation (C.V.) were 3.3 and 2.1% for the (*R*)-(—) enantiomer of TOC at concentrations of 0.125 and 1.25 μg/ml, respectively, and 3.4 and 2.4% for the (*S*)-(+) enantiomer at the same concentrations. Day-to-day

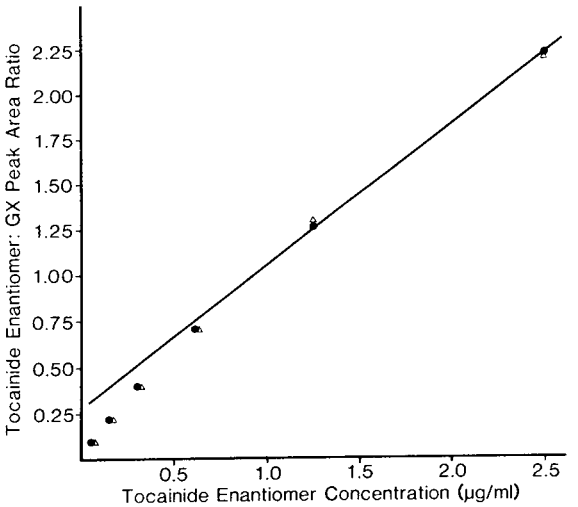

Fig. 3. Test of electron-capture detector response linearity. MTPA derivatives of (*R*)-(—)-TOC (•); (*S*)-(+)-TOC (△). The line was drawn arbitrarily to illustrate curvilinearity of the data.

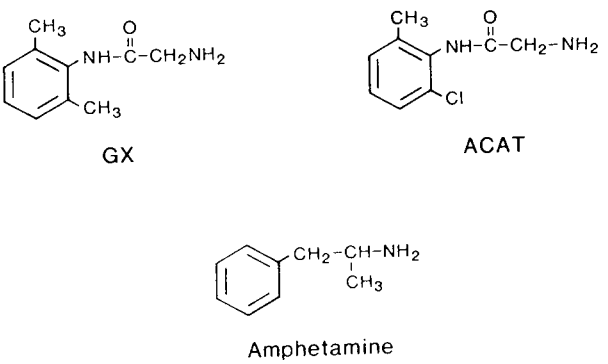

Fig. 4. Chemical structures of the internal standards and amphetamine.

C.V. values were 2.5 and 3.0% for the (*R*)-(—) and (*S*)-(+) enantiomers, respectively, at a concentration of 1.25 μg/ml. The lower limit of sensitivity of the assay for each enantiomer was found to be 10 ng/ml.

Derivatization of (*R,S*)-1-phenyl-2-aminopropane (amphetamine, Fig. 4) with (*S*)-MTPA-Cl and chromatography of the derivatives revealed that the response of the electron-capture detector to these derivatives is ca. 100-fold less than the response to the derivatives of tocainide.

The retention times of diazepam and nordiazepam were 8.1 and 12.8 min, respectively, under the chromatographic conditions used. The acid wash in the procedure removed 80% and 90% of diazepam and nordiazepam, respectively.

DISCUSSION

Several different approaches are available for the simultaneous determination of enantiomers in biological fluids [7]. We selected derivatization with a chiral

reagent and GLC separation of the resulting diastereomers for the analysis of the enantiomers of TOC. The chiral reagent chosen, (*S*)-MTPA-Cl, (Fig. 1) has been shown in our laboratory to be useful in the GLC resolution of TOC [3] and a variety of other chiral amines [5, 7, 8], and was used successfully in studies of the stereoselective disposition of TOC [3]. In these previous studies, flame ionization [5, 8] or nitrogen–phosphorus detection [3] was employed. The sensitivity provided by these detectors, however, was insufficient for studies of the pharmacokinetics of TOC in man. The observation that the chemical structure of the MTPA moiety includes the trifluoromethyl group (Fig. 1) suggested that the electron-capture detector would respond well to the MTPA derivatives. This expectation was realized, and excellent sensitivity was achieved in the assay of the enantiomers. Surprisingly, however, when the MTPA derivatives of amphetamine (Fig. 4) were chromatographed, the detector response was ca. 100-fold less than that for the TOC derivatives. It is clear that structural elements other than the trifluoromethyl group are also involved in the high response of the electron-capture detector to the MTPA derivatives of tocainide.

The use of MTPA-Cl for the resolution of the TOC enantiomers offers advantages over a recently published procedure [9] based on derivatization of TOC with heptafluorobutyric anhydride followed by GLC resolution of the derivatives on a glass capillary column coated with a chiral stationary phase. Both procedures include a derivatization step before chromatography, but the MTPA derivatives are resolved on a conventional (non-chiral) stationary phase in a packed column, a significant advantages over the use of chiral glass capillary columns in terms of ease of handling and cost. MTPA-Cl, having no exchangeable hydrogen at the chiral center, is stereochemically extremely stable, and the MTPA derivatives of TOC also have excellent chemical stability.

After considering several compounds, GX (Fig. 4) was selected to serve as the internal standard. GX is a primary amine with a chemical structure (Fig. 4) very similar to that of TOC (Fig. 1), and under the GLC conditions used, the retention time of its MTPA derivative is ideal (Fig. 2). Another advantage of GX, a non-chiral compound, is that it gives a single derivative with the chiral derivatizing agent. GX can be readily prepared in one step from commercially available starting materials. The response of the electron-capture detector to the MTPA derivative of GX is similar in magnitude to its response to the derivatives of TOC. GX is a metabolite of lidocaine [10], but this presents no problem in our pharmacokinetic studies, since in this controlled setting the absence of lidocaine in the study subjects is assured. If the assay procedure were to be used for the determination of TOC in samples from a patient receiving TOC and lidocaine simultaneously — an unlikely event — the use of GX as internal standard may result in spuriously low TOC concentrations. The presence in the sample of GX resulting from metabolism of lidocaine can be readily determined by processing the sample through the present procedure without the addition of internal standard. To circumvent the problem of the presence of lidocaine-derived GX in the sample, an alternative internal standard, ACAT (Fig. 4) may be used (data not shown). The only disadvantage of ACAT is a longer than optimum R_T (19 min). In all other respects, i.e., chemical structure, extractability, derivatization, electron-capture detector response, and com-

mercial availability, ACAT is well suited for the role of internal standard.

Since several benzodiazepines have been shown to elicit a high response from the electron-capture detector [11], potential interference from diazepam and nordiazepam was studied. It was found that extraction with 1 *M* hydrochloric acid, a step carried out after derivatization of TOC with MTPA-Cl, removes 80—90% of these benzodiazepines present in the ethyl acetate solution. The serum concentration of diazepam during chronic administration of therapeutic doses is in the range 0.7—1.5 μg/ml [12]. Since 80% of the amount present is removed during the assay procedure for TOC, and since the peak due to diazepam is adequately separated from the peaks of interest in the chromatogram, it is clear that this drug does not interfere with the analysis of TOC in the therapeutic range. If, however, low TOC concentrations (< 1 μg/ml) are measured in the presence of relatively high concentrations (> 1.5 μg/ml) of diazepam, interference by the latter drug may occur, inasmuch as the peak due to diazepam may partially overlap with the peak due to (*R*)-(−)-TOC. Serum concentrations of nordiazepam, the major circulating metabolite of diazepam, are in the range 35—52 ng/ml [12]. Since these already low concentrations are reduced by 90% during the assay procedure, nordiazepam does not interfere in the analysis, despite having a retention time nearly coinciding with that of GX. Other commonly used benzodiazepines, e.g., flurazepam, oxazepam and chlordiazepoxide, have retention times shorter than that of diazepam or longer than that of nordiazepam [11], and thus do not interfere with the determination of TOC. A previously published procedure did not evaluate potential interference from other drugs [9].

The chromatograms obtained display good peak shapes, and baseline resolution of the diastereomers was achieved (Fig. 2). The procedure provides high sensitivity and good accuracy and precision. The method was developed for the 1-ml sample size, but recent experiments in our laboratory (data not shown) indicate that smaller sample sizes (200—300 μl) may be used if necessary, with only a small loss of sensitivity (lower limit ca. 20 ng/ml). The procedure displays good selectivity as a result of several organic—aqueous partitioning steps, specific derivatization, selective detection, and chromatographic separation.

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DETERMINATION OF ISOSORBIDE DINITRATE AND ITS MONONITRATE METABOLITES IN HUMAN PLASMA USING EXTRELUT® PURIFICATION AND CAPILLARY COLUMN GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, accurate and sensitive method for the determination of isosorbide dinitrate (internal standard: isomannide dinitrate) and its 2- and 5-mononitrate metabolites (internal standard: isoidide mononitrate) in 1.0 ml of human plasma has been developed. Before chromatographic quantitation by gas—liquid chromatography with electron-capture detection, isosorbide nitrates were purified by Extrelut® chromatography (recovery about 90%), eliminating most of the endogenous interferences. Routine limit of quantitation and reproducibility were 0.5, 2.0 and 10.0 ng/ml and 6, 8 and 7% for isosorbide dinitrate and the 2- and 5-mononitrates, respectively. This method allowed the behaviour of this vasodilating drug and its metabolites to be studied in humans.

INTRODUCTION

Isosorbide dinitrate (ISDN) is an organic nitrate widely used for its vasodilating properties in the treatment of angina pectoris and refractory congestive heart failure. ISDN is rapidly metabolized in humans to 2-isosorbide mononitrate (2-ISMN) and 5-isosorbide mononitrate (5-ISMN) which are pharmacologically active, sharing the action of the unchanged drug [1, 2]. Pharmacokinetic studies of isosorbide nitrates (ISN) therefore require the plasma determination of ISDN, 2-ISMN and 5-ISMN. Available methods for determination of ISDN [3—8] or its metabolites [9] or the three products [10—14] in plasma following administration of therapeutic doses of ISDN (ng/ml range) use gas—liquid chromatography with electron-capture detection (GLC—ECD) or high-performance liquid chromatography with detection by

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thermal energy analysis [15, 16]. The methods whose technological features have been provided are highly sensitive and accurate, although time consuming [11] and/or poorly reproducible in the lower concentration range [10, 12] and/or require a large plasma sample [10, 11, 15, 16].

For determination of ISN by GLC–ECD, the use of packed columns is hampered by the high background response interfering with the analysis of the metabolites and, in our hands, by irreproducibility of mononitrates. GLC capillary columns appear to overcome these drawbacks although data available from the literature are scarce [11, 17].

The present paper describes a GLC–ECD capillary column procedure for the determination of ISDN and its 2- and 5-mononitrate metabolites in a 1.0 ml plasma sample. The extraction procedure involves chromatography on an Extrelut® column eliminating laborious extraction and most of the background response but keeping a good extraction yield.

EXPERIMENTAL

ISDN, isomannide dinitrate (IMDN), 2-ISMN, 5-ISMN and isoidide mononitrate (IIMN) were provided by Merrell, Paris, France. All reagents used were analytical grade and water was double glass distilled. Hexane and ethyl acetate were from Carlo Erba, Milan, Italy. Extrelut was obtained from E. Merck, Darmstadt, F.R.G., and washed with hexane–ethyl acetate mixture (1:1) and then dried at 80°C for 48 h. Stock standard solutions of ISDN and IMDN (the internal standard for ISDN) were prepared at a 10 mg per 100 ml concentration in hexane. 2-ISMN, 5-ISMN and IIMN (the internal standard for mononitrates) stock standard solutions were prepared at a 10 mg per 100 ml concentration in ethyl acetate. Working standard solutions were prepared in hexane at 0.1 mg/ml concentration for ISDN, IMDN, IIMN and 2-ISMN and 1 mg/ml for 5-ISMN by dilution of the respective standard solutions. All these solutions were stored at −20°C. Under these conditions, ISN were found to be stable for several weeks. All glassware (columns and conical tubes) were washed with an ionic detergent, rinsed with double glass distilled water and silanized by a toluene solution of trimethylchlorosilane (5%).

Blood collection

Blood samples (10 ml) were drawn into Vacutainer® tubes (Becton-Dickinson A3200 XF 713) and immediately centrifuged at 4500 *g* for 10 min at 4°C. Separated plasmas were frozen and stored as 1.1 ml aliquots at −20°C until processing.

Extraction procedure

Aliquots (1.0 ml) of plasma samples were introduced into 10 ml conical extraction tubes fitted with glass caps. Internal standard solutions were added to all tubes giving a concentration of 20 ng/ml plasma for IMDN and 25 ng/ml plasma for IIMN. Then 1.0 ml of water was added. The tubes were capped and mixed for 10 sec. Diluted plasma was transferred with a silanized Pasteur pipette to the top of a column prepared as follows: 1.2 g of washed Extrelut were packed into a silanized glass column (height 30 cm, internal diameter 0.6 cm;

solvent tank 15 ml) fitted with silane glass wool and left to equilibrate for 20 min. Dinitrate compounds were eluted from the column by 10 ml of hexane (elution time was about 10 min). Thereafter, mononitrates were eluted by 9 ml of ethyl acetate—hexane mixture (70:30); elution time was about 5 min. The respective fractions were evaporated under a gentle nitrogen stream at room temperature until approximately 15 μl of dinitrate fraction and 75 μl of mononitrate fractions were obtained. The tubes were immediately frozen and stored at −20°C prior to chromatography; 1.0 μl was injected into the chromatograph.

Chromatography

Chromatography was performed on an HP 5700 gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a ^{63}Ni electron-capture detector, direct silanized glass injector (length 11 cm, internal diameter 1 mm) and an OV-17/01 wall-coated open tubular fused-silica capillary column (length 12.5 m, I.D. 0.32 mm) obtained from Girdel, Suresnes, France. Chromatographic conditions were different for dinitrate or mononitrate analysis. Temperature settings were detector 200°C, injection port 150°C (dinitrates) or 200°C (mononitrates), oven 140°C (dinitrates) or 130°C (mononitrates). Helium was used as carrier gas at a flow rate of 6 ml/min. Flow rate of make-up gas (argon—methane, 90:10) was 25 ml/min.

RESULTS AND DISCUSSION

Calibration

Calibration samples were prepared using drug-free plasma. Aliquots (1.0 ml) were spiked by addition of ISDN, 2-ISMN and 5-ISMN working standard solutions to produce concentration ranges of 1—60 ng/ml, 5—60 ng/ml and 25—250 ng/ml, respectively. IMDN (20 ng/ml) and IIMN (25 ng/ml) were added according to the routine extraction procedure. Standard calibration curves were obtained by plotting the peak height ratios (measured by a 3388 A Hewlett-Packard integrator) of ISDN and its metabolites versus internal standard against the concentration in the calibration standards. Linear regression analysis of typical calibration curves were found to be:

$y = 0.0686x + 0.035$ ($r = 0.9994$) for ISDN

$y = 0.0796x + 0.071$ ($r = 0.9980$) for 2-ISMN

$y = 0.0263x + 0.109$ ($r = 0.9998$) for 5-ISMN

These results demonstrate that the calibrations are linear over the concentration range. Day-to-day chromatographic variations may occur spontaneously, thus inducing changes in the parameters of the calibration curves. However, these changes do not influence accuracy since calibration curves were run every day.

Reproducibility and accuracy

The reproducibility and accuracy were determined for the three compounds in series consisting of five, seven or ten spiked plasma samples with respect to

a standard calibration curve (three or five points of increasing concentration and a blank). From results shown in Tables I, II and III, coefficients of variation were found not to exceed 6%, 7% and 6.5% for ISDN, 2-ISMN and 5-ISMN, respectively. However, the coefficient of variation for ISDN was 8.0% at the routine limit of detection. As shown in Tables I–III, accuracy did not exceed 10%, whatever the ISN molecule.

TABLE I

REPRODUCIBILITY AND ACCURACY OF ISOSORBIDE DINITRATE ASSAY

Spiked value (ng/ml)	Number of samples	Assayed value (ng/ml) (mean ± S.D.)	Coefficient of variation (%)
Within-day			
0.5	5	0.62 ± 0.05	8.0
1	10	0.97 ± 0.04	4.1
2	5	2.00 ± 0.04	2.0
5	5	5.50 ± 0.09	1.6
10	10	10.22 ± 0.40	3.9
10	7	10.56 ± 0.41	3.9
20	10	20.38 ± 0.44	2.2
20	7	20.79 ± 0.67	3.2
20	5	19.70 ± 0.50	2.5
Day-to-day			
2	5	1.98 ± 0.08	4.0
4	5	4.21 ± 0.22	5.2
6	5	6.43 ± 0.22	3.4

TABLE II

REPRODUCIBILITY AND ACCURACY OF 2-ISOSORBIDE MONONITRATE ASSAY

Spiked value (ng/ml)	Number of samples	Assayed value (ng/ml) (mean ± S.D.)	Coefficient of variation (%)
Within-day			
2	5	1.7 ± 0.1	5.2
5	10	4.8 ± 0.2	4.1
5	5	5.5 ± 0.1	1.8
10	10	10.5 ± 0.7	6.6
10	7	10.9 ± 0.5	4.6
10	5	10.4 ± 0.4	3.8
25	10	23.8 ± 1.5	6.3
40	7	38.7 ± 1.4	3.5
40	5	41.1 ± 1.1	2.6
Day-to-day			
5	5	4.9 ± 0.3	6.1
10	5	10.1 ± 0.7	6.9
20	5	19.9 ± 1.3	6.5

TABLE III

REPRODUCIBILITY AND ACCURACY OF 5-ISOSORBIDE MONONITRATE ASSAY

Spiked value (ng/ml)	Number of samples	Assayed value (ng/ml) (mean ± S.D.)	Coefficient of variation (%)
Within-day			
10	5	9.3 ± 0.5	5.3
25	10	24.9 ± 1.4	5.6
25	5	22.8 ± 0.7	3.0
50	5	51.5 ± 1.7	3.3
100	10	101.4 ± 3.9	3.8
100	7	108.5 ± 7.1	6.5
200	10	197.7 ± 6.3	3.2
250	7	242.6 ± 3.2	1.3
250	5	246.5 ± 2.8	1.1
Day-to-day			
25	5	22.5 ± 0.9	4.0
50	5	49.1 ± 2.5	5.1
200	5	205.3 ± 5.9	2.9

Extraction yield

Extraction yield was evaluated as follows (Table IV). Internal standards were added to ten plasma samples as described above. Of these, seven were spiked with ISDN, 2-ISMN and 5-ISMN before extraction and three received the three organic nitrates just before evaporation. Extraction yield was calculated as the ratio of mean peak height ratio (PHR) of the seven plasma samples over mean PHR of the three samples. It should be noted that a similar recovery (around 90%) was obtained for each of the isosorbide nitrates.

TABLE IV

EXTRACTION YIELD OF ISDN, 2-ISMN AND 5-ISMN FROM SPIKED PLASMA SAMPLES

ISDN			2-ISMN			5-ISMN		
Spiked value (ng/ml)	Number of samples	Extraction yield (%)	Spiked value (ng/ml)	Number of samples	Extraction yield (%)	Spiked value (ng/ml)	Number of samples	Extraction yield (%)
2	7	92.0	5	7	90.4	25	7	89.9
10	7	91.7	10	7	89.8	100	7	87.8
20	7	92.6	40	7	90.5	250	7	92.6

Chromatography

Typical chromatograms of healthy subjects receiving a single oral dose of a 40 mg sustained release form of ISDN are shown in Fig. 1. Retention times of ISDN and IMDN were 5.5 and 7.6 min, respectively, and 2.6, 5.0 and 7.1 min for 2-ISMN, IIMN and 5-ISMN, respectively.

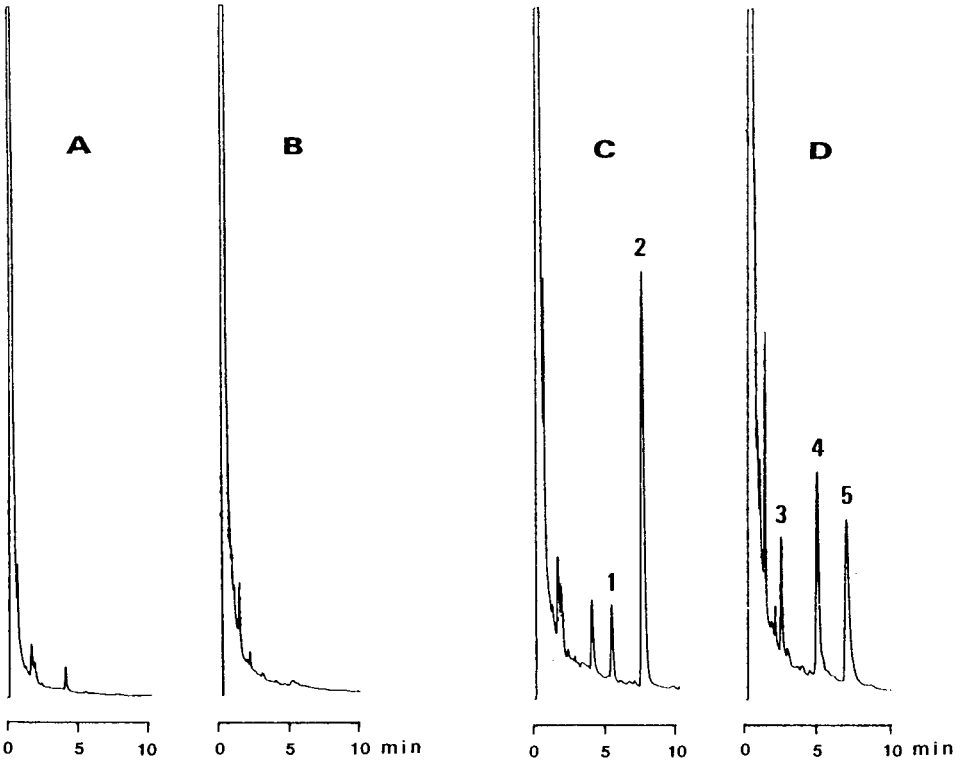

Fig. 1. Chromatograms of plasma extracts from a healthy subject (LAP. . .) before administration (A, B) and in receipt of 40 mg of ISDN (C, D). A = dinitrate fraction. B = mononitrate fraction. C = dinitrate fraction: 1 = isosorbide dinitrate (2 ng/ml); 2 = isomannide dinitrate (20 ng/ml), internal standard. D = mononitrate fraction: 3 = 2-isosorbide mononitrate (6.5 ng/ml); 4 = isoidide mononitrate (25 ng/ml), internal standard; 5 = 5-isosorbide mononitrate (30 ng/ml).

Limits of quantitation

The limit of quantitation of routine assays was 0.5, 2 and 10 ng/ml for ISDN, 2-ISMN and 5-ISMN, respectively, with reproducibility better than 10% at these concentrations. It should be emphasized that detection limits were often half of these values.

Kinetic studies in healthy human subjects

The proposed method was used for kinetic studies of ISDN and metabolites using sustained release forms of ISDN with different dosages (40, 60, 80 mg). Fig. 2 shows a concentration—time curve after administration to a healthy subject.

CONCLUSION

This paper describes a new capillary GLC—ECD method for the determination of ISDN and its two mononitrate metabolites, which represents a significant advance over previous techniques [3—17]. The Extrelut extraction

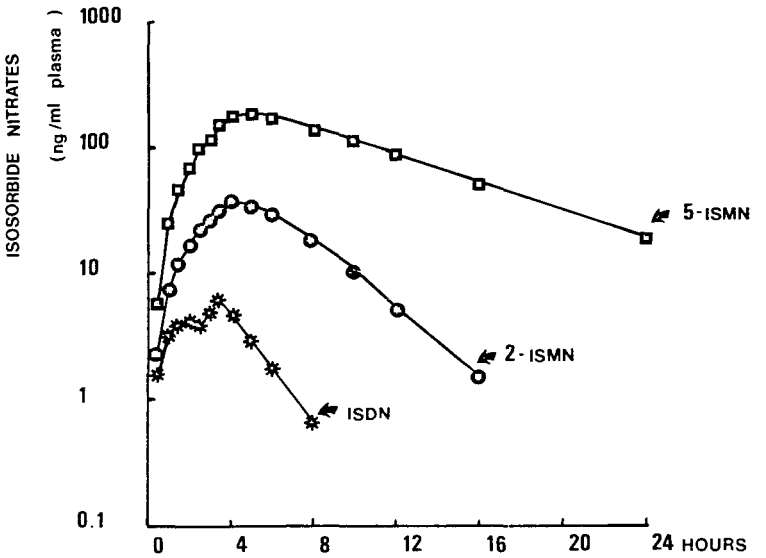

Fig. 2. Plasma ISDN, 2-ISMN and 5-ISMN concentration—time curves in a healthy subject (REV.) following a single oral dose of a 40 mg sustained release form (Langoran® 40 mg, Merrell).

procedure permits a high extraction yield (90% and over) for the three ISN molecules with only a 1.0 ml plasma sample. The specific quantitation of the mononitrate derivatives has been performed eliminating significantly the endogenous interferences. Furthermore, this method is very sensitive, specific, and reproducible, and the determination of the unmetabolized drug and its metabolites allows a study of the kinetic behaviour of this vasodilating drug. In addition, it should be noted that this technique can be used routinely and 15—20 plasma samples can be easily analyzed every day.

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DETERMINATION OF COCAINE IN PLASMA BY AUTOMATED GAS CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive gas chromatographic method is described for the determination of cocaine in plasma. A close structural analogue of cocaine, *m*-toluylecgonine methyl ester, is used as an internal standard. The simplicity of the extraction scheme and the use of an automatic sampler makes the method convenient for large numbers of samples generated in pharmacokinetic studies.

INTRODUCTION

Despite the interest in cocaine, both as a drug of abuse and as a useful local anesthetic, relatively little is known about its pharmacokinetics and metabolism in humans. This is in part because until recently sufficiently sensitive methods [1] for cocaine determination in biological fluids have been lacking.

In studies of cocaine pharmacokinetics in humans, we required a method of quantitation suitable for large numbers of samples and sensitive enough to measure plasma concentrations several hours after a single dose. Published methods with adequate sensitivity for plasma include gas chromatography (GC) with nitrogen—phosphorus [2, 3] or electron-capture [4] detection and gas chromatography—mass spectrometry (GC—MS) [1, 5]. GC—MS is a highly selective, sensitive technique; however, the expensive instrumentation and highly trained personnel required are serious drawbacks for routine applications. High-performancce liquid chromatography [6—8] has been used for the quantitation of cocaine in biological fluids, but the sensitivity is inadequate for low concentrations in small plasma samples.

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In recent years, the nitrogen—phosphorus detector has been extensively utilized in the GC determination of basic drugs, including cocaine [2, 3]. The very high selectivity of this detector for organic compounds containing nitrogen and/or phosphorus minimizes interference from extraneous substances and frequently allows determination of subnanogram quantities. This paper describes a sensitive, precise method for the determination of cocaine in plasma using GC with nitrogen—phosphorus detection. A close structural analogue of cocaine is used as an internal standard. The extraction scheme has been designed so that large numbers of samples may be processed simultaneously and analyzed in large batches by automated GC. The assay is considerably faster and more convenient than previously reported methods.

MATERIALS AND METHODS

Chemicals and reagents

Cocaine hydrochloride was from Mallinckrodt (St. Louis, MO, U.S.A.). The internal standard, *m*-toluylecgonine methyl ester, was synthesized as described below. Aqueous reagent solutions were prepared from analytical reagent grade chemicals and water distilled in glass. Isoamyl alcohol and *tert.*-amyl alcohol were reagent grade; *n*-butyl acetate and toluene were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Each new bottle of solvent was checked by GC for interfering substances.

Instrumentation

GC used a Hewlett-Packard Model 5711A Instrument, configured for on-column injection and equipped with nitrogen—phosphorus detectors. Flow-rates for carrier gas (nitrogen), detector air and hydrogen were 30, 50 and 5 ml/min, respectively. Columns were 1.8 m × 2 mm I.D. glass, packed with 3% OV-101, 0.1% potassium hydroxide on 100—120 mesh Chromosorb W HP (Alltech Assoc., Deerfield, IL, U.S.A.), conditioned overnight at 250°C. Chromatograms were recorded on a Hewlett-Packard 3390A plotter/integrator interfaced with a Hewlett-Packard 7672A automatic sampler. The automatic sampler parameters were as follows: number of sample pumps, 3; number of sample prewashes, 3; number of solvent postwashes, 5; injection volume, 5 μl. A toluene—isoamyl alcohol (90:10) postwash reduced ghosting to non-detectable levels.

Analyses were at column oven temperatures ranging from 200 to 220°C, depending upon the column condition. Clean separation of cocaine and internal standard was achieved with the retention of internal standard relative to cocaine of 1.35.

Preparation of glassware

Screw-top glass culture tubes (13 × 100 mm) and autosampler vials were washed with a phosphate-free detergent, soaked overnight in 4 *M* hydrochloric acid, rinsed several times with water and then dried at 80°C. The PTFE-lined tube caps were soaked overnight in dilute hydrochloric acid, rinsed with distilled water and dried. Autosampler vials were further soaked overnight in a 0.1% tetrasodium EDTA solution and then oven-dried.

Extraction procedure

Plasma (1 ml) and internal standard (100 ng in 100 μl of 0.01 *M* sulfuric acid) were pipetted into culture tubes and briefly mixed. Samples were alkalinized with a potassium carbonate—bicarbonate buffer pH 9.5 (0.5 ml, 1 *M*). Two ml of toluene—*tert.*-amyl alcohol (90:10) were added, the tubes were fitted with PTFE caps, and vortex-mixed for 6 min with a multiple tube vortex mixer. The tubes were centrifuged at 0°C for 15 min at 700 *g* to break emulsions and then placed in a dry ice—acetone bath to freeze aqueous layers. Organic layers were poured into clean tubes containing 0.5 ml of 0.1 *M* sulfuric acid, which were then vortex-mixed, centrifuged and frozen as above. The organic phase was poured off and discarded, the aqueous layer was thawed and then washed with 2 ml toluene—*tert.*-amyl alcohol (90:10). The extraction could be interrupted at this point if necessary and the samples stored frozen. Buffer, pH 9.5 (0.5 ml, 1 *M*) and butyl acetate (0.5 ml) were added, the tubes were vortex-mixed, centrifuged, and frozen in dry ice—acetone to separate the aqueous layers. The butyl acetate extracts were poured into EDTA-treated autosampler vials, which were left uncapped and loaded into the autosampler cassette for GC analysis.

Calibration procedure

Stock solutions (1 mg/ml as the base) of cocaine hydrochloride and of internal standard, *m*-toluylecgonine methyl ester hydrochloride, were prepared in 0.01 *M* sulfuric acid and stored frozen. Fresh cocaine solutions were prepared every four weeks. These solutions were diluted to appropriate concentrations with 0.01 *M* sulfuric acid and added (as 100-μl aliquots) to blank plasma obtained from drug-free volunteers to provide several concentrations spanning the range 0—500 ng/ml cocaine and 100 ng/ml internal standard.

The samples were taken through the extraction procedure and analyzed by GC as described above. Standard curves were constructed by plotting the peak height ratio of cocaine versus internal standard, and were linear over the range 0—1000 ng/ml. A standard in the middle of the expected concentration range was reinjected and the computing integrator was calibrated using the internal standard method.

After several runs, we found aqueous standard curves were always identical to those obtained from spiked plasma and, consequently, aqueous standard curves were used for calibration in future runs. As controls, plasma spiked with 50, 100 and 500 ng/ml cocaine as well as blank plasma were included in each run.

Sample collection and storage

Cocaine undergoes both spontaneous [9, 10] and enzymatic hydrolysis [11] in plasma at physiologic pH. Special precautions are necessary to avoid losses. Blood (approximately 6 ml) from experimental subjects was drawn into heparinized tubes on ice containing 0.2 ml of saturated sodium fluoride to inhibit plasma esterases [5, 12]. The tubes were immediately vortexed and placed back on ice until centrifuged at 0°C to separate the plasma. Immediately following centrifugation, the plasma samples were frozen and stored at −10°C until analysis. Blank plasma used for standards and control samples was like-

wise spiked with sodium fluoride (0.2 ml, saturated) prior to adding cocaine. As a check for stability during storage, samples were reanalyzed over periods ranging from two weeks to one year.

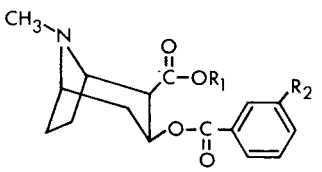
Synthesis of the internal standard, m-toluylecgonine methyl ester hydrochloride

A solution of ecgonine methyl ester hydrochloride [13] (235 mg, 1 mmol in 25 ml water) was made basic with potassium carbonate and extracted with 50 ml of methylene chloride. The extract was evaporated to dryness on a rotary evaporator, reconstituted in 10 ml of toluene, and refluxed for 3 h with *m*-toluic anhydride (300 mg). The mixture was cooled, extracted with 50 ml of 0.5 *M* sulfuric acid, and the resulting aqueous extract washed with two 25-ml portions of diethyl ether. Potassium carbonate was added to bring the pH to 10, and the product was extracted with two 25-ml portions of diethyl ether. The extract was dried over anhydrous potassium carbonate and evaporated to give an oil, which was purified by column chromatography on silica gel (Merck, 70—230 mesh, 6 × 1.5 cm column), eluting with ethyl acetate—methanol—58% ammonia (85:10:0.5). Fractions (4 ml) were taken and monitored by thin-layer chromatography (TLC) for product. The impurities, which included unreacted ecgonine methyl ester, had lower R_F values and were readily separated. Those fractions containing product were combined and evaporated with a rotary evaporator. Isopropyl alcohol (2 ml) was added followed by 3 drops of concentrated hydrochloric acid, which produced an acidic solution, pH < 2 by damp, universal pH paper. The solution was slowly diluted with anhydrous diethyl ether, with stirring and scratching with a glass rod, which caused the product to crystallize. The salt was collected by filtration, washed with anhydrous diethyl ether and air-dried to give 55 mg of white crystalline powder, m.p. 180—181°C. The product was homogeneous by TLC (silica gel) and GC (OV-101). A microanalysis for carbon, hydrogen and nitrogen was within ± 0.2% of theory.

RESULTS AND DISCUSSION

Hydrolytic instability of cocaine and internal standard

The ease with which cocaine hydrolyzes, particularly under alkaline conditions, presents special problems in its quantitative analysis. Cocaine is a basic compound, and extraction from aqueous solution into organic solvents must be carried out at alkaline pH, a condition which will lead to some unavoidable losses. For example, Fletcher and Hancock [9] found that even at



1, $R_1 = CH_3$; $R_2 = H$

2, $R_1 = CH_3$; $R_2 = CH_3$

3, $R_1 = \underline{n}\text{-}C_3H_7$; $R_2 = H$

Fig. 1. Structures of cocaine (1), *m*-toluylecgonine methyl ester (2), and benzoylecgonine propyl ester (3).

pH 8, cocaine was hydrolyzed to the extent of 17% in 1 h at room temperature. At pH 9.4 the loss was 41%.

In chromatographic assays, errors due to loss of the analyte can often be minimized by the use of an internal standard with similar chemical and physical properties. The site of lability in the cocaine molecule is the methyl ester grouping, which is hydrolyzed to the carboxylic acid derivative, benzoylecgonine [9, 10]. For this reason, we synthesized an internal standard, *m*-toluylecgonine methyl ester (2, Fig. 1) which maintains this critical functionality. During the alkaline extraction steps, hydrolysis of this close structural analogue proceeds at a rate similar to that of cocaine hydrolysis. This results in constancy of the ratio of the two substances following extraction even if significant hydrolysis has occurred. Aliquots of a solution containing 100 ng/ml of both compounds in pH 9.5 buffer extracted at 15, 30, 60, 90 and 120 min gave peak height ratios that differed by only ± 2% from the mean, although the decrease in magnitude of peak heights was about 30% over the 2-h period. Consequently, allowing samples to stand in alkaline solution does not lead to serious errors. To obtain maximum sensitivity, however, the samples should be extracted without delay. Previously reported methods for the GC determination of cocaine have utilized either unrelated compounds or the propyl ester analogue (3, Fig. 1) as internal standards [1—3]. These compounds would be expected to be stable or less vulnerable to hydrolysis than cocaine.

Stability of stored plasma samples

Due to the hydrolytic lability of cocaine, special precautions were necessary for sample storage. Blood was drawn into cold tubes containing sodium fluoride to inhibit hydrolysis catalyzed by pseudocholine esterases [5, 12] and immediately centrifuged in the cold to separate the plasma. Samples were stored frozen at −10° C until analyzed. To check stability, split samples were reanalyzed at intervals from one week to three months. In addition, a batch of plasma was spiked with cocaine, stored frozen, and analyzed on successive runs as a control. The data (Table I) indicated that losses are insignificant for storage periods as long as one year, if the samples are kept frozen. We observed that a sample repeatedly thawed and frozen over a period of several weeks lost significant amounts of cocaine.

Extraction

The extraction scheme simplifies the handling of large numbers of samples to make efficient use of the automatic sampler. Organic solvents liquid at −80° C facilitate separation of aqueous layers by freezing in a dry ice—acetone bath. A multi-tube vortex mixer allowed the simultaneous extraction of 24 samples. Using this simple extraction procedure (Fig. 2), one technician can extract as many as 60 samples in a day.

Gas chromatography

The GC analysis was carried out on a 1.8-m column packed with 3% OV-101 and 0.1% KOH on 100—120 mesh Chromosorb W. Baseline separation of cocaine and the internal standard was readily achieved, and co-extracted

TABLE I

STABILITY OF COCAINE IN FROZEN PLASMA

Sodium fluoride added to plasma (10 mg/ml) prior to addition of cocaine. Set 1 was outdated plasma from a local blood bank; sets 2, 3 and 4 were freshly prepared plasma specimens from three drug-free volunteers.

	Set 1	Set 2	Set 3	Set 4
Concentration given (ng/ml)	70.0	85.0	85.0	85.0
Concentration found, mean	70.2	88.9	84.3	81.4
Number of samples	8	6	6	5
Standard deviation	6.68	5.30	10.4	5.20
Coefficient of variation	9.5	6.0	12.3	6.4
Time period* (month)	18	2	2	2

*Time between spiking and analysis of last sample. All samples within a given set were analyzed on different days.

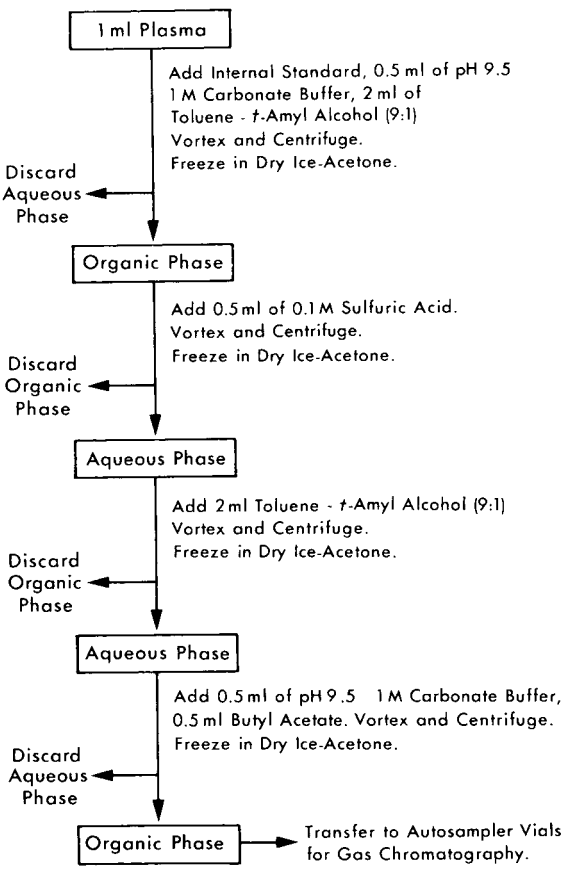


Fig. 2. Flow diagram of the extraction procedure.

endogenous substances did not lead to interfering peaks (Fig. 3). The cleanness of the extracts allowed injection of samples at short intervals (run time = 5 min), and automated analysis could proceed unattended. Some care was necessary to avoid extraneous peaks from solvent impurities or contaminated glassware. An occasional bottle of butyl acetate contained a substance with a retention time similar to cocaine. Consequently, all solvents were checked by GC before using. The caps for autosampler vials were another source of interfering peaks, presumably derived from the PTFE-lined rubber septa. This problem was solved by using a high boiling solvent (butyl acetate, b.p. 127° C) to minimize evaporation and by leaving the vials uncapped. All glassware was meticulously acid-washed including several rinses with distilled water prior to oven-drying at 80° C.

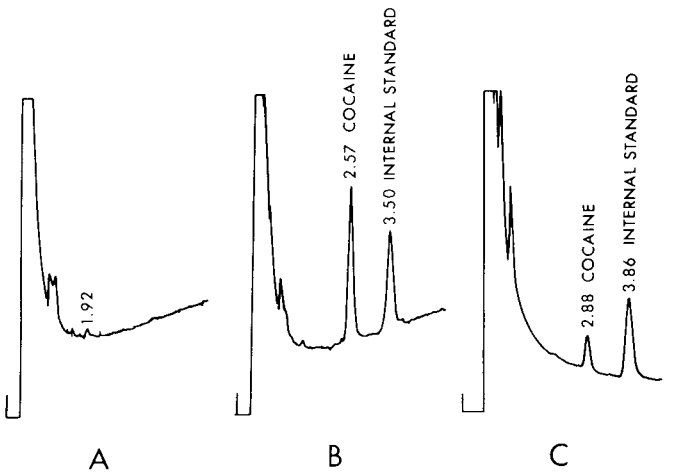


Fig. 3. Gas chromatograms obtained from extracts of human plasma. (A) Extract of drug-free plasma; (B) extract of plasma spiked with 100 ng/ml cocaine and 100 ng/ml internal standard; (C) extract of plasma containing 30 ng/ml cocaine from a subject following intravenous injection.

Initially, we encountered difficulty with losses due to adsorption on the surfaces of the borosilicate glass autosampler vials. After standing several hours, the peak heights for both cocaine and the internal standard were greatly diminished when the sample was reinjected. We had previously encountered a similar problem with nicotine [14], and found that the addition of basic substances (ammonia or triethylamine) or base-washing the vials helped somewhat, but did not eliminate the problem. On the assumption that metal ions on the glass surface could form a complex with tertiary amines, such as nicotine or cocaine, we added a final rinse with a chelating agent, 0.1% aqueous ethylenediamine tetraacetic acid tetrasodium salt (EDTA). This resulted in a two-fold increase in peak heights compared to identical samples in untreated vials. Reinjection of samples after 24 h resulted in no significant change in peak heights, which meant that samples could be run overnight on the autosampler.

Precision and sensitivity

Within-run precision was determined by running in duplicate randomly chosen samples from clinical studies. All samples were given code numbers. The person carrying out the extraction and GC had no knowledge of which samples were duplicates. As can be seen from Table II, duplicates agreed quite closely, the average variation being < 4%. Between-run precision was determined analogously (Table III).

No difficulty was encountered in measuring cocaine at concentrations as low as 3 ng/ml. Good precision was obtained for duplicates both within-run and between-run for concentrations in the range 0—25 ng/ml.

TABLE II

WITHIN-RUN PRECISION: PERCENT DEVIATION FOR DUPLICATE ANALYSES

Concentration range (ng/ml)	0—25	25—50	50—100	100—200	200—400
Number of samples	7	8	10	26	13
Mean concentration (ng/ml)	14.0	34.6	74.3	156	250
Mean percent deviation*	3.6	2.0	3.0	2.5	2.5

*Computed by determining percent deviation from the mean for each pair, and then averaging the deviations for all pairs within the specified concentration range.

TABLE III

BETWEEN-RUN PRECISION: PERCENT DEVIATION FOR DUPLICATE ANALYSES

Concentration range (ng/ml)	0—25	25—50	50—100	100—200	200—400
Number of samples	16	11	8	11	8
Mean concentration (ng/ml)	12.0	37.3	65.4	143	304
Mean percent deviation*	6.3	7.5	5.8	5.0	2.9

*Calculated as in Table II.

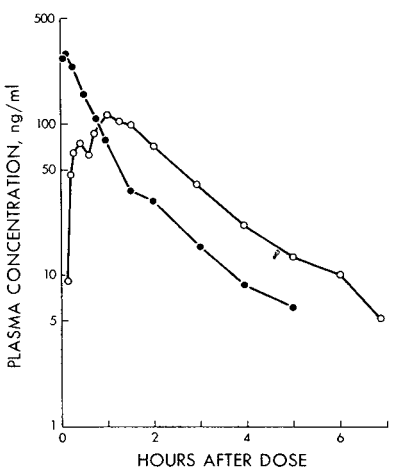


Fig. 4. Plasma cocaine concentrations in human subjects following intravenous administration of 0.4 mg/kg (•, I.H.), and intranasal administration of 2 mg/kg (○, I.P.).

Pharmacokinetic studies

The assay has been in use for over a year in studies of the pharmacokinetics of cocaine in humans. Data from two representative subjects was used to construct semilogarithmic plots of concentration versus time shown in Fig. 4. One subject (I.H.) had received a 0.4 mg/kg intravenous bolus, the other (I.P.) a 2 mg/kg intranasal dose. Readily measurable quantities of cocaine were present at 6 h, about four half-lives in these subjects.

CONCLUSION

A sensitive and precise method for the GC determination of cocaine in plasma has been developed. The operational simplicity of the procedure and use of an automatic sampler make the method well suited for the analysis of large numbers of samples generated in pharmacokinetic studies.

ACKNOWLEDGEMENTS

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COMBINATION OF LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION AND GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION FOR THE DETERMINATION OF Δ^9-TETRAHYDROCANNABINOL-11-OIC ACID IN URINE

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SUMMARY

A method is described for the determination of Δ^9-tetrahydrocannabinol-11-oic acid (Δ^9-THC-11-oic acid) in urine by using a combination of liquid chromatography and glass capillary gas chromatography with electron-capture detection.

Prior to extraction, the glucuronide conjugate of Δ^9-THC-11-oic acid was enzymatically converted to the free acid and Δ^8-THC-11-oic acid added as an internal standard. An aliquot of the extract was separated by liquid chromatography and one eluate fraction containing Δ^8-THC-11-oic and Δ^9-THC-11-oic acids was collected. Before gas chromatographic analysis the acids were converted to their pentafluoropropyl-pentafluoropropionyl derivatives. Authentic urine samples, positive with the EMIT® cannabinoid assay, were analysed and the results compared with those obtained from an earlier described gas chromatographic—mass spectrometric method. The detection limit for the overall method was approximately 20 ng/ml. The precision was 8% for a sample concentration of 90 ng/ml of Δ^9-THC-11-oic acid in urine.

INTRODUCTION

To detect the abuse of cannabis, sensitive and selective or specific analytical methods are needed for the determination of tetrahydrocannabinol (THC) and its metabolites in body fluids. Most widely used is the EMIT® cannabinoid assay [1], which is rapid but cross-reacts with a variety of THC metabolites; thus, positive results need verification by an independent method. For this purpose analysis has been directed towards the determination of the acid metabolite, Δ^9-tetrahydrocannabinol-11-oic acid (Δ^9-THC-11-oic acid), present in urine after cannabis smoking. A number of gas chromatographic—mass

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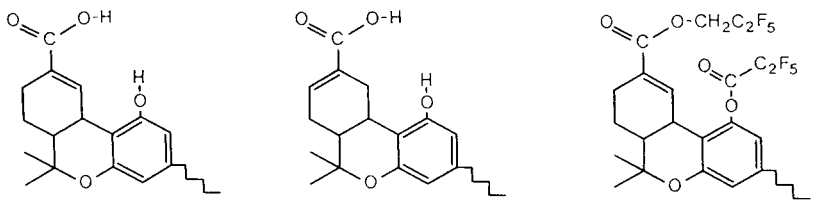

a b c

Fig. 1. Structure of (a) Δ^9-THC-11-oic acid, (b) Δ^8-THC-11-oic acid, and (c) pentafluoropropyl-pentafluoropropionyl derivative of Δ^9-THC-11-oic acid.

spectrometric (GC—MS) methods are available [2—7], but to date only a few techniques other than GC—MS have been used for this purpose. One gas chromatographic (GC) method has been presented [8] where the THC-11-oic acid was converted to its methyl ester, methyl ether derivative, prior to analysis with flame ionization detection, resulting in a comparatively high detection limit. Although suitable only for fairly high concentrations, thin-layer chromatography (TLC) has also been used [9]. High-performance liquid chromatography (HPLC) has been used in combination with radioimmunoassay for the determination of cannabinoids in human urine [10] and as a clean-up step for GC—MS analysis of cannabinoid metabolites [4]. Methods for the determination of both THC and its metabolites have been extensively reviewed elsewhere [11].

The present paper describes a method for the determination of Δ^9-THC-11-oic acid in urine which utilizes a combination of liquid chromatography (LC) with ultraviolet (UV) detection and GC with electron-capture detection. The isomer, Δ^8-THC-11-oic acid, was used as an internal standard, and the THC-11-oic acids were converted to their pentafluoropropyl-pentafluoropropionyl derivatives prior to GC. Structures of Δ^8-THC-11-oic acid, Δ^9-THC-11-oic acid, and the derivative of Δ^9-THC-11-oic acid are shown in Fig. 1. Qualitative and quantitative results obtained for a number of authentic urine samples were compared with those obtained with an independent GC—MS method [7].

EXPERIMENTAL

Chemicals

Δ^9-Tetrahydrocannabinol-11-oic acid was purchased from the Faculty of Pharmacy (University of Uppsala, Sweden), and Δ^8-THC-11-oic acid from the National Institute of Drug Abuse (Research Triangle Institute, NC, U.S.A.). Mirex (1,1a,2,2,3,3a,4,5,5,5a,5b,6-dodecachloro-octahydro-1,3,4-metheno-1H-cyclobuta [*cd*] pentalene) from Poly Science (U.S.A.) was used as an alternative internal standard. Pentafluoropropionic anhydride (PFPA) was purchased from Reagenta (Uppsala, Sweden) and pentafluoropropanol (PFPOH) from ICN Pharmaceuticals (Plainview, NY, U.S.A.). The enzyme β-glucuronidase was purchased from Sigma (lot No. G-0251) (St. Louis, MO, U.S.A.). Acetonitrile, far-UV grade and HPLC grade, from Fisons (Loughborough, U.K.) was used in the mobile phase. Other organic solvents

used throughout were *n*-hexane p.a. (pro analysi) and cyclohexane p.a. from Merck (Darmstadt, F.R.G.), diethyl ether analytical reagent grade from Mallinckrodt (Paris, KY, U.S.A.), and methanol p.a. from May and Baker (Dagenham, U.K.). All other chemicals were of analytical reagent grade.

Sample preparation

One millilitre of urine was transferred to a 16 × 100 mm glass tube with PTFE-lined screw cap, adjusted to pH 4–5 with 0.8 ml of 0.5 *M* acetate buffer, pH 4.5, and incubated with β-glucuronidase (2300 U) at 56°C for 30 min [8]. Thereafter, 130 ng of Δ^8-THC-11-oic acid were added as an internal standard and the sample was shaken with 4.0 ml of diethyl ether—*n*-hexane (1:1) for 10 min, centrifuged, and 3 ml of the organic phase were evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 μl of acetonitrile–water (1:1) and an aliquot of 100 μl was further used for LC analysis (for details see below). One eluate fraction from the LC analysis containing both the THC acids was collected and reduced from 2 ml to 200–300 μl by evaporation before being shaken with 1 ml of diethyl ether—*n*-hexane (1:1), which alternatively contained Mirex (42 ng/ml) as an internal standard. After centrifugation, the organic phase was transferred to a 1.0-ml silanized Reacti-vial (Pierce) with a PTFE-lined screw cap and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl of PFPA—PFPOH (4:1) and heated at 70°C for 15 min. Before GC analysis the reagent mixture was evaporated and the residue dissolved in 50 μl of cyclohexane.

Liquid chromatography

Liquid chromatography was performed with an SP 8000 liquid chromatograph (Spectra Physics) equipped with a variable UV detector (Model 770, Schoeffel). Separation was accomplished on a Hibar® column (Merck), 125 × 4 mm, packed with LiChrosorb (5 μm) RP-8. The mobile phase was acetonitrile—0.05 *M* ammonium dihydrogen phosphate (50:50, v/v) with a flow-rate of 1.0 ml/min. The UV detector was operated at 220 or 225 nm depending on the UV cut-off value of the acetonitrile used.

Gas chromatography

GC analyses were made on a Fractovap 4160 gas chromatograph (Carlo Erba) equipped with an electron-capture detector. The GC conditions used were as follows: fused-silica column, 25 m × 0.3 mm I.D., with SE-54 as stationary phase (Hewlett-Packard); helium carrier gas flow-rate approximately 40 cm/sec. Nitrogen was used as make up gas to the electron-capture detector with a flow-rate of 46 ml/min; GC temperatures are given in the figure captions. One microlitre of the cyclohexane extract was injected with a preset split ratio of 1:30.

Gas chromatography—mass spectrometry

GC—MS analyses were carried out as described in an earlier reported method for the determination of Δ^9-THC-11-oic acid in urine [7]. By this method Δ^9-THC-11-oic acid was extracted from enzymatically hydrolysed urine with

hexane—ether, followed by evaporation of the organic solvent and derivatization with a mixture of PFPA and PFPOH as described in the present paper. The d_3-labelled Δ^9-THC-11-oic acid was used as internal standard. GC—MS was carried out by chemical ionization with methane as reactant gas and by selective detection of negative ions.

RESULTS

Combined HPLC and GC

Fig. 2a and b shows an example from analysis of a pool of authentic urine samples which were found positive by EMIT assay. The concentration of

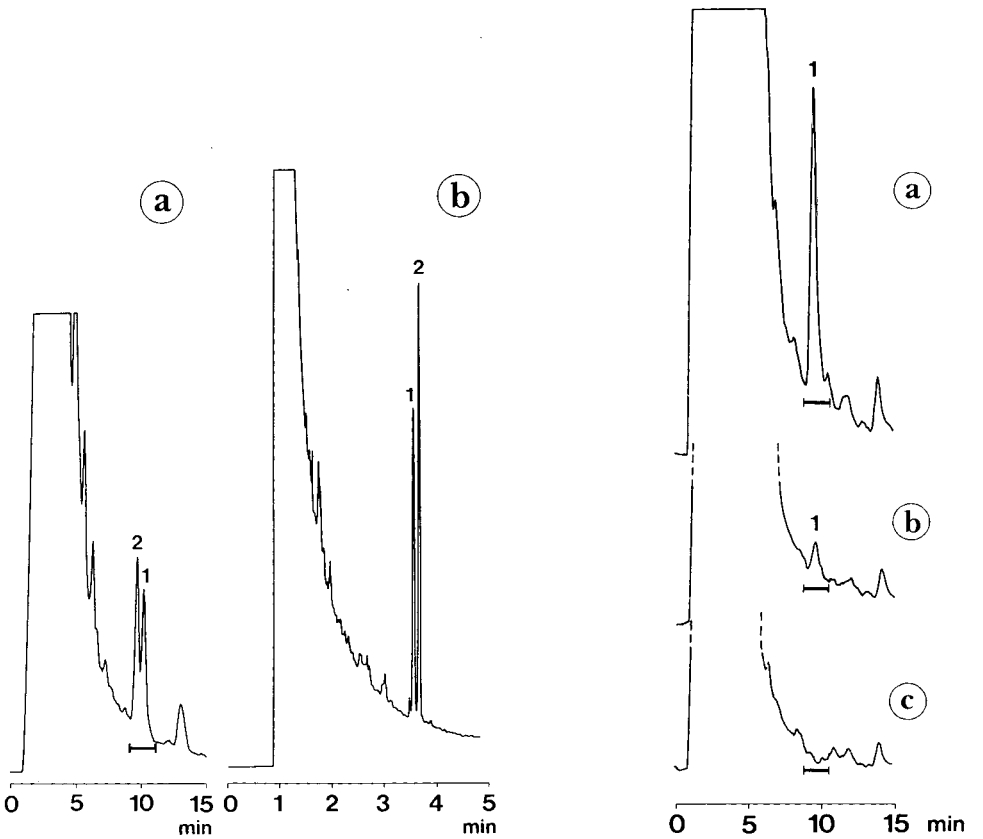

Fig. 2. Analysis of an authentic pool of urine, which was found positive with the EMIT cannabinoid assay, containing 1 = Δ^9-THC-11-oic acid (90 ng/ml) and 2 = Δ^8-THC-11-oic acid (130 ng/ml) used as internal standard. (a) LC chromatogram. UV conditions: 220 nm, 0.04 a.u.f.s. (b) GC chromatogram of derivatized LC fraction (see Experimental). Conditions: oven temperature 220—280°C, 5°C/min; injection temperature 250°C; detector temperature 300°C; helium carrier gas flow-rate 45 cm/sec; attenuation × 128; chart-speed 2 cm/min.

Fig. 3. LC chromatograms obtained from the analysis of three urine samples (no internal standard added). 1 = Δ^9-THC-11-oic acid. (a) 530 ng/ml, (b) 35 ng/ml, (c) negative. UV conditions: 225 nm, 0.04 a.u.f.s.

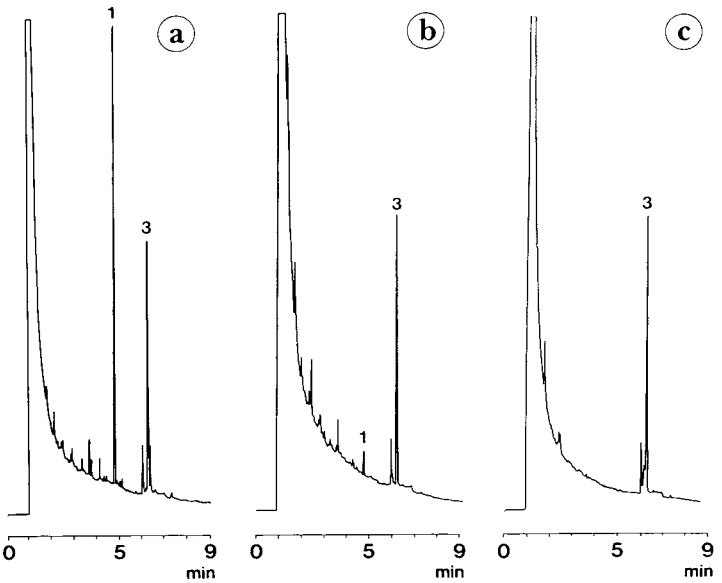

Fig. 4. GC chromatograms of derivatized LC fractions (see Experimental) from the samples in Fig. 3. 1 = Δ^9-THC-11-oic acid, 3 = Mirex (internal standard). (a) 530 ng/ml, (b) 35 ng/ml (c) negative. Conditions: oven temperature 200—280°C, 10°C/min; injection temperature 250°C; detector temperature 300°C; helium carrier gas flow-rate 40 cm/sec; attenuation × 256; chart-speed 1 cm/min.

Δ^9-THC-11-oic acid was 90 ng/ml. Both the liquid chromatogram and the gas chromatogram are shown. The latter was obtained by collecting the eluate fraction (as marked in the chromatogram) containing both the THC acids and further treating it as described in the experimental section.

As an alternative to Δ^8-THC-11-oic acid as internal standard, Mirex (useful only in the GC step) was used, and two examples from analysis of authentic urine samples and one blank urine are presented. The liquid chromatograms Fig. 3a—c and gas chromatograms 4a—c are shown in each case. The three examples represent concentrations of 530 ng/ml (a), 35 ng/ml (b) and a blank urine (c). Chromatographic data are given in the figure captions and in the experimental section.

Linearity, precision, recovery, and detection limit

The linearity for the overall method was determined by analysing blank urines to which known amounts of Δ^9-THC-11-oic acid had been added. Peak heights in the gas chromatograms were measured and related to the peak height of the internal standard (Mirex). The resulting calibration plot was linear from 16 ng/ml up to 1020 ng/ml with a correlation coefficient of 0.998.

The precision for the method using Δ^8-THC-11-oic acid as internal standard was determined by analysing a pool of authentic urine samples with a measured Δ^9-THC-11-oic acid concentration of 90 ng/ml. The relative standard deviation (R.S.D) obtained was thus calculated as 8.0% (n = 5). The precision for the method by using Mirex as internal standard was similarly determined by analysing a pool of urine samples with a measured concentration of 235 ng/ml which resulted in an R.S.D of 10.3% (n = 5).

The recovery through the entire method was measured after analysis of blank urines to which known amounts of Δ^9-THC-11-oic acid (68 ng) had been added. After adjusting for aliquots this result was compared with GC analysis of the same amount of Δ^9-THC-11-oic acid subjected only to the derivatization and the GC step with Mirex as internal standard. The recovery was measured on two different occasions as 77% and 79%, respectively.

The detection limit for the overall method was approximately 20 ng/ml.

Correlation with the GC—MS method

The results obtained by the described method with Δ^8-THC-11-oic acid or Mirex as internal standards were compared with the results obtained by the GC—MS method [7] for 27 authentic urine samples which were found positive by EMIT assay. The concentration of Δ^9-THC-11-oic acid in these samples ranged from 8 to 850 ng/ml determined by GC—MS. By comparing the results for seventeen samples with Δ^8-THC-11-oic acid as internal standard, a correlation coefficient of 0.990 was obtained. The corresponding value for ten samples with Mirex as internal standard was 0.969. When all 27 samples were included the correlation coefficient was 0.974 and the correlation plot is shown in Fig. 5. To visualize the correlation over the wide concentration range, logarithmic values were used in the plot shown Fig. 5.

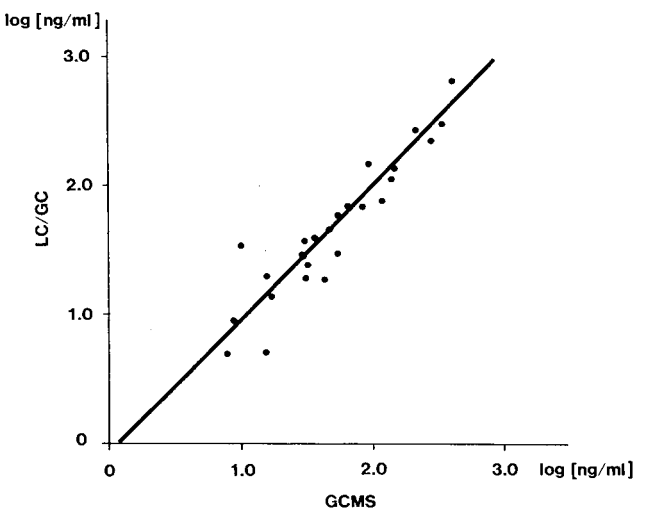

Fig. 5. Plot of the correlation (logarithmic values) between LC/GC and GC—MS for 27 samples.

DISCUSSION

The combination of LC and GC served two functions. First, the determination of the THC acid in high concentrations (> 100 ng/ml) could be made already in the LC step alone by monitoring the UV response of the liquid effluent which is shown in Figs. 2a and 3a. Second, a selective clean-up was accomplished for the GC analysis. Furthermore, a high resolution was achieved in the GC step and the high selective response to the derivative improved the detectability. An approach was made to use the GC technique alone but a

large number of interfering peaks resulted in unreliable results. Most of the urine samples which were found positive with the EMIT cannabinoid assay yielded an amount of THC acid which could be detected with the described method.

The UV detection of Δ^9-THC-11-oic acid in the LC step was limited by the background from the urine sample and also by instrumental sensitivity. By monitoring the UV response, large variations in the urinary background were observed in different samples and, if the LC step was used alone, background peaks could interfere with the determination of Δ^9-THC-11-oic acid. Although a comparatively sophisticated sample clean-up step was used before the GC analysis, the detectability was still limited by background peaks. The electron-capture detector showed high response to the derivative. The example shown in Fig. 4b corresponded to about 10 pg of derivative injected on the column.

In the raw material of cannabis, minute amounts of Δ^8-THC relative to that of Δ^9-THC may be found [12]. Therefore, one could expect small amounts of Δ^8-THC-11-oic acid appearing as a metabolite in the urine. If so, this amount can be considered negligible compared to the amount of Δ^8-THC-11-oic acid added as internal standard provided that the content of Δ^9-THC-11-oic acid is not too high. We have, however, not been able to detect this metabolite in any of the samples analysed so far. The obtained results showed acceptable correlation with those from the GC—MS method. The use of Δ^8-THC-11-oic acid as internal standard did not significantly improve the correlation of the quantitative results compared with those samples in which Mirex was used as internal standard. If the Δ^8 analogue is unavailable, Mirex can be used as internal standard, but it fails to compensate for losses during the extraction step or for incomplete derivatization. It is suitable as a GC internal standard to control the injected amount, also when the Δ^8 analogue is used as internal standard.

In conclusion, the described method offers a less expensive alternative to GC—MS and is useful for confirmation of positive findings made by EMIT assay. By combining LC and GC a high degree of selectivity was obtained.

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SIMULTANEOUS DETERMINATION OF CAFFEINE AND ITS N-DEMETHYLATED METABOLITES IN UMBILICAL CORD PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A convenient high-performance liquid chromatographic method for the simultaneous determination of caffeine and its N-demethylated metabolites in plasma is described. Separation is achieved by reversed-phase chromatography using a mobile phase consisting of 0.01 *M* sodium acetate buffer, pH 5.0—methanol—tetrahydrofuran (95:4:1) in conjunction with a μBondapak C_{18} column protected by a guard column containing Bondapak C_{18}/Corasil. With a flow-rate of 3 ml/min, levels in the region of 50 ng/ml for the dimethylxanthines and 100 ng/ml for caffeine can be determined by ultraviolet detection at 254 nm. The method was used clinically for measuring cord blood samples to provide information regarding fetal exposure to caffeine and its N-demethylated metabolites during late pregnancy.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) has been referred to as the most common drug of abuse in our society [1]. Although its pharmacologic and toxicologic effects are not fully understood the U.S. Food and Drugs Administration in 1980 advised pregnant women to reduce their caffeine consumption [2]. This was prompted by indications of decreased intra-uterine fetal growth, lower birth weight and skeletal abnormalities induced by the drug in animal studies [3—6]. However, in spite of the demonstrated teratogenicity of caffeine in animals [7, 8] the implications for humans are unclear since the mode and level of exposure, and metabolism of the drug differ widely [9]. Conflicting reports associating increased fetal risk with caffeine consumption during pregnancy [10, 11] only complicate the issue. The problem in these studies is compensating for the known adverse effects of cigarette smoking and alcohol

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consumption during pregnancy [12], in order to assess the effects of caffeine ingestion. Unfortunately, neither of the previously mentioned reports [10, 11] included details of circulating plasma caffeine levels to confirm the extent of coffee drinking or the degree of fetal exposure.

In studies aimed at providing such information, carried out in Vermont [13], Montreal [14] and London [15], transplacental passage of caffeine was assessed by measuring umbilical cord blood levels from unselected infants. These, however, did not attempt to measure the N-demethylated metabolites of caffeine namely, theophylline (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine). Since these compounds are also pharmacologically active [16] it would seem advantageous to be able to measure the individual metabolites and assess their contribution to the total xanthine level.

The increasing use of xanthines in the treatment of apnoèa of prematurity [17, 18] and growing concern regarding fetal exposure to caffeine during pregnancy [2, 10, 19] indicate the need for a sensitive and specific method for the simultaneous determination of caffeine, theophylline, theobromine and paraxanthine in plasma. Previous high-performance liquid chromatographic (HPLC) assays have only been partially successful because some earlier workers [20, 21] appear to have overlooked the contribution of paraxanthine whilst Sved and Wilson's method [22] for mono- and dimethylxanthines cannot quantitate caffeine without further modification. More recently, however, improved HPLC assays have become available which will permit the simultaneous determination of caffeine and its N-demethylated metabolites in plasma [23—25].

This paper describes an alternative, more sensitive method based on the technique employed by Miksic and Hodes [26] for measuring theophylline in biological fluids.

TABLE I

EXTRACTION RECOVERIES OF CAFFEINE AND ITS N-DEMETHYLATED METABOLITES FROM 3% BOVINE SERUM ALBUMIN (n = 4)

Component	Standard solutions					Extracted samples
	Concn. (μg/ml)	Volume injected (μl)	Quantity on column (ng)	Mean peak height (mm)	C.V. (%)	Concn. (μg/ml)
Caffeine	10	30	300	76.38*	3.60	2
	10	90	900	109.17**	0.70	10
Theophylline	10	7.5	75	48.38*	2.29	0.5
	10	45.0	450	130.38**	0.73	5.0
Theobromine	10	7.5	75	57.13*	5.82	0.5
	10	45.0	450	159.67**	1.30	5.0
Paraxanthine	10	7.5	75	43.88*	2.85	0.5
	10	45.0	450	132.63**	2.21	5.0

*Detector sensitivity 0.01 a.u.f.s.
**Detector sensitivity 0.02 a.u.f.s.

EXPERIMENTAL

Reagents

Dichloromethane (laboratory reagent grade) and isopropyl alcohol, methanol, tetrahydrofuran, sodium acetate (Analar quality) were all purchased from BDH Chemicals (Poole, U.K.). Caffeine, paraxanthine, theophylline, theobromine and β-hydroxyethyltheophylline were supplied by Sigma London (Poole, U.K.).

Equipment

The Waters Assoc. (Hartford, U.K.) high-performance liquid chromatograph consisted of a constant volume Model M6000A pump in conjunction with a U6K injector and a Model 440 UV detector operating at 254 nm. The signal from the UV monitor was linked to a 10-mV Linseis Model LS 24/80/80 two-pen recorder adjusted to give a chart speed of 200 mm/h for the purpose of this assay.

Chromatography

The analytical column, a 30 cm × 3.9 mm I.D. μBondapak C_{18} (10-μm reversed-phase packing) together with a guard column containing Bondapak C_{18}/Corasil were supplied by Waters Assoc. This was used in conjunction with a mobile phase consisting of 0.01 *M* sodium acetate buffer, pH 5.0—methanol—tetrahydrofuran (95:4:1) which was filtered through a 0.45-μm Millipore filter (type HA) and degassed prior to use. Chromatography was performed at ambient temperature using a flow-rate of 3 ml/min producing a back pressure of approximately 25.5 MPa (3750 p.s.i.). The eluent was monitored at 254 nm with a detector sensitivity of 0.01 a.u.f.s.

Volume injected (μl)	Quantity on column (ng)	Mean peak height		C.V. (%)	Percent recovery corrected for volume losses
		Uncorrected (mm)	Corrected (mm)		
100	300	56.83	68.2*	4.68	89.29
60	900	81.08	97.3**	4.06	89.13
100	75	35.83	43.0*	2.91	88.88
60	450	104.00	124.8**	1.44	95.72
100	75	39.50	47.4*	7.05	82.97
60	450	126.50	151.8**	1.37	95.07
100	75	32.5	39.0*	2.67	88.88
60	450	98.75	118.5**	2.53	89.35

Procedure

A 300-μl aliquot of plasma containing 1200 ng of the internal standard (incorporated by adding 20 μl of a solution containing 60 μg/ml β-hydroxyethyltheopylline in ethanol) was extracted by shaking for 10 min with 6 ml of dichloromethane—isopropyl alcohol (90:10). Following centrifugation at 2000 rpm (approximately 600 *g*) for 10 min the organic phase was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 55° C. The residue was then reconstituted in 200 μl of mobile phase and 70—100 μl were injected onto the column.

Preparation of standard curves

A series of calibration standards were prepared in 3% bovine serum albumin (BSA). Each standard contained all four xanthine components. Concentrations of 2, 4, 6 and 10 μg/ml were adopted for caffeine whilst levels of 0.5, 1, 2.5 and 5 μg/ml were most suitable for paraxanthine, theophylline and theobromine. These standards were subjected to the previously described procedure alongside patient samples and graphs comparing the peak height ratio with the actual concentration of the xanthine present were then constructed.

Extraction recovery experiment

Samples were prepared in 3% BSA to give concentrations of 2 and 10 μg/ml for caffeine and 0.5 and 5 μg/ml for the dimethylxanthines representing values at the lower and higher extremities of the calibration graphs. These were subjected to the previously described extraction procedure; to facilitate quantitation of the extraction recovery, however, only a 5-ml aliquot of the original 6 ml of extractant was prepared for analysis. Extracted samples were injected and the peak heights measured; these were multiplied by 6/5 to adjust for the above volume differences giving rise to the corrected peak heights referred to in Table I. The corrected peak heights were compared with those obtained from injections of standard solutions containing 10 μg/ml caffeine and the dimethylxanthines in 3% BSA and the percentage recovery calculated.

RESULTS

The chromatogram obtained following injection of a solution of authentic components in 3% BSA is illustrated in Fig. 1. Caffeine, β-hydroxyethyltheophylline (internal standard), theophylline, paraxanthine and theobromine are well separated producing sharp, symmetrical peaks with retention times of 12.75, 8.55, 7.05, 6.45 and 4.35 min, respectively. Fig. 2 is a typical chromatogram of extracted xanthine-free plasma obtained from an adult female volunteer after abstaining from caffeine-containing beverages for five days. This trace clearly indicates the absence of endogenous components which could interfere with the quantitation of caffeine and the dimethylxanthines. The chromatogram shown in Fig. 3 is representative of the many extracted cord plasma samples. Peaks with retention times corresponding to caffeine, theophylline, paraxanthine and theobromine together with the internal standard can be observed.

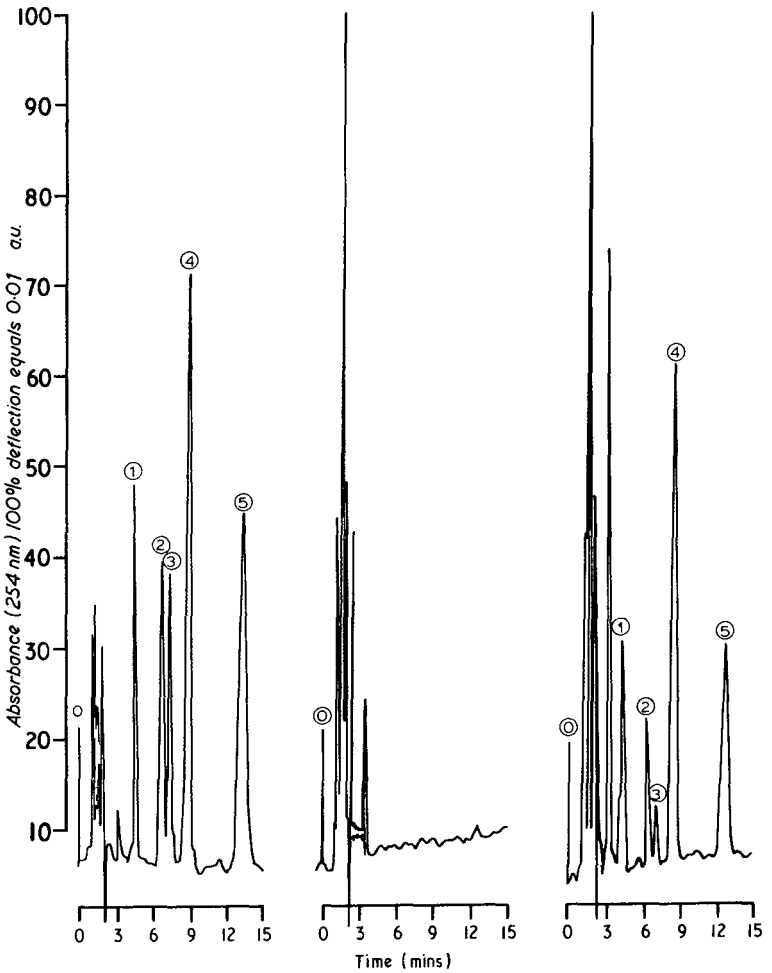

Fig. 1. Chromatogram of authentic components in 3% BSA. Peaks: 0 = injection; 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = β-hydroxyethyltheophylline (internal standard); 5 = caffeine.

Fig. 2. Chromatogram of extracted xanthine-free plasma obtained from an adult female volunteer after abstaining from caffeine-containing beverages for five days. Peaks: 0 = injection.

Fig. 3. Chromatogram of a typical extracted cord plasma sample obtained at delivery. Peaks: 0 = injection; 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = β-hydroxyethyltheophylline (internal standard); 5 = caffeine.

The adoption of 3% BSA as an alternative to plasma for calibration, recovery and storage studies was based on results (previously unpublished) obtained from comparisons using theophylline.

Theophylline was extracted from both plasma and 3% BSA at concentrations of 5 and 40 μg/ml. Recoveries of 91.85% and 93.01% with coefficients of variation (C.V.) of 9.66% and 6.85% were obtained for the low and high

TABLE II

EFFECTS OF STORAGE ON REPRODUCIBILITY OF MEASUREMENTS OBTAINED FROM SAMPLES PREPARED IN 3% BOVINE SERUM ALBUMIN

Samples stored at −20° C and assayed at weekly intervals up to six weeks followed by a final determination after six months.

Component	Concn. added (μg/ml)	Intra-batch variation*				Inter-batch variation**			
		Mean peak height ratio	Concn. determined	C.V. (%)	Recovery (%)	Mean peak height ratio	Concn. determined	C.V. (%)	Recovery (%)
Caffeine	2	0.36	1.90	3.10	95	0.35	1.85	9.83	92.5
	10	1.84	9.45	1.79	94.5	1.88	9.65	9.02	96.5
Theophylline	0.5	0.24	0.50	2.91	100	0.26	0.53	7.50	106
	5	2.44	5.0	1.60	100	2.49	5.1	4.63	102
Theobromine	0.5	0.33	0.505	9.82	101	0.33	0.505	7.10	101
	5	3.05	4.90	2.02	98	3.09	4.98	3.53	99.6
Paraxanthine	0.5	0.272	0.525	5.15	105	0.26	0.5	6.33	100
	5	2.45	4.85	1.03	97	2.49	4.93	3.44	98.6

*$n = 5$.
**$n = 11$.

plasma levels, respectively. Values for similar concentrations prepared in 3% BSA were 101.42% and 99.12% with respective coefficients of variation of 5.22% and 3.24%.

The effect of sample storage on reproducibility of results was also examined. Samples were prepared in both plasma and 3% BSA by adding aliquots of theophylline to give final concentrations of 5 and 40 μg/ml. These samples were stored at −20° C and assayed at weekly intervals for six weeks. The intra-assay results (i.e. replicate samples determined at zero time) gave mean plasma concentrations of 38.75 and 5.2 μg/ml with coefficients of variation of 1.46% and 2.61% for the high and low levels, respectively. Values obtained from equivalent samples prepared in 3% BSA were 40.25 and 5 μg/ml with respective coefficients of variation of 1.07% and 2.62%. The accumulated data obtained from the weekly analysis of replicate samples stored at −20° C over a period of six weeks were used to provide inter-assay results. The mean plasma concentrations were 39.5 and 5 μg/ml with coefficients of variation of 6.27% and 5.20% for the high and low levels, respectively. Similar sample concentrations made up in 3% BSA gave values of 40.75 and 5 μg/ml with respective coefficients of variation of 4.22% and 7.34%.

Calibration curves were obtained by comparing the peak height ratio (theophylline/internal standard) with the actual concentration of theophylline in spiked aliquots of plasma or 3% BSA. In both cases the relationship was linear over the concentration range 0—40 μg/ml. Slope values were 0.043 and 0.05 with correlation coefficients (r) of 0.984 and 0.993 for plasma and 3% BSA, respectively.

On comparison, the results obtained from these calibration, recovery and storage trials using theophylline samples prepared in both plasma and 3% BSA are very similar. This is a strong indication that, for the purposes specified above, 3% BSA is a suitable alternative to plasma. Since there is no reason to believe that caffeine or the other dimethylxanthines should behave differently, the assumption that 3% BSA can be used to obviate the need for xanthine-free plasma is not without foundation.

It is apparent from Table I that the extraction efficiency (in the region of 89 ± 7% for all components) and reproducibility (coefficients of variation $< 8\%$ in all cases) are independent of the xanthine concentration at the levels determined.

The effect of sample storage on reproducibility of results was also examined. Analyses were carried out on samples prepared in 3% BSA which concentrations were 0.5 and 5.0 μg/ml for the dimethylxanthines or 2 and 10 μg/ml in the case of caffeine. These samples were stored at −20° C and assayed at weekly intervals for six weeks followed by a final determination after six months. The findings (listed in Table II) indicate that intra-batch variation, i.e. the variation in results encountered when a batch of n replicate samples are assayed simultaneously on the same day, is generally very good (coefficients of variation $< 6\%$ with one exception). Furthermore, the inter-batch coefficients of variation are less than 10% for all the compounds measured. Clearly, with recoveries $> 92\%$ in all instances and good reproducibility (coefficients of variation $< 10\%$) for both fresh and stored samples, storage for up to six months at −20° C has had no serious adverse effect.

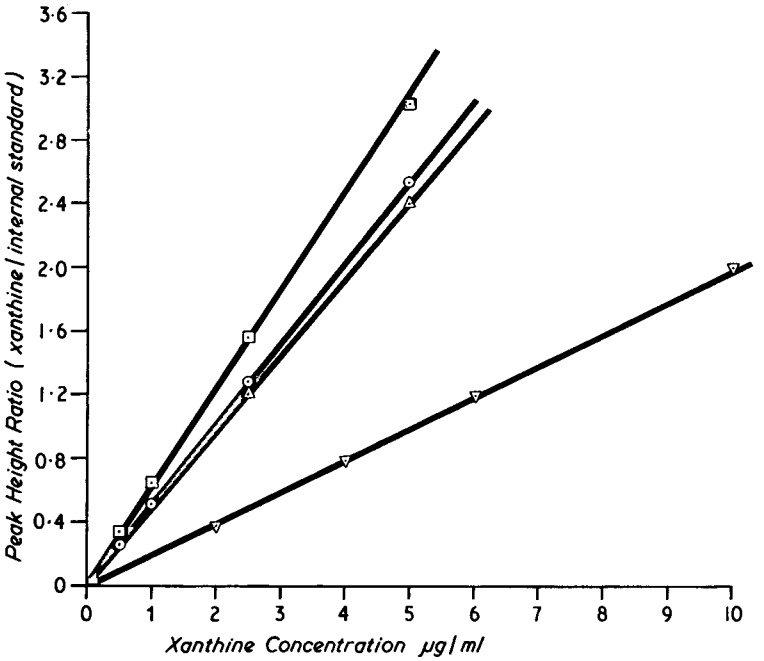

Fig. 4. Calibration graphs for caffeine and its N-demethylated metabolites. Samples prepared in 3% BSA. (▽) caffeine; (△) theophylline; (○) paraxanthine; (□) theobromine.

TABLE III

MEAN PLASMA CONCENTRATIONS OF CAFFEINE AND ITS N-DEMETHYLATED METABOLITES OBTAINED FROM UNSELECTED CORD BLOOD SAMPLES AT DELIVERY

Component	Mean concn.	S.D.	No. of patients (n)
Caffeine	2.85	2.309	113
Theobromine	1.05	1.253	111
Paraxanthine	0.67	0.476	113
Theophylline	0.287	0.174	113
Total (caffeine and dimethylxanthines)	5.146	3.227	111

Calibration curves were obtained by comparing the peak height ratio (xanthine/internal standard) with the actual concentration of xanthine in spiked aliquots of 3% BSA. The relationships were linear over the working range 0—5 µg/ml for the dimethylxanthines and 0—10 µg/ml in the case of caffeine as shown in Fig. 4. The correlation coefficients (r) and corresponding slope values are 0.98 and 0.197, 0.998 and 0.482, 0.999 and 0.501, 0.999 and 0.626 for caffeine, theophylline, paraxanthine and theobromine respectively (n = 24 in all cases).

The method was applied to measuring caffeine and its N-demethylated metabolites in 113 unselected cord blood samples obtained from The Leeds

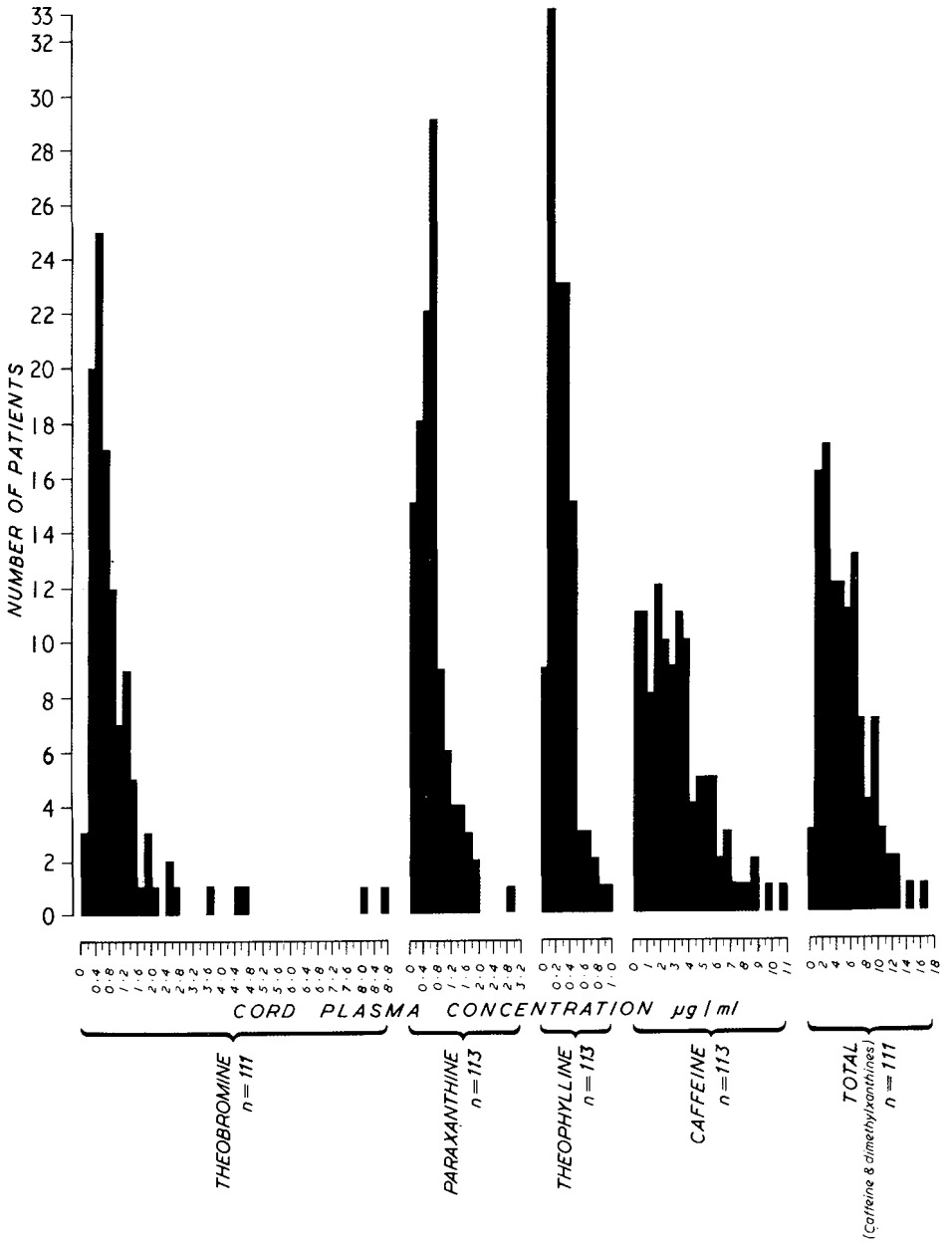

Fig. 5. Frequency distribution of cord plasma concentrations of caffeine and its N-demethylated metabolites among 113 unselected patients at delivery.

Maternity Hospital (Leeds, U.K.). The mean cord plasma concentrations were found to be 2.85, 0.29, 1.05, 0.67 and 5.15 µg/ml for caffeine, theophylline, theobromine, paraxanthine and total (caffeine + dimethylxanthines), respectively. These are summarised in Table III and the frequency distribution graphs for the individual components along with total levels are illustrated in Fig. 5.

DISCUSSION

In addition to the possibility of teratogenic effects [2], excessive caffeine ingestion has been associated with the development of cancers of the urinary tract [27, 28], coronary heart disease arising from hyperlipaemia [29] and anxiety states [30—32]. Clearly, caffeine is not the harmless substance once assumed and further investigation of its pharmacological and toxicological effects would seem justified. The method described is sensitive, selective, relatively quick and ideally suited to such investigations. The advantage of this method over earlier techniques [20—22] is readily apparent; by permitting the simultaneous determination of caffeine and all three N-demethylated metabolites in plasma, it provides additional information regarding circulating levels of pharmacologically active xanthines.

Comparison of the more recent HPLC assays [23—25] reveals similar analysis times (10—14 min) although completely different chromatographic conditions have been used to achieve separation. The technique employed by Van Aerde et al. [23] using normal-phase conditions provides the shortest analysis time (10 min), requires the smallest sample volume (100 μl of plasma) and will permit theophylline plasma concentrations as low as 200 ng/ml to be measured (value for caffeine not quoted). Tse and Szeto [24] used reversed-phase chromatography to achieve better sensitivity (100 ng/ml and 200 ng/ml for theophylline and caffeine, respectively) but with a much larger sample (500 μl of plasma) and a longer retention time (14 min). The ion-pair approach devised by Muir et al. [25] requires a similar sample volume to the previous method but the chromatographic separation is faster (10 min). Unfortunately, the detection limits of the assay were not indicated.

Although there is no improvement in the speed of the analysis, the present method is more sensitive than that of Tse and Szeto [24]. It is capable of detecting concentrations in the region of 100 ng/ml for caffeine and 50 ng/ml for the dimethylxanthines in only 300 μl of plasma compared with concentrations of 200 ng/ml and 100 ng/ml for caffeine and theophylline, respectively, in 500 μl of plasma using the latter technique.

There are probably numerous HPLC methods which may be suitable, or could be modified, for the purpose of simultaneously measuring caffeine and its N-demethylated metabolites in plasma. Many of these, however, have been primarily concerned with eliminating interferences in the estimation of individual xanthine components. The separation of paraxanthine from theophylline in biological fluids, for example, has attracted a lot of attention [26, 33—37]. Indeed, the present method and that of Tse and Szeto [24] are based on the conditions used by Miksic and Hodes [26]. The possibilities of some of these alternative methods are obviously limited; this is illustrated by the assay of Haughey et al. [38] for the determination of caffeine in plasma. Although the technique is very sensitive, separation of theophylline and paraxanthine is not achieved, making it unsuitable for the type of study reported here.

However, considering only those methods which are capable of measuring caffeine and its N-demethylated metabolites simultaneously [23—25], the present approach is clearly the most sensitive and has the additional advantage

of smaller sample requirement compared with the methods of Tse and Szeto [24] and Muir et al. [25].

This exploratory assessment of fetal exposure to caffeine and its N-demethylated metabolites in late pregnancy illustrates the clinical application of the technique. Mean cord plasma concentrations obtained from 113 unselected patients were 2.85, 0.29, 1.05, 0.67 and 5.15 μg/ml for caffeine, theophylline, theobromine, paraxanthine and total (caffeine and dimethylxanthines), respectively. The appreciably lower theophylline level probably reflects the lower dietary intake of this component compared with caffeine and theobromine [39]. The intermediate concentration established for paraxanthine supports an earlier observation that in human adults this is the major metabolite of caffeine [40].

On subjecting the data to linear-regression analysis, significant correlations between cord plasma concentrations of caffeine, theophylline and paraxanthine (at the 99% level of significance) were obtained. This seems to confirm that both dimethylxanthines are principally derived from the metabolism of caffeine [40]. Theobromine, however, only correlates with caffeine and paraxanthine at the 95% significance level and even less with theophylline, although a positive coefficient is still evident. This may be interpreted as an indication that the primary source of theobromine is dietary rather than metabolic and, since the major metabolites of caffeine in man are considered to be paraxanthine and theophylline [40], the comparatively higher mean plasma level of theobromine supports this view.

The mean caffeine level established in this study is approximately double previously published values found in Vermont and Marburg [13] and in Montreal [14] but compares favourably with that obtained in a London survey [15]. The latter study, which included an assessment of caffeine intake during pregnancy, showed that the daily consumption in pregnant British women was twice the value reported by Graham [39] in a survey of pregnant American women. The difference in mean cord plasma levels between the North American studies and the values obtained in England may be due, therefore, to national differences in dietary intake of caffeine during pregnancy.

These findings suggest that in Britain fetal exposure to caffeine and the pharmacologically active dimethylxanthine components may be much higher than previously indicated [13—15]. In Leeds, 46% of the babies were exposed to plasma levels in excess of the minimum concentration for respiratory stimulation by caffeine, which is 3 μg/ml, whilst total (caffeine and dimethylxanthine) plasma values exceeded this level in 67.6% of the cases. The significance of these elevated levels of circulating methylxanthines, in terms of effects on the outcome of the pregnancy, are unclear since they may not be associated with any adverse effects.

However, this technique is being used in continuing studies regarding fetal exposure to caffeine and its N-demethylated metabolites in late pregnancy in an attempt to identify relationships with parameters used to measure the successful outcome of pregnancy, e.g. birth weight, apgar score etc. These results will be reported on completion of the study.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POLAROGRAPHIC AND VOLTAMMETRIC ANODIC DETECTION: SIMULTANEOUS DETERMINATION OF ALLOPURINOL, OXIPURINOL AND URIC ACID IN BODY FLUIDS

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SUMMARY

Allopurinol, oxipurinol and uric acid have been determined in human serum and urine by liquid chromatography with electrochemical detection. In particular the use of a polarographic detector operating in the oxidative mode, whose principle of detection is based on the property of allopurinol, oxipurinol and uric acid to form insoluble anodic films on mercury, is described. The performance of such a detector is compared with that of a glassy carbon wall-jet detector. Different procedures for sample pretreatment have been evaluated.

INTRODUCTION

Allopurinol (1H-pyrazolo[3,4-*d*]pyrimidin-4-ol) and its major active metabolite oxipurinol (1H-pyrazolo[3,4-*d*]pyrimidin-4,6-diol) are powerful xanthine oxidase inhibitors [1] extensively used in the treatment of purine (and pyrimidine) metabolic disorders which lead to the formation of an excess of uric acid in humans.

Procedures capable of detecting simultaneously the primary drug (allopurinol), its metabolite (oxipurinol) and uric acid (effect) in body fluids are of considerable interest for pharmacokinetics and/or clinical studies. Several high-performance liquid chromatographic (HPLC) methods have been devised for the determination of allopurinol and oxipurinol in body fluids: reversed-phase HPLC [2], ion-exchange HPLC [3] and ion-exchange HPLC in combination with purification on Chelex-100 resin [4]. At the same time, several HPLC methods are available for the determination of uric acid separately [5—7] or simultaneously [8] with other pyrimidines and purines. Recently

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an HPLC method (with UV detection at 254 nm) for the simultaneous determination of allopurinol, oxipurinol and uric acid in human plasma has been reported by Nissen [9]. However, unidentified compounds were found to interfere with allopurinol and/or oxipurinol in six of eleven blank plasma samples taken from different subjects. Moreover, since no sample pretreatment was adopted the renewal of the precolumn after 50—70 applications was necessary because of clogging.

We have recently undertaken [10] an electrochemical study on the anodic behaviour of allopurinol and oxipurinol on mercury and glassy carbon electrodes. Both compounds (and uric acid) can be electrooxidized on glassy carbon; at mercury electrodes they give rise to anodic waves due to the formation of sparingly soluble compounds with mercury. This latter peculiarity is of a certain interest from an analytical point of view since it permits the use of highly sensitive electrochemical techniques such as differential pulse cathodic stripping voltametry (DPCSV). However, in spite of the great sensitivity of DPCSV (detection limits typically in the order of 10^{-9} mol/l), the direct determination of the investigated drugs in complex matrices such as body fluids is not possible owing to severe interference effects. Evidently electrochemical methods of analysis could be employed only if coupled to a separating technique such as liquid chromatography.

In this paper we wish to report an HPLC method for the simultaneous determination of allopurinol, oxipurinol and uric acid in both human serum and urine, which makes use of a polarographic (dropping mercury electrode) detector operating in the oxidative mode; the detection principle is based on the peculiar anodic behaviour shown by these compounds (e.g. the formation of an insoluble anodic film). The use of a glassy carbon electrochemical detector (wall jet type) is also described and the relative merits of both detectors are discussed. Different procedures for sample pretreatment have been also evaluated.

EXPERIMENTAL

Apparatus

A Perkin Elmer Model 3B pump module equipped with a Rheodyne 7125 injector and a reversed-phase column (Perkin Elmer RP-8, 10 μm, 250 × 4.6 mm) was used as the chromatographic system. When necessary a Brownlee RP-GU guard cartridge (30 × 4.6 mm), fitted into a Brownlee MPLC holder, was used to protect the analytical column.

A home-made pulse dampener (consisting of an air damping device and a Bourdon tube both in a tee configuration) was placed between the pump outlet and the injector to ensure a pulseless delivery of the chromatographic eluate to the flow-sensitive electrochemical detectors.

A PAR Model 310 detector (EG&G, Princeton Applied Research, Princeton, NJ. U.S.A.) was used as polarographic detector. The previously described [10] glassy carbon wall jet electrode was used as voltammetric detector. Both detectors, operating in the amperometric mode, were controlled by a PAR 174 A polarographic analyser.

Materials

Solvents used were HPLC grade (Carlo Erba). Allopurinol, oxipurinol and uric acid were obtained from Sigma. All the other chemicals were analytical reagent grade.

The phosphate buffer (pH 6.1, 0.025 *M*) used in the mobile phase was filtered through a 0.45 μm membrane before use.

Chromatographic conditions

The mobile phase was a 0.025 *M* phosphate buffer pH 6.1 with 6—8% of methanol added unless otherwise specified. It was degassed, before use, by conventional methods.

The flow-rate was normally 1.5 ml/min and the injection volume 20 μl. All separations were run at room temperature. Quantitations were done by an external standard method. This choice was conditioned by the fact that it was not easy to find a compound with the same polarographic behaviour as the studied drugs that could act as internal standard.

Sample preparation

A sample clean-up procedure similar to that described in ref. 11 for seven model drugs has been found to work satisfactorily in the present case.

Urine. A 2-ml volume of urine was mixed with 0.1 ml of 15% (w/w) zinc sulphate, 0.4 ml of saturated barium hydroxide and 2 ml of methanol. After each reagent addition the sample was vortex-mixed for a few minutes and was finally centrifuged at about 1200 *g* for 5 min. The supernatant was diluted, as necessary, with the mobile phase and injected. (Unless otherwise specified the final dilution ratios were 1:40 for voltammetric detection and 1:20 for polarographic detection.) Alternatively, urine could be injected directly into the chromatographic system after dilution with the mobile phase. In this case, however, the use of a precolumn is recommended to prolong the life of the analytical column.

Serum (or plasma). To 1 ml of serum the following reagents were added in sequence: 0.1 ml of 15% (w/w) zinc sulphate, 0.2 ml of saturated barium hydroxide, 0.1 ml of 0.5 mol/l phosphate buffer pH 6.1, 1 ml of methanol. After each addition the sample was briefly vortex-mixed and finally centrifuged at approximately 1200 *g* for 5 min. The supernatant was ready for direct chromatographic analysis. Alternatively (e.g. when a preconcentration of the sample was required) the following procedure was adopted: 0.5 ml of serum was extracted twice with 5 ml of diethyl ether—isopropanol (6:1, v/v) mixture. The combined extracts were concentrated to dryness, reconstituted with 50 μl of mobile phase and 20 μl were injected directly.

RESULTS AND DISCUSSION

HPLC with electrochemical detection

Allopurinol, oxipurinol and uric acid give rise [10] to anodic waves at mercury electrodes due to a depolarization effect on the mercury oxidation caused by the formation of sparingly soluble compounds with mercury ions. This peculiarity can permit the use of an HPLC polarographic detector operat-

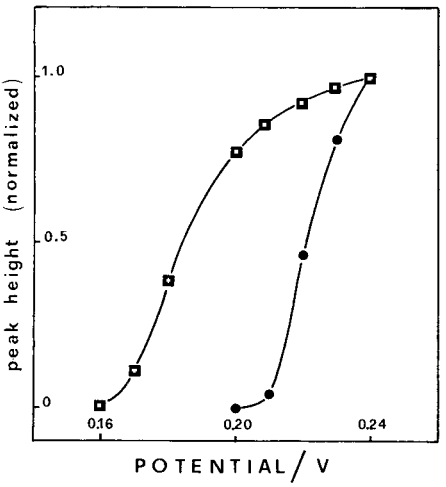

Fig. 1. Current—potential profiles (hydrodynamic conditions) for allopurinol (160 ng) and oxipurinol (220 ng). Injected volume, 20 μl; column, C_8 10 μm (250 × 4.6 mm I.D.); mobile phase, phosphate buffer—methanol (92:8); drop time, 1 sec; flow rate, 1.5 ml/min; detection mode, sampled d.c.

ing in the oxidative mode. In this way the unique advantage of a polarographic detector, i.e. the continuously renewed electrode surface, can be coupled to the main advantage of the oxidative operating mode, i.e. no requirement for the deoxygenation of the mobile phase (the main deterrent in using an electrochemical detector in the reductive mode).

The current—potential profile for allopurinol and oxipurinol obtained at a dropping mercury electrode detector in hydrodynamic conditions is shown in Fig. 1. An applied potential higher than +0.245 V caused an abnormal increase of the noise, which lead to a decrease of the signal-to-noise (S/N) ratio; the peak currents were then normalized to the value obtained at +0.24 V which represents the optimum value for the applied potential. From Fig. 1 it can be seen that, if desired, oxipurinol may be selectively determined by setting the potential at +0.20 V with only a 20% decrease in sensitivity. This kind of selectivity can be rarely obtained with other detectors such as the UV detector.

Detection limits calculated at S/N = 2 (in the following experimental conditions: flow-rate 1.5 ml/min; drop time 1 sec; drop size setting at the PAR 310 "small"; applied potential +0.24 V vs. Ag/AgCl) are around 0.8 ng. This value can be lowered by a factor of approximately 2 by using a mercury drop of higher area (drop size setting "medium").

Calibration plots, generated in the range 2—2000 ng, were found to be linear with regression coefficients higher than 0.999 for both compounds.

Allopurinol and oxipurinol were both found [10] to be electroactive on the glassy carbon electrode and a mechanistic pathway for their oxidation has been suggested. At the same time it is known that uric acid can be easily oxidized on glassy carbon and can be determined by HPLC with electrochemical detection [6].

The analytical applications of an HPLC method with voltammetric and/or polarographic detection to the analysis of real matrices are described below.

Analysis of urine and serum samples

The procedure described in the experimental section for the deproteinization of urine and serum has been found effective, simple and inexpensive. The resultant supernatant is free of visible residues and both types of sample can be analyzed without a guard column. No change in the performance or in the working pressure of the column has been observed in the course of the present work.

The recovery of the drugs under examination increases on increasing the quantity of methanol added to the sample until a plateau is reached. Fig. 2 illustrates the pattern trend in urine. In this case recoveries were determined on urine containing added allopurinol and oxipurinol, by comparison of the chromatograms obtained on treated and untreated urine aliquots both at the same final dilution ratio (1:20) obtained by adding mobile phase. The recovery from serum was estimated on spiked samples by direct comparison with drug standards. Blank (control) samples of serum and urine taken from different subjects showed no interference from endogenous compounds for either allopurinol or oxipurinol. Other drugs such as probenecide and phenylbutazone, which gouty patients could take concurrently with allpurinol, cannot interfere with the present assay because of their electrochemical inactivity. Theobromine and theophylline which are voltammetrically, but not polarographically, electroactive were also found not to interfere.

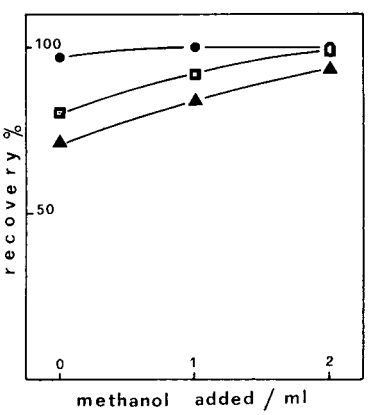

Fig. 2. Recovery of the studied compounds from urine as a function of the quantity of methanol added. The volume of treated urine is 2 ml; pretreatment was as described in the experimental section. (■), oxipurinol; (●), allopurinol; (▲), uric acid.

Figs. 3 and 4 show typical chromatograms of urine samples, taken from a patient under treatment with 300 mg/day of allopurinol, recorded using a voltammetric detector and a polarographic detector, respectively. Under the given experimental conditions the polarographic detector appears more selective in particular for the first part of the chromatogram where the uric acid peak (retention time ca. 2.8 min) is eluted. Moreover, in the case of polarographic detection, no major peak appears after the elution of allopurinol while in the case of voltammetric detection the last eluting peak occurs at about 19.5 min.

Improved resolution of the uric acid peak (in the case of voltammetric

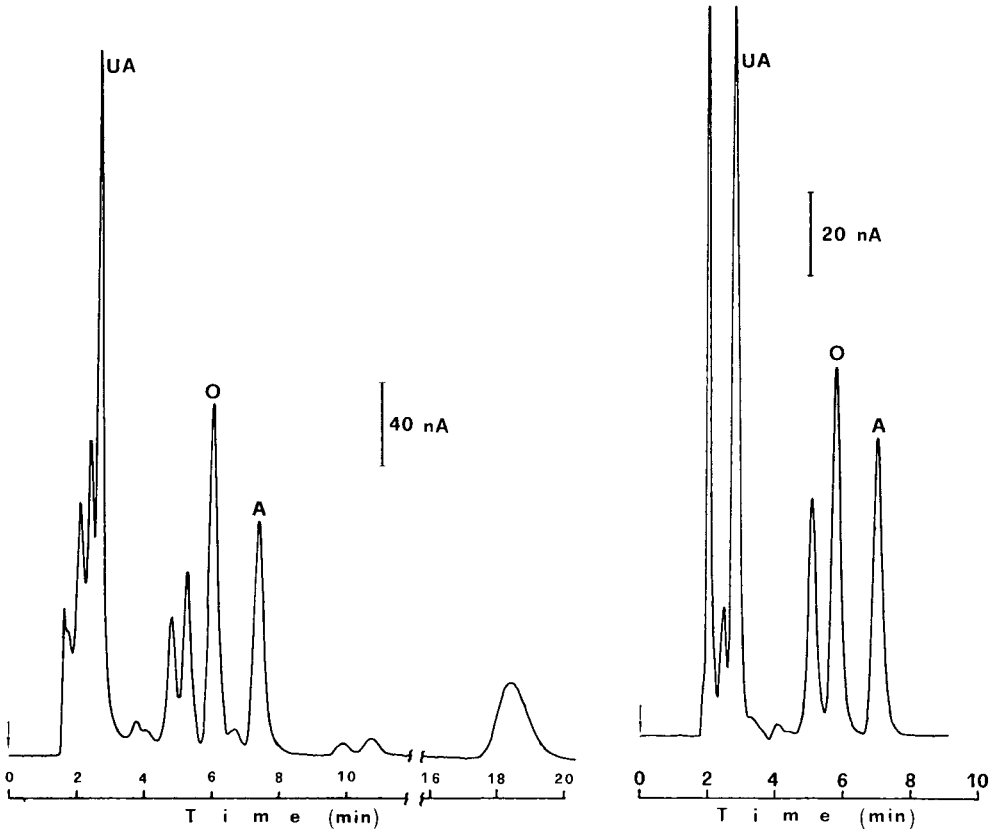

Fig. 3. Chromatogram of a urine sample from a patient under treatment with 300 mg/day of allopurinol (the final dilution ratio of urine was 1:40). Glassy carbon wall jet detector operating at +1.2 V vs. SCE in the d.c. mode. Mobile phase, phosphate buffer—methanol (94:6); flow-rate, 1.5 ml/min. UA = uric acid; O = oxipurinol; A = allopurinol.

Fig. 4. Chromatogram of a urine sample from a patient under treatment with 300 mg/day of allopurinol obtained by a polarographic detector operating in the oxidative mode (the final dilution ratio of urine was 1:20). Experimental conditions: applied potential, +0.24 V vs. Ag/AgCl; other conditions as in Fig. 1. Peak notation as in Fig. 3.

detection) can be achieved simply by changing the applied potential. Profile A in Fig. 5, for example, shows how the chromatogram of Fig. 3 changes when the applied potential is reduced from +1.2 V to +0.7 V vs. standard calomel electrode (SCE). The uric acid peak is now completely resolved but, at the same time, any signal of allopurinol and oxipurinol is completely lost. Intermediate potential values gave a different degree of selectivity: for example, at +1.0 V uric acid and oxipurinol were determined simultaneously. Of course the simultaneous determination of allopurinol, oxipurinol and uric acid in urine by HPLC—voltammetric detection can be obtained by the most suitable choice of the mobile phase (e.g. using an ion-pair agent) which in the present case has been optimized for polarographic detection. Alternatively, a dual-electrode (parallel configuration) cell could be used in which the first electrode is held a +0.7 V (uric acid) and the second at +1.2 V (allopurinol and oxipurinol).

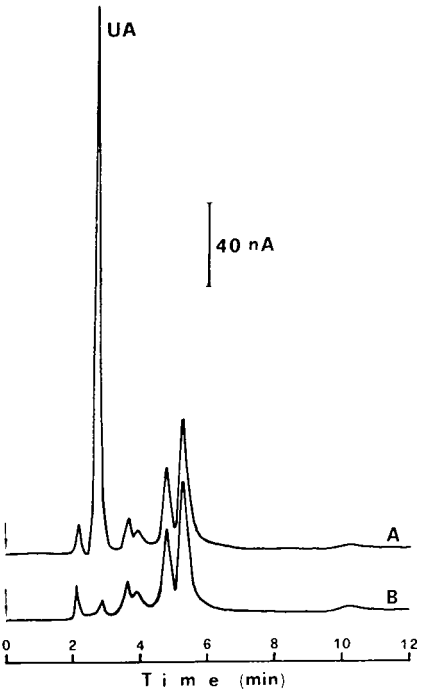

Fig. 5. (A) Chromatogram obtained in the same conditions as Fig. 4 except for the applied potential which, in the present case, is +0.7 V vs. SCE. (B) Chromatogram obtained in the same conditions as chromatogram A on a urine sample treated, before the deproteinization step, with the enzyme uricase.

Since uric acid is an endogenous component of human body fluids it was necessary to ascertain the purity of the relevant chromatographic peak (for both detection methods); this was simply done by treating the sample, before the deproteinization step, with the enzyme uricase. After this treatment a profile such as B in Fig. 5 was obtained which shows a nearly flat baseline at the retention time of uric acid. In addition, the urinary, or serum (see later), uric acid concentrations obtained with the HPLC method were found to be in agreement (within ± 8%) with those obtained by a conventional enzymatic method [12]. Figs. 6 and 7 show typical chromatograms relevant to serum samples containing allopurinol and oxipurinol recorded with a polarographic and voltammetric detector, respectively. Again the polarographic detector appears more specific even if, in this case, the uric acid peak appears well resolved also on the voltammetric detector response.

To evaluate precision known amounts (2—10 μg/ml) of allopurinol and oxipurinol were added to urine and serum samples which were then treated as described in the experimental section. The coefficient of variation observed within-day for both compounds in serum and urine ranged from 3% to 4%; the day-to-day precision was in the range 3.5—5%.

Detection limits for allopurinol and oxipurinol with both methods of detection and for both matrices were found to be practically the same: ca. 0.2 μg/ml. This value is well below the usual serum [2] or urinary concentrations of the studied drugs associated with the usual therapeutic doses.

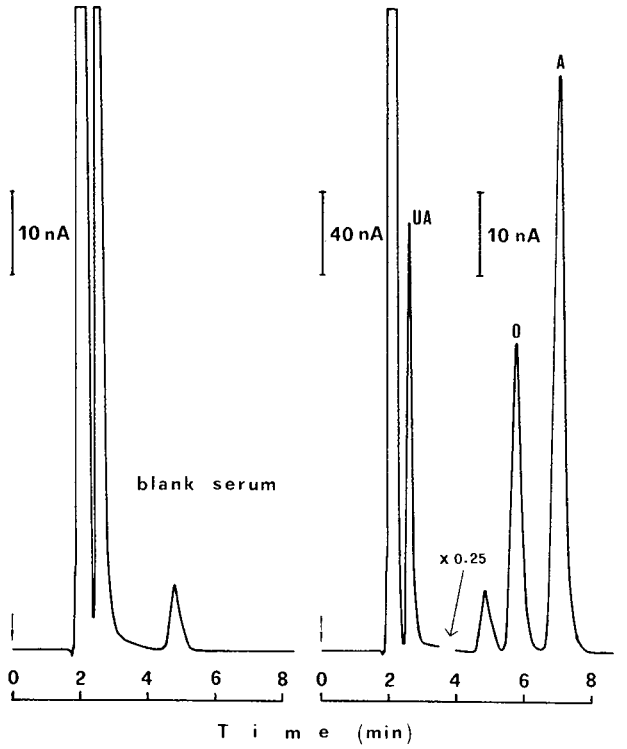

Fig. 6. Chromatograms relevant to a blank serum sample (left) and to a serum sample with added oxipurinol and allopurinol (right). Polarographic detector. Experimental conditions as in Fig. 5. Peak notation as in Fig. 3. The arrow at about 3.5 min indicates a sensitivity change. Injected quantities: allopurinol 120 ng; oxipurinol 80 ng.

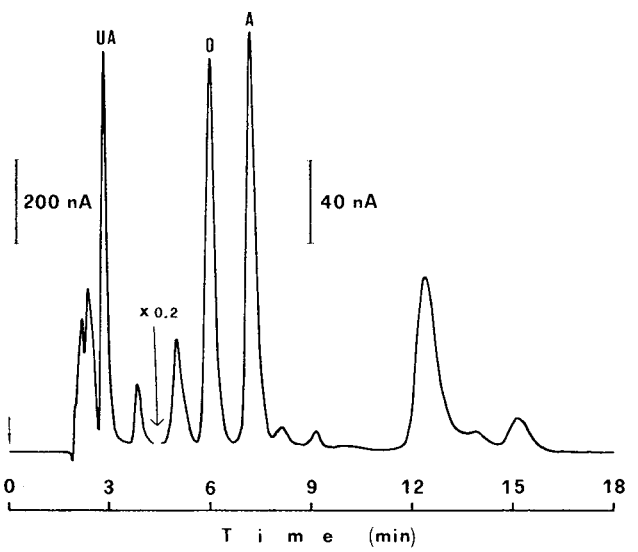

Fig. 7. Chromatogram of a serum sample with added oxipurinol and allopurinol. Voltammetric detector. Experimental conditions and peak notation as in Fig. 3. The arrow at about 4 min indicates a sensitivity change. Injected quantities: allopurinol 130 ng; oxipurinol 138 ng.

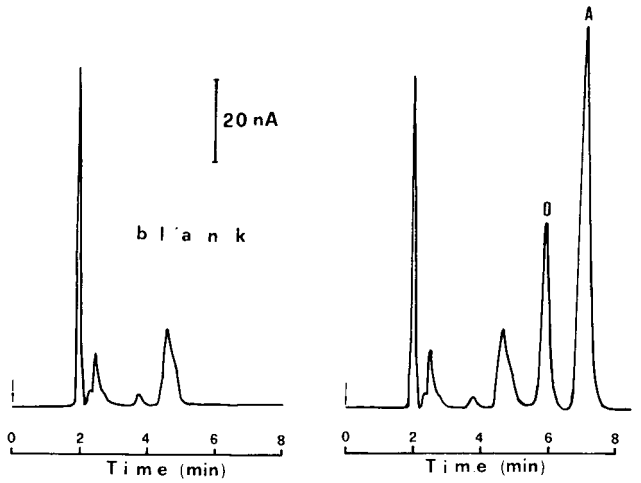

Fig. 8. Chromatograms relevant to serum extracts. Polarographic detector. Left: chromatogram relevant to the extract from a blank serum sample. Right: chromatogram relevant to the extract from a serum sample containing 0.45 μg/ml of oxipurinol and 0.7 μg/ml allopurinol. Experimental conditions and peak notation as in Fig. 6.

However, if desired, the previously reported detection limits can be lowered by about one order of magnitude (i.e. to about 10—20 ng/ml) by means of the extraction procedure described in the experimental section. This procedure has been evaluated only for serum because it presents a lower concentration of the drugs (with respect to urine) and is more important from a clinical point of view. Fig. 8 shows typical chromatograms (polarographic detector) relevant to an extract from a serum blank sample and from a serum sample containing 0.45 μg/ml of oxipurinol and 0.7 μg/ml of allopurinol. The recovery of the proposed procedure (estimated at the above concentration levels by direct comparison to standards that were not extracted) was found to be quite satisfactory, for both compounds being 95 ± 4%.

CONCLUSION

The results presented in this paper clearly indicate that a polarographic detector can be successfully employed in the oxidative mode for those compounds which form insoluble salts with mercury. The detector appears even more specific than a voltammetric detector and possesses the unique peculiarity of a continuously renewable electrode surface. The only disadvantage is perhaps represented by the necessity of handling mercury which could discourage some potential users. It is interesting to note that an anodic behaviour similar to that of allopurinol, oxipurinol and uric acid is presented [13] by a considerably large number of compounds of biological or pharmaceutical interest such as most purines and pyrimidines, thiocompounds (like cysteine, cystine, glutathione, thiobarbiturates, etc.), sulphadrugs, porphyrins and so on. A possible renewed interest could be given to polarographic detectors (whose main use has been, up to now, for reducible compounds) since the detection mode presented here can extend considerably the utilization of mercury in

In this laboratory a study is in progress [14] dealing with the detection of antineoplastic agents such as 5-fluoro- and 5-bromouracil, methotrexate, 5-mercaptopurine and 2-thioguanine.

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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF URINARY PARACETAMOL METABOLITES USING RADIALLY COMPRESSED COLUMNS

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SUMMARY

Methods have been adapted for the high-performance liquid chromatographic (HPLC) analysis of urinary paracetamol metabolites on radial compression columns. Enhanced resolution and decreased analysis time were two major advances. Various modifications to existing methods were made to counter the effect of the different C18 surface. Thus in ion suppression HPLC the addition of triethylamine at pH 3.0 (phosphate buffer) was necessary to block residual hydroxyl sites, while in ion-pair HPLC a higher tetrabutylammonium hydroxide concentration of 0.01 *M* at pH 5.0 was used to enhance selectivity. The methods were successfully applied to the study of the metabolism of paracetamol, its glutathione conjugate and 3-thiomethylparacetamol in Sprague—Dawley rats. 3-Thiomethylparacetamol sulphoxide and its glucuronide and sulphate conjugates were shown to be metabolites of both 3-thiomethylparacetamol and paracetamol. 3-Thiomethylparacetamol sulphate was unresolved from the sulphates of paracetamol and 3-methoxyparacetamol in ion-pair HPLC. This raises a previously unrecognised problem in which the peak normally attributed to paracetamol sulphate contains metabolites arising from an oxidative metabolic pathway. Elevated levels of 3-methoxyparacetamol conjugates were found in human overdose urine and to some extent in analgesic nephropathy.

INTRODUCTION

There has been great interest over the past few years in the development of chromatographic methods capable of separating paracetamol and its metabolites [1—4]. As methods have become more refined it has become possible to separate previously unidentified metabolites such as the conjugates of 3-thiomethylparacetamol [5], a metabolite which has previously been detected only by following enzymatic hydrolysis of urine [6]. The same workers [6] have unequivocally identified 3-thiomethylparacetamol sulphoxide

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as a further metabolite, presumably present in urine in conjugated form, although no high-performance liquid chromatographic (HPLC) analysis has been presented to date. Wilson et al. [7] have recently reported on the use of electrochemical detection for paracetamol and its major metabolites, the glucuronide and sulphate, as well as the unconjugated 3-methoxy-, 3-hydroxy- and 3-thiomethylparacetamols following enzymatic hydrolysis of urine containing conjugates of all these.

We have previously discussed the development of ion suppression and ion-pairing techniques for reversed-phase HPLC of urinary paracetamol [1]. Using stainless-steel columns packed with 10-μm particles of C18 reversed-phase silica, optimum solvent systems were developed with the emphasis on speed for the isocratic ion suppression and maximum resolution for the gradient programmed ion-pair methods, respectively. Regrettably the benefits of both high resolution and short analysis time (< 20 min) were not available within the one separation.

The introduction of radial compression technology [8] suggested that the same separations could be achieved quickly without any sacrifice in resolution. There was an immediate problem however; the packing in conventional stainless-steel columns is usually treated in situ with trimethylchlorosilane to cap residual silica hydroxyls on the C18 silica whereas this treatment is omitted for Radial Pak™ columns. Furthermore the percentage of C18 coating of the silica used for Radial Pak columns is greater than that used for stainless-steel columns which leads to increased k' values. Thus solvent systems have to be adapted to the different C18 surface and reoptimized to obtain a similar separation. Two such modifications are described in this paper.

The methods were applied in the continuing study of the metabolism of paracetamol and of 3-thiomethylparacetamol and paracetamol glutathione conjugate, and in the course of their development 3-thiomethylparacetamol sulphoxide and its glucuronide and sulphate conjugates were detected and identified as metabolites of paracetamol.

EXPERIMENTAL

Apparatus

A Spectra-Physics 3500B dual-pump liquid chromatograph fitted with a solvent programmer, a Waters Assoc. U6K loop injector and Model 440 dual-channel UV detector (filters 254 and 280 nm) were used for all HPLC analyses. Waters Assoc. Rad-Pak A columns were used (10 cm × 8 mm I.D., particle size 10 μm) and contained unsilanized C18 packing. These were operated in a Waters Assoc. radial compression module (RCM). Conventional stainless-steel μBondapak C18 columns (30 cm × 3.9 mm I.D., particle size 10 μm; Waters Assoc.) were used for comparison of selectivity and efficiency.

Solvents

Distilled water purified through a Milli Q ion-exchange system (Millipore) was obtained from the Marine Chemistry Laboratory (University of Melbourne, Australia). Methanol (AR) was used without further treatment.

Mobile phases

Water—methanol (1:1) was used to establish retentions of a test mixture of non-ionized paracetamol derivatives on both column types.

Ion suppression solvents

Initially 15% methanol in 0.05 *M* potassium phosphate buffer at pH 2.3 was used on Rad-Pak A columns. Subsequent modifications involved the addition of triethylamine (TEA) (AR, Aldrich, Milwaukee, WI, U.S.A.) at concentrations of 0.005 *M* and adjustment of pH with H_3PO_4 or KOH maintaining the phosphate concentration at 0.05 *M*.

Ion-pair solvents

An aqueous solution of tetrabutylammonium (TBA) hydroxide (0.4 *M* solution, Eastman-Kodak, Rochester, NY, U.S.A.) was diluted to 0.005 *M* or 0.01 *M* in water or water—methanol (1:1). The pH was adjusted with H_3PO_4 or with Tris (tris-hydroxymethylaminomethylaminomethane) (Sigma, St. Louis, MO, U.S.A.) and H_3PO_4. In some cases ethylenediaminotetraacetic acid disodium salt (EDTA) was added to the eluent at a concentration of 0.005 *M*. Gradient programming was used to enhance resolution. Details of gradient programming on μBondapak C18 columns have been given previously [1]. Program times on Rad-Pak A columns were generally shorter as a result of faster flow-rates on these columns. Typically, program times of ca. 9–12 min were used.

Standard compounds

Paracetamol was obtained from Aldrich. 3-Methylcholanthrene (3MC) was purchased from Sigma. 3-Methoxyparacetamol (3OMeP) was synthesised in our laboratory. 3-Thiomethylparacetamol (3SMeP) was prepared synthetically [5] and contained no trace of paracetamol by HPLC. Paracetamol 3-glutathione conjugate [5-(5′-acetamido-2′-hydroxyphenyl)-L-glutathione (PSG)] was synthesised by the general method of nucleophilic addition of a sulphhydryl group, in this case L-glutathione, to the quinoneimine of paracetamol [9,10]. Paracetamol 3-cysteinylglycine conjugate [3-(5-acetamido-2-hydroxyphenylthio)alanylglycine) (PCG)] was prepared analogously to the PSG conjugate [10].

Urine samples

These were obtained from Sprague—Dawley albino rats following administration of paracetamol, 3-thiomethylparacetamol, 3-methoxyparacetamol or synthesised paracetamol glutathione conjugate. Details of the method of administration and urine collection have been given elsewhere [11]. In some cases rats were pre-treated with 3-methylcholanthrene, a known cytochrome P450 mixed function oxidase inducer, for two days prior to administration of paracetamol. Samples of human urine containing metabolites from a paracetamol overdose were kindly supplied by Dr. G. Duggin, Royal Prince Alfred Hospital, Sydney, Australia. A sample of human urine containing metabolites of paracetamol from a patient with suspected analgesic nephropathy was kindly supplied by St. Vincent's Hospital, Melbourne,

Australia. Urine from a healthy person was collected following the ingestion of a normal therapeutic dose of paracetamol (1 g). Urine samples were filtered through 0.5-μm cellulose filters (Millipore, Bedford, MA, U.S.A.) following the addition of methanol up to 20%. Filtered urine samples were kept frozen at −20°C in acid-washed screw-capped vials until ready for analysis.

Perfusion urines were obtained by techniques previously described [10—13] where paracetamol glutathione conjugate was added to the perfusion medium at a concentration of 1.6 m*M*. Perfusion urines were injected without further treatment onto the HPLC column because of their small volumes.

Bile duct cannulation

Female Sprague—Dawley rats (250—300 g) were anaesthetised with diethyl ether. A midline incision was made and the right kidney was exposed. The ureter was cannulated (Portex tubing, size PP10) and the tubing held with two ligatures around the ureter. The common bile duct was then exposed and cannulated. The incision was closed and the animals placed in restraining cages. A single dose of paracetamol (15.0 mmol/kg) was administered as a slurry (15 g per 100 ml) by oral gavage.

Samples of urine and bile were collected with a fraction collection at 2-h intervals over 24 h and immediately frozen. Animals were then killed. The urine and bile were analysed for paracetamol metabolites.

RESULTS AND DISCUSSION

k' Values

Apart from the obvious mechanical differences in using radially compressed columns, the packing presents major differences by comparison with μBondapak C18 columns. The higher percentage C18 loading on the particles causes difficulty in wetting and thus it is necessary to first treat the column thoroughly with methanol before lowering the methanol concentration. With a given solvent composition it is found that k' values on the Rad-Pak A columns are greater than on μBondapak C18 columns as illustrated by paracetamol,

TABLE I

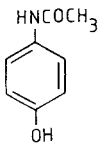
COMPARISON OF k' VALUES AND EFFICIENCIES ON μBONDAPAK C18 AND RADIAL PAK COLUMNS

Solvent: methanol—water (1:1).

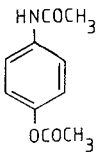
Solute	μBondapak C18			Rad-Pak A				
	Flow-rate			Flow-rate				
	2 ml/min			2 ml/min			4 ml/min	
	k'	Nf	Nf/t*	k'	Nf	Nf/t	Nf	Nf/t
Phenacetin	1.79	1069	4.6	2.78	1138	3.6	807	5.5
Paracetamol acetate	0.87	421	2.7	1.26	653	3.4	373	4.2
Paracetamol	0.14	–	–	0.29	–	–	–	–

*Nf/t = number of effective plates per second.

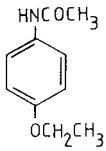
its O-acetate and phenacetin which provide a convenient series of non-ionic compounds of decreasing polarity to monitor k' values in water–methanol mixtures (Table I).



Paracetamol



Paracetamol acetate



Phenacetin

Likewise in a buffered solvent system containing 15% methanol the retention of paracetamol was greater on the Rad-Pak A column (Table II).

TABLE II

COMPARISON OF k' VALUES AND EFFICIENCIES ON μBONDAPAK C18 AND RADIAL PAK COLUMNS

Solvent: 15% methanol in 0.05 *M* potassium phosphate buffer, pH 2.8.

Solute	μBondapak C18 (2 ml/min)		Rad-Pak A (4 ml/min)	
	k'	Nf	k'	Nf
Paracetamol	1.85	985	4.1	2309

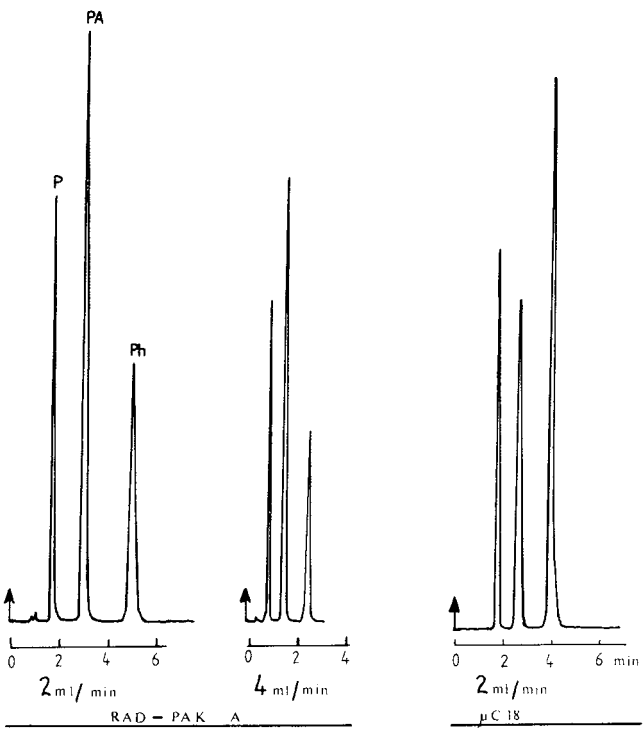


Fig. 1. Resolution of the test mixture, paracetamol (P), paracetamol acetate (PA) and phenacetin (Ph) on Rad-Pak A and μBondapak C18 columns using methanol–water (1:1) at the flow-rates indicated.

Efficiency

Tables I and II also list the efficiency of each column at different flow-rates. The effective number of plates per column (Nf) is calculated as $5.54 \times \left[\frac{t_r - t_0}{W_{\frac{1}{2}h}}\right]^2$. At a flow-rate of 2 ml/min on both columns (this represents a lower linear flow on the 8-mm I.D. Rad-Pak A column) similar efficiencies are obtained for a given k' value and the elution time for a given compound is approximately the same, e.g. 5 min for phenacetin. As the flow-rate on the Rad-Pak A column is increased to 4 ml/min the elution time is halved and although the efficiency drops off it is still competitive with conventional columns at 2 ml/min for peaks where k' is greater than 2. It is not possible to increase the flow-rate on μBondapak C18 columns much past 2 ml/min because the pressure drop becomes excessive. Fig. 1 shows the resolution of the test mixture on the two columns at different flow-rates. It is possible to achieve greater resolution on both the μBondapak C18 columns and Rad-Pak A columns at low flow-rates but such separations would be unnecessarily long.

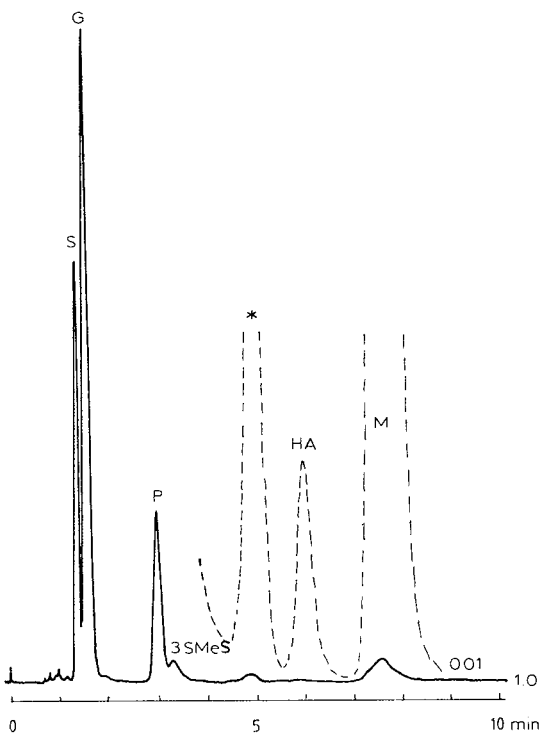


Fig. 2. Ion suppression HPLC of paracetamol metabolites on a Rad-Pak A column. Eluent, 15% methanol in 0.05 *M* phosphate buffer, pH 2.3; flow-rate, 4 ml/min; detector 254 nm at two sensitivities as indicated. Peaks: S = paracetamol sulphate; G = paracetamol glucuronide; P = paracetamol; 3SMeS = 3-thiomethylparacetamol sulphate; HA = hippuric acid (endogenous urine constituent); M = paracetamol mercapturic acid; (*) composite peak subsequently identified as a mixture of 3-thiomethylparacetamol sulphoxide and other metabolites (see text).

Analysis of urinary paracetamol metabolites by ion suppression HPLC

The direct application of the ion suppression system devised for μBondapak C18 columns [1] to a Rad-Pak A column gave a different result (Fig. 2). Sulphates eluted early resulting in poor resolution of paracetamol metabolites. Addition of TEA, as recommended by the manufacturers, increased sulphate retentions (Fig. 3) presumably by an ion-pair reaction while other compounds eluted earlier since active adsorption sites on the column were now masked. Resolution was still not acceptable since paracetamol glucuronide (G) and paracetamol sulphate (S) were too close together, a criticism of earlier ion suppression methods, and 3-thiomethylparacetamol sulphate (3SMeS) and hippuric acid (HA) were unresolved. Variation of the three parameters, pH, methanol concentration and TEA concentration, enabled a wide range of

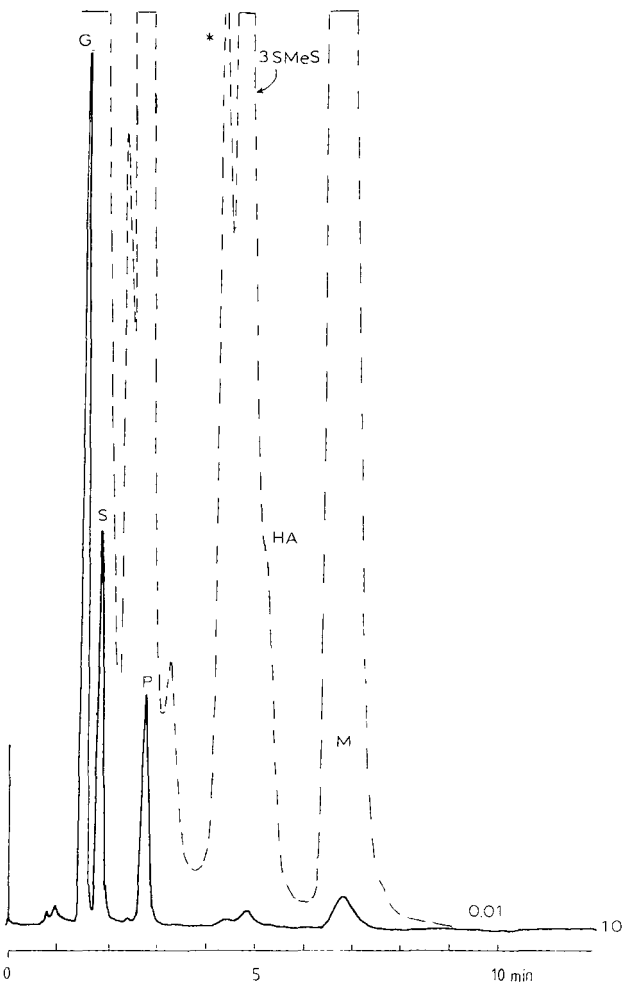


Fig. 3. The effect of 0.005 *M* TEA on ion suppression HPLC of paracetamol metabolites. Eluent, 0.005 *M* TEA, 15% methanol in 0.05 *M* potassium phosphate buffer, pH 2.3; flow-rate, 4 ml/min; detector 254 nm at two sensitivities as indicated. For peak identification see Fig. 2.

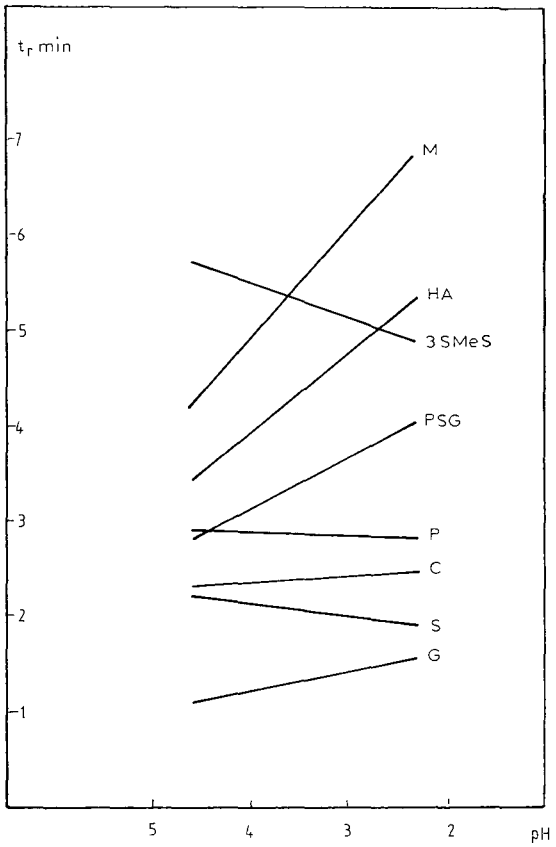


Fig. 4. Effect of pH on the selectivity of the separation of paracetamol metabolites by ion suppression HPLC on a Rad-Pak A column. Eluent, 0.005 *M* TEA, 15% methanol in 0.05 *M* potassium phosphate buffer, pH varied; flow-rate, 4 ml/min. For abbreviations see Fig. 2; PSG = paracetamol glutathione conjugate.

separation alternatives. Increasing methanol concentration past 15% was unfavourable since all metabolites eluted too quickly. Therefore it was more profitable to adjust pH and TEA concentration.

The effect of changing pH on a relatively new column is shown in Fig. 4 where it is noted that sulphates behave differently to the other metabolites. A TEA concentration of 0.005 *M* (0.07%) pH 3.2 was chosen as optimum.

A number of urine samples containing different proportions of various metabolites were run in this system so that detailed information on retention times could be obtained. These are tabulated in Table III. Metabolites of 3-thiomethylparacetamol were obtained from animals administered the parent compound [5] and the same metabolites were observed in animals induced with 3-methylcholanthrene and receiving high doses of paracetamol. Metabolites of 3-methoxyparacetamol were likewise obtained by the administration of the parent compound to Sprague—Dawley rats. The presence of these metabolites was noted in a sample of human urine obtained from a patient with analgesic nephropathy (Fig. 5a). The levels of 3-methoxypara-

TABLE III

RETENTION TIMES FOR PARACETAMOL METABOLITES IN ION SUPPRESSION CHROMATOGRAPHY

HPLC conditions as for Fig. 5.

Metabolite	Abbreviation	Retention time (min)
Paracetamol glucuronide	PG	1.2
Paracetamol sulphate	PS	1.9
3-Methoxyparacetamol glucuronide	3OMeG	2.2
Paracetamol cysteine conjugate	C	2.2
3-Methoxyparacetamol sulphate	3OMeS	2.4
Paracetamol	P	2.6
Paracetamol cysteinylglycine conjugate	PCG	2.6
Paracetamol glutathione conjugate	PSG	3.2
3-Thiomethylparacetamol glucuronide	3SMePG	3.6
3-Thiomethylparacetamol sulphoxide	3SOMeP	4.1
3-Methoxyparacetamol	3OMeP	4.2
Hippuric acid	HA	4.6
3-Thiomethylparacetamol sulphate	3SMePS	5.0
Paracetamol mercapturic acid	M	5.8
3-Thiomethylparacetamol	3SMeP	>8.0

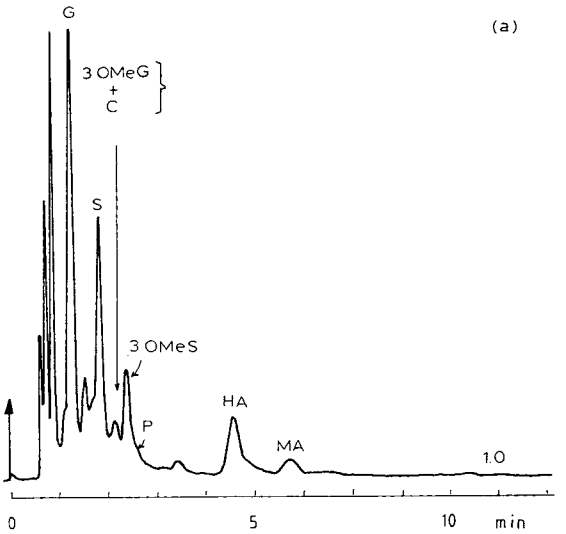
cetamol metabolites are slightly exaggerated by comparison with the metabolism of a therapeutic dose of paracetamol in a healthy volunteer (Fig. 5b).

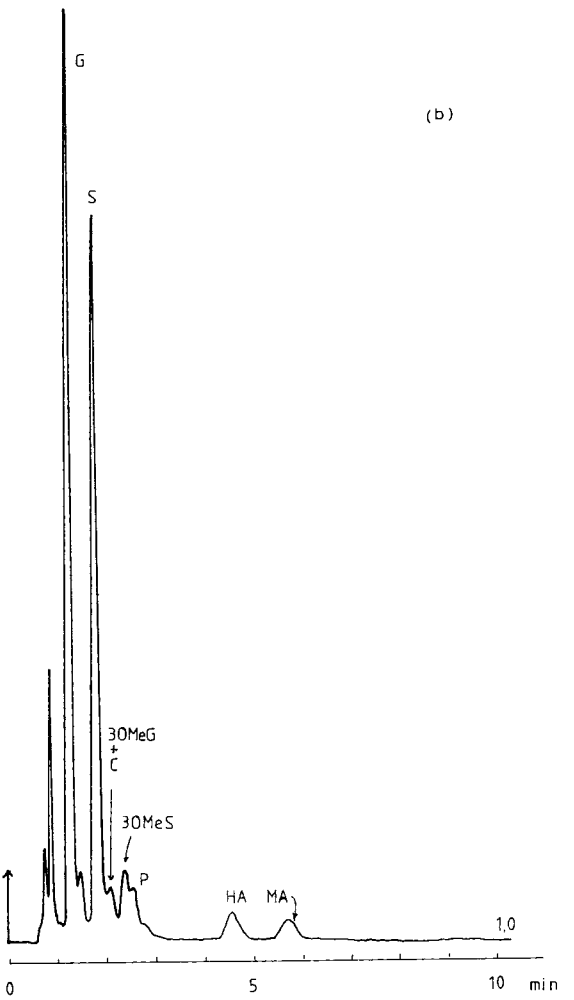
The same chromatographic system was used successfully to monitor the metabolism of paracetamol glutathione conjugate both in vivo, in bile-cannulated animals and in the isolated perfused kidney [12,13]. While the major metabolites in vivo were the cysteine and mercapturic acid, perfusion urines and bile samples yielded an intermediate metabolite in the breakdown of the glutathione conjugate, that is paracetamol cysteinylglycine. The latter compound was prepared synthetically and found to run at the same retention time as paracetamol. Increasing the TEA concentration enabled resolution from P but care was needed to avoid the metabolite co-eluting with C.

It became apparent that the ion suppression separation was not adequate for the large number of metabolites now being studied. Furthermore, there was an inherent lack of stability in the system with the injection of a large number of urine samples and especially perfusion urines. The graph of t_R versus pH is quite different on an old column (Fig. 6) compared to a new one (Fig. 4). Sulphates were most particularly affected suggesting that urinary amine constituents became bound to the reversed-phase packing thus creating an anion exchange surface to which sulphates are strongly attracted at low pH. The end of useful column life occurred when the metabolite pairs S-C and 3SMePS-M coalesced.

Analysis of urinary paracetamol metabolites by ion-pair HPLC

The increased complexity of paracetamol metabolism made it clear that an ion-pair reagent with a higher log E_{QX} [14] than TEA would be more


G
(a)
3 OMeG
+
C
S
3 OMeS
P
HA
MA
1.0
0
5
10
min


G
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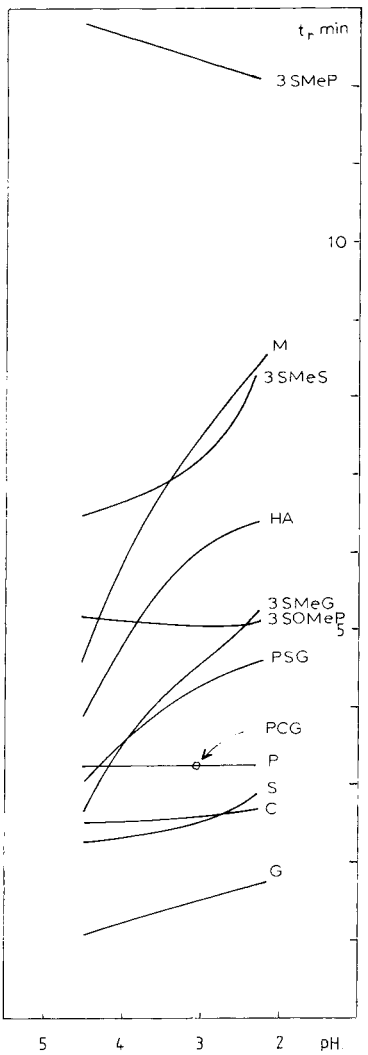

Fig. 6. Effect of pH on the selectivity of the separation of paracetamol metabolites by ion suppression HPLC on a Rad-Pak A column. Conditions as for Fig. 4 except that the experiment repeated on an older column subjected to numerous urine injections. For abbreviations see Table III.

successful in providing the necessary selectivity. Initially TBA at 0.005 *M*, pH 5.0 in 20% methanol was used. Gradient programming was necessary because the retention of 3SMePS was prohibitively long (60 min) and poor resolution occurred between HA, M and S. The solvent system 0.005 *M* TBA,

Fig. 5. Ion suppression HPLC of urinary paracetamol metabolites on a Rad-Pak A column. Eluent, 0.005 *M* TEA, 15% methanol in 0.05 *M* potassium phosphate buffer, pH 3.2; flow-rate 4 ml/min; detector 254 nm, two sensitivities as indicated. (a) Sample of human urine following a therapeutic dose of paracetamol from a patient with suspected analgesic nephropathy. (b) Sample of human urine from a healthy volunteer who ingested 1 g of paracetamol (i.e. therapeutic dose). For abbreviations see Table III.

0.01 *M* Tris, 0.005 *M* EDTA at pH 7.2 with gradient programming as previously developed for stainless-steel μBondapak C18 columns [1] was compared with the same solvent on a Rad-Pak A column and a different result was obtained. In addition to this expected change in selectivity the new metabolites had to be taken into account and it was found that insufficient resolution was available to separate the greater number of metabolites.

Various alterations to the solvent system were made in order to improve resolution. Dropping the pH to 5.0 enabled the cysteine conjugate to be eluted as a symmetrical peak without the need to add EDTA to the solvent. Omission of the EDTA caused all glucuronides, M and HA to elute much later thus illustrating its role not simply as a column deactivator but also as a competitive ion-pair substrate for TBA at the expense of the more water-

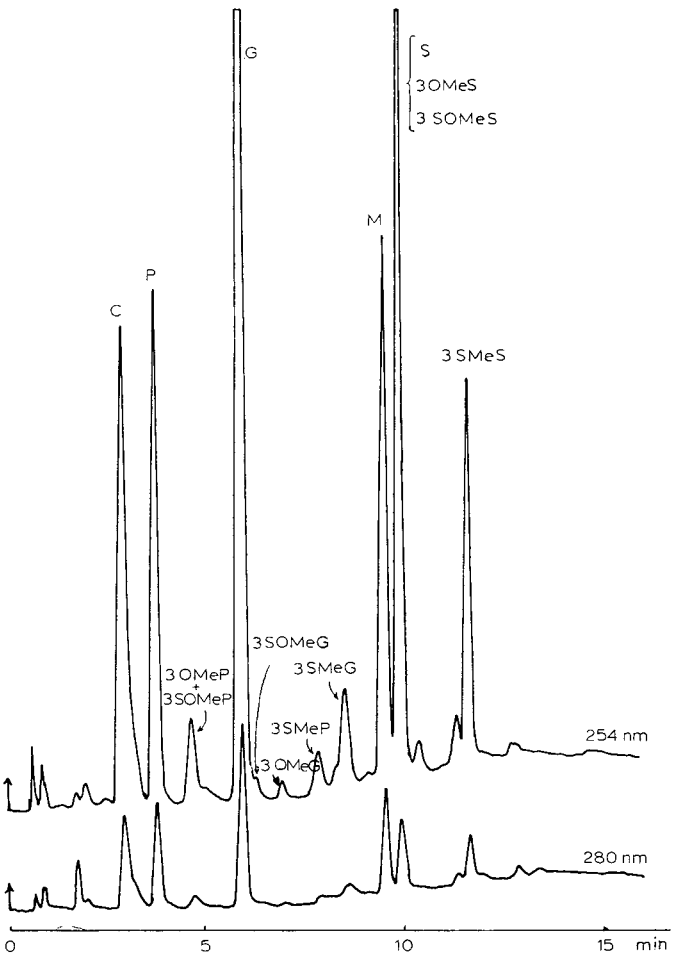

Fig. 7. Optimum separation of urinary paracetamol metabolites by ion-pair HPLC on Rad-Pak A column. Conditions: solvent, 0.01 *M* TBA, 0.01 *M* Tris, pH 5.0 (H_3PO_4) programmed from 10—50% methanol over 12 min; flow-rate, 4 ml/min; detector: 254 nm, 1.0 a.u.f.s.; 280 nm, 0.5 a.u.f.s.; sample, urine from Sprague—Dawley rat pretreated with 3-methylcholanthrene and given 15 mmol/kg paracetamol. Urine sample spiked with C.

soluble anions. Doubling the TBA concentration ensured that glucuronides were moved well past their parent compounds thus enhancing overall selectivity and resolution. Further fine tuning of the separation was achieved by adjusting the initial methanol concentration and the gradient sweep time. The optimized separation is shown in Fig. 7. A re-equilibration time of 9 min has been found to be ample thus allowing the analysis of a sample in 20 min. The previous gradient programmed separation took 1 h [1].

The method has so far proved useful in the study of paracetamol metabolism. In particular it has allowed the isolation and spectroscopic identification of the conjugates of 3SOMeP following Sephadex LH-20 separation of urine containing 3-thiomethylparacetamol metabolites and enabled the identification of these compounds as products of paracetamol metabolism (unpublished results). 3SOMeP has been previously reported only as the free compound in enzymatically hydrolysed urines [6]. The most significant finding was the presence of the metabolite 3SOMeS and the difficulty of its separation from S. In previous studies the peak assigned to S in paracetamol urines has always been assumed to be the result of non-toxic metabolism. From our investigations

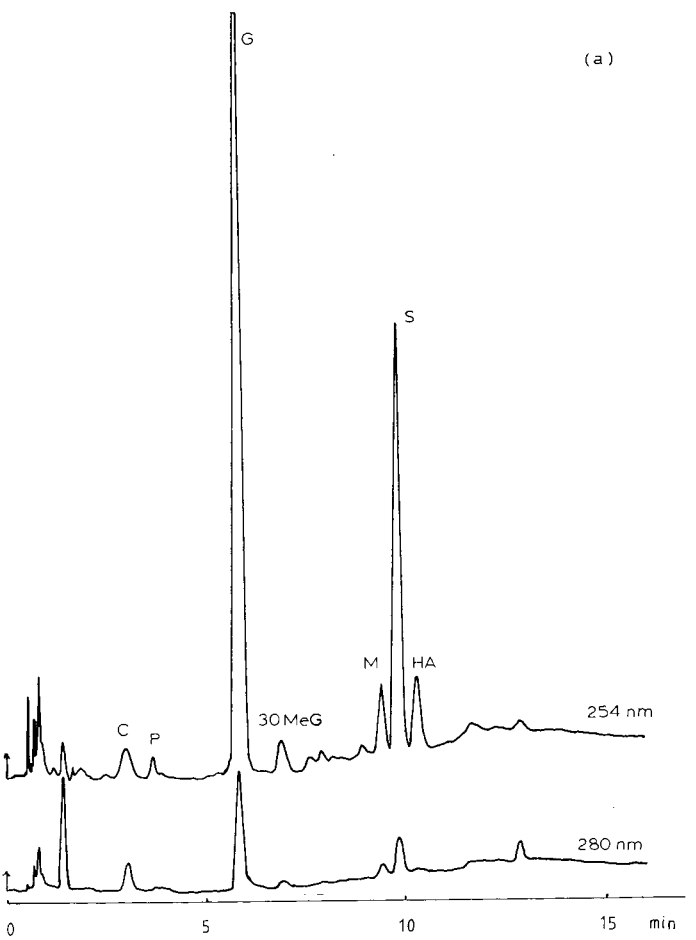

Fig. 8.

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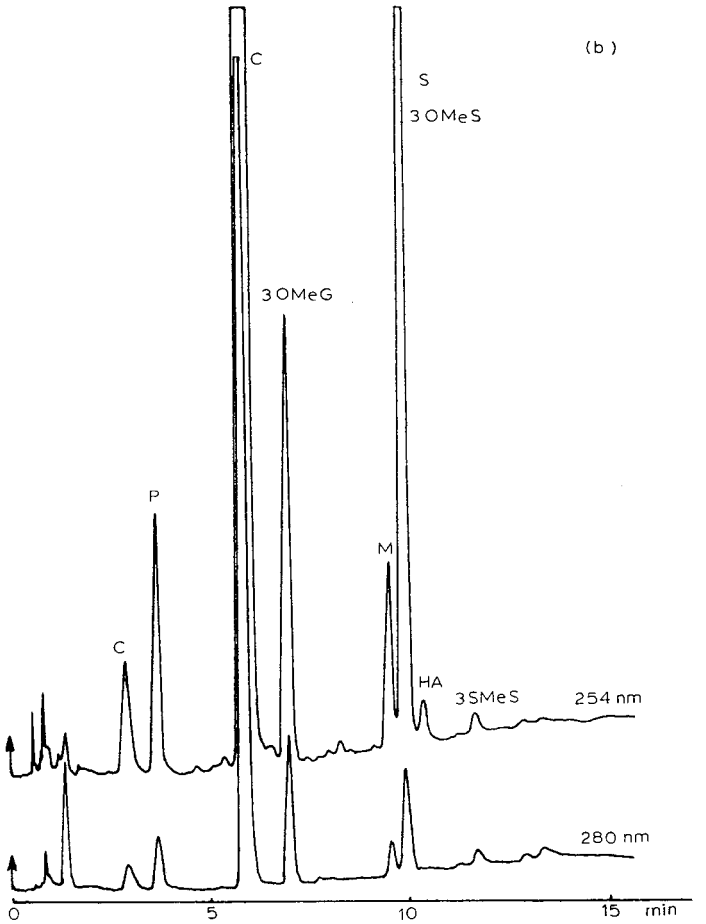

Fig. 8. Separation of human urinary paracetamol metabolites. Conditions as in Fig. 7. (a) Sample of urine taken following therapeutic administration of 1 g paracetamol to a healthy adult (same sample as shown in Fig. 5b). (b) Sample of urine taken following a fatal overdose of paracetamol. Note relatively high concentration of 3-methoxyparacetamol derivatives relative to M.

with experimental animals it appears that varying proportions of the 3SOMeS conjugate are produced following paracetamol administration as determined by UV ratio since in most of these cases 3-methoxyparacetamol glucuronide, and by inference its sulphate analogue, were absent. However, our work has shown that in other instances, e.g. in the study of human paracetamol metabolism, 3-methoxyparacetamol formation is significant as determined by UV ratio (Fig. 8). This creates a major problem when the trio of metabolites S, 3SOMeS, 3OMeS are all present as would be the case under conditions of human overdose.

The formation of 3-methoxy, 3-thiomethyl and 3-thiomethylsulphoxide derivatives of paracetamol appears to be inextricably linked to paracetamol-induced toxicity. Until a better separation of the three sulphate metabolites is obtained these results are presented to illustrate the complex nature of paracetamol metabolism.

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CHROMBIO. 1954

SEPARATION, ISOLATION AND IDENTIFICATION OF OPTICAL ISOMERS OF 1,4-BENZODIAZEPINE GLUCURONIDES FROM BIOLOGICAL FLUIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of four separate 1,4-benzodiazepine glucuronides in urine, plasma and bile is presented. We succeeded not only in determining the single glucuronides but also in separating the enantiomers (optical isomers) of the 1,4-benzodiazepine glucuronides. The optical isomers of the glucuronides of oxazepam and cinolazepam and of two other glucuronides of benzodiazepine metabolites could be well separated. The ratio of the isomers could be evaluated. An octadecyl reversed phase was used with a mobile phase of acetonitrile and 0.01 *M* orthophosphoric acid. After the initial separation, the isomers were fractionated by HPLC. After treatment with β-glucuronidase to yield the aglycone, the separated fractions were hydrolysed to the corresponding benzophenones whose identity was confirmed by HPLC. Gas chromatography and gas chromatography—mass spectrometry demonstrated that the separated glucuronides corresponded to the enantiomeric benzodiazepines. Human urine and plasma as well as rabbit urine, plasma and bile were examined.

INTRODUCTION

The determination of glucuronides has been extensively described in the literature [1—7]. Glucuronides of drugs [1—3] as well as glucuronides of steroids [4, 5] have been determined. Baker [6] gave indices to some drugs and their glucuronides depending on the capacity factors of 2-ketoalkanes. This method can be helpful for finding the unknown glucuronide in the chromatogram of a known drug.

Hydrolysis of glucuronides under either acidic or enzymatic conditions does not always lead to the desired result. Goenechea et al. [7] found that under the usual acidic conditions the hydrolysis of codeine glucuronide was either incomplete or that codeine was destroyed to the extent of 8—83%.

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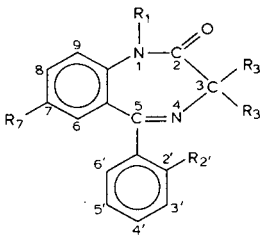
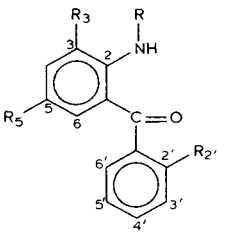


Ring nomenclature for 1,4-benzodiazepines



Benzophenones (all structures verified by MS)

		R_1	$R_{2'}$	R_3	R_7	R	$R_{2'}$	R_3	R_5
Oxazepam	I	H	H	H,OH	Cl	H	H	H	Cl
Cinolazepam	II	CH_2CH_2CN	H	H,OH	Cl	CH_2CH_2CN	H	H	Cl
N-1-Desalkyl--flurazepam	III	H	F	H,OH	Cl	H	F	H	Cl
SAS 643 [17]	IV	CH_2CH_2OH	F	H,OH	Cl	CH_2CH_2OH	F	H	Cl

Fig. 1. Structural formulae of the benzodiazepines and the corresponding benzophenones.

Oxazepam (Fig. 1, I) and cinolazepam (Fig. 1, II) and several other benzodiazepines too have an optically active center. These drugs also have a hydroxyl group in position 3 for glucuronidation. Glucuronidation takes place in vivo and a similar procedure involving chiral reagents was used by some authors [8—11] for the determination of enantiomeric drugs through precolumn derivatization. The separation of the diastereoisomeric glucuronides of oxazepam without chemical derivatization, involving preparative-scale ion-exchange chromatography for isolation and stereoselective enzymatic hydrolysis to yield the pure isomers of oxazepam, was first described by Ruelius et al. [12].

In this study we describe a procedure for the separation and identification of optical isomers of 1,4-benzodiazepines using a mobile phase in which the glucuronides had a capacity factor of 5—10. We analyzed both human plasma and urine, and rabbit plasma, urine and bile after oral administration of I or II.

MATERIALS AND CHEMICALS

The high-performance liquid chromatographic (HPLC) system was either a Spectra Physics 8000 system or was built up by an LDC Constametric III pump in connection with a Rheodyne 7126 injector. Detection was performed by an LDC Spectromonitor III at 230 nm or 380 nm. We used loops of 10, 20, 50 and 500 μl. The integration and plotting of the chromatograms were done by the SP 8000 or by a Shimadzu C-R1A integrator. We usually used Knauer columns (250 × 4 mm or 40 × 4 mm) filled normally with LiChrosorb RP-18 (7 μm) or Polygosil C 18 (7.5 μm). We also used LiChrosorb RP-18 cartridges (125 × 4 mm). For the gas chromatographic (GC) analysis of the benzophenones we used a Dani 6800 gas chromatograph with an SE 52 glass capillary column (25 m × 0.32 mm I.D.), and a Varian CH7 for the mass spectrometric (MS) analysis.

Additional reagents used were: buffer pH 7 (1.82 g of $Na_2HPO_4 \cdot 2H_2O$ + 0.88 g of KH_2PO_4 per litre of water); LiChrosorb RP-18, RP-8, NH_2, each 7 μm

particle size; RP-18 cartridges; acetonitrile p.a.; chloroform p.a.; phosphate buffer (Merck, Darmstadt, F.R.G.); methanol p.a.; extraction columns ODS, 1 ml (Baker, Deventer, The Netherlands); PRP-1 (10 μm particle size) column 150 × 4.1 mm (Hamilton, Bonaduz, Switzerland); Partisil 10 ODS-2 (Whatman, Maidstone, U.K.); HPX 87H column (300 × 7.8 mm) (Bio-Rad Laboratories, Vienna, Austria); Polygosil 60-7 C 18 (Macherey-Nagel, Düren, F.R.G.); β-glucuronidase from *Helix pomatia* and *Escherichia coli* (Boehringer, Mannheim, F.R.G.); β-glucuronidase from marine mollusc and beef liver (P-L Biochemicals, Milwaukee, WI, U.S.A.).

EXPERIMENTAL

Separation of the optical isomeric benzodiazepine glucuronides

For separation we usually used LiChrosorb RP-18 or Polygosil C_{18} packings with ultraviolet (UV) detection at 230 nm. To achieve good separation from other endogenous material it was necessary to keep the capacity factor of the glucuronides greater than 5.

The composition of the mobile phase for the separation of I, II, III and IV glucuronides was acetonitrile—0.01 *M* orthophosphoric acid (20:80). The mobile phase was changed to acetonitrile—0.01 *M* orthophosphoric acid (30:70) for the separation of the optical isomeric glucuronides together with the corresponding free benzodiazepines of I and II.

Identification of the optical isomeric benzodiazepine glucuronides

Although we tried to obtain a glucuronide through a chemical derivatization step we were not successful in obtaining a reference standard.

Identification of the optical isomeric benzodiazepine glucuronides was performed according the following procedure. Several 500-μl aliquots of human or animal urine were injected onto the HPLC system and the optical isomeric glucuronides were separated by fractionation. The single fractions were checked by HPLC. After evaporating the acetonitrile, the benzophenones of the benzodiazepine glucuronides were made by treating the samples with 1 *M* hydrochloric acid for 30 min at 100°C. The resulting peaks are identical with the corresponding benzophenones by HPLC; showed from the fractions only one benzophenone was found, verifying the separation of the optical isomers.

The same samples were made alkaline and extracted with chloroform. After preparation and evaporation of the solvent the residue was redissolved in a small volume of ethanol and 2 μl were injected in the capillary gas chromatograph. As with HPLC the corresponding benzophenones are identical.

In addition, mass spectra of the above samples also confirmed the presence of the corresponding benzophenones of the two optical isomeric benzodiazepine glucuronides.

The fractions were also examined after treatment with β-glucuronidase on a HPX 87H column with a mobile phase of 0.005 *M* sulphuric acid and UV detection at 195 nm. Glucuronic acid was found only after treatment with β-glucuronidase. We used a LiChrosorb RP-18 (7 μm) column with a mobile phase of methanol—buffer, pH 7 (70:30) to separate the benzophenones with UV detection at 380 nm.

Routine determination of glucuronides

Determination of I and II as their optical isomeric glucuronides was performed as follows. Usually urine was injected following centrifugation without further purification. Plasma and bile were pretreated either on a Baker extraction column C_{18} or on-line on a precolumn as described by Roth et al. [13] or Voelter et al. [14]. Detection limits for benzodiazepine glucuronides were better than 50 ng/ml in urine, plasma or bile.

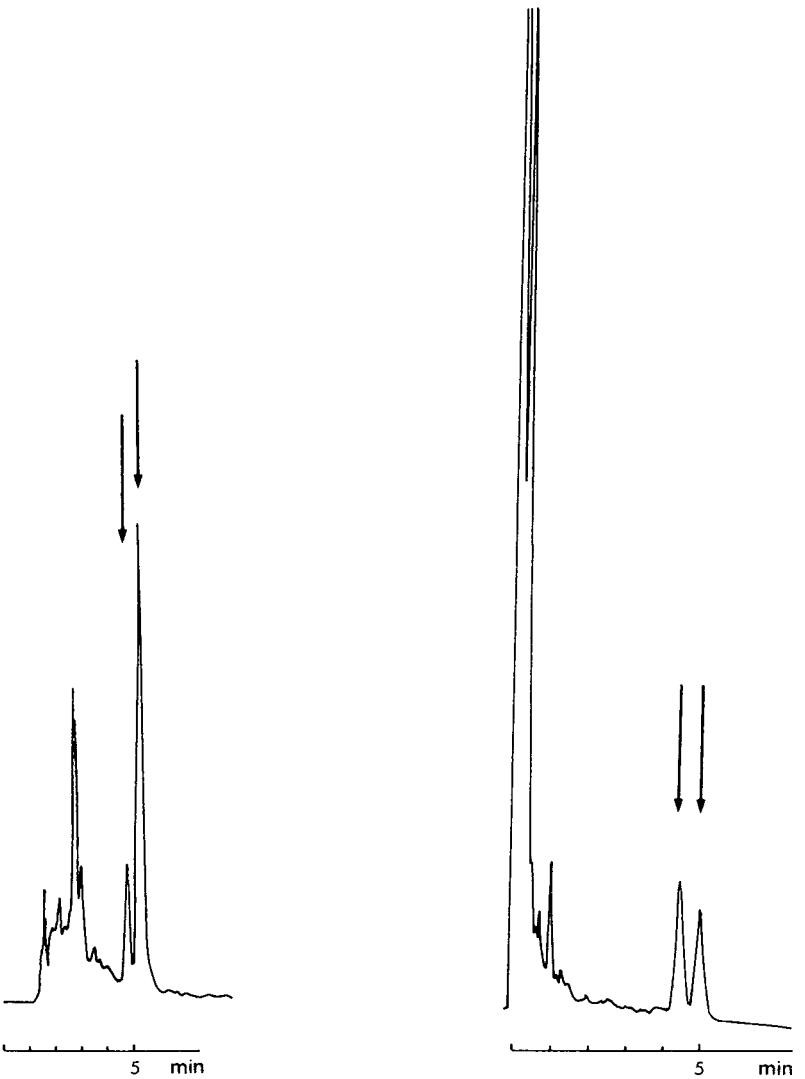


Fig. 2. Separation (Polygosil ODS) of optical isomeric 1,4-benzodiazepine glucuronides in human urine 2 h after oral administration of 20 mg of I. The arrows indicate the two glucuronides.

Fig. 3. Separation (LiChrosorb RP-18) of optical isomeric 1,4-benzodiazepine glucuronides in human urine 4 h after oral administration of 30 mg of II. The arrows indicate the two glucuronides.

For quantitation of I or II glucuronides some of the samples were determined as glucuronides and after hydrolysis with β-glucuronidase as free benzodiazepines. Since sulphate conjugates are not hydrolyzed by β-glucuronidase, showing also a different chromatographic behaviour compared to the glucuronides, any sulphate conjugate present can be excluded. We also checked the efficiency of the hydrolysis by determining the residual glucuronides. Hydrolysis was usually complete in all samples. A conversion factor for the intact glucuronides to aglycone was determined for quantitative analysis. Detection was at 230 nm.

RESULTS

Fig. 2 shows the separation of I glucuronides in human urine after oral administration of 20 mg of I. The separation of II glucuronides in human urine after oral administration of 30 mg of II is shown in Fig. 3. In Fig. 4 the

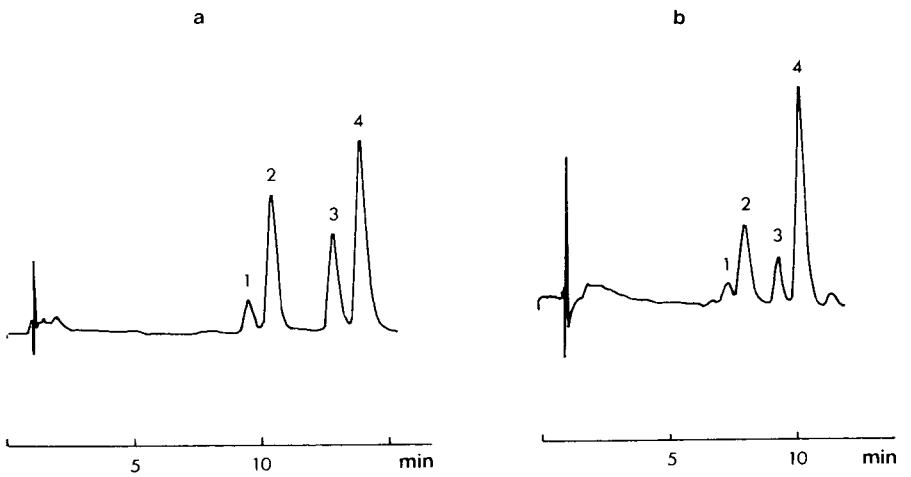


Fig. 4. Separation (Polygosil ODS) of optical isomeric glucuronides isolated from urine. Peaks: (a) 1 and 2 = I glucuronides; 3 and 4 = II glucuronides. (b) 1 and 2 = IV glucuronides; 3 and 4 = III glucuronides.

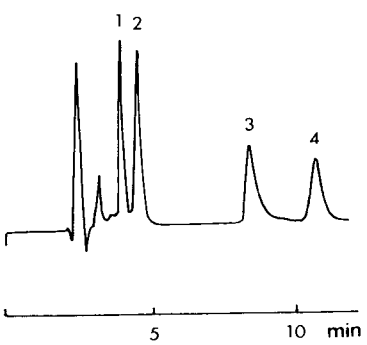


Fig. 5. Chromatogram of the unseparated glucuronides, extracted from human urine, together with the corresponding free benzodiazepines. Peaks: 1 = I glucuronides, 2 = II glucuronides, 3 = I, 4 = II.

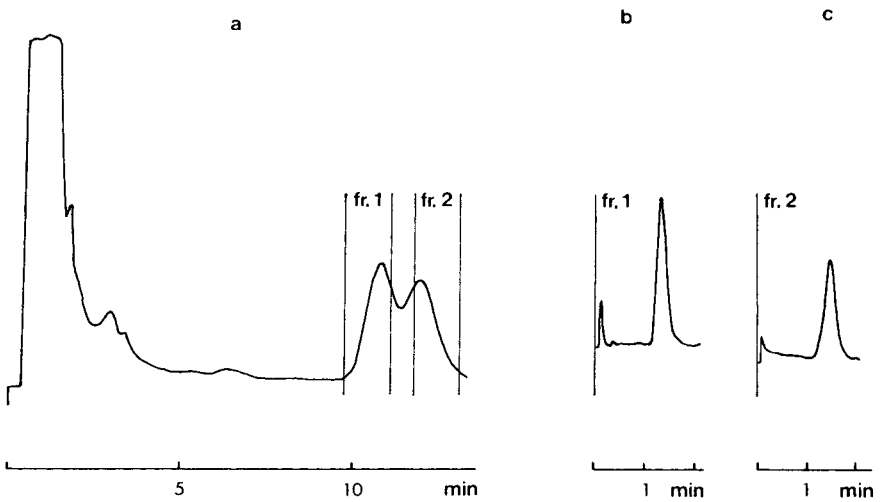


Fig. 6. (a) Chromatogram of the separated II glucuronides indicating which parts of the peaks were fractionated. (b, c) Separate chromatograms of the isolated fractions of the two diastereoisomeric II glucuronides (retention times: fr. 1, 1.32 min; fr. 2, 1.44 min).

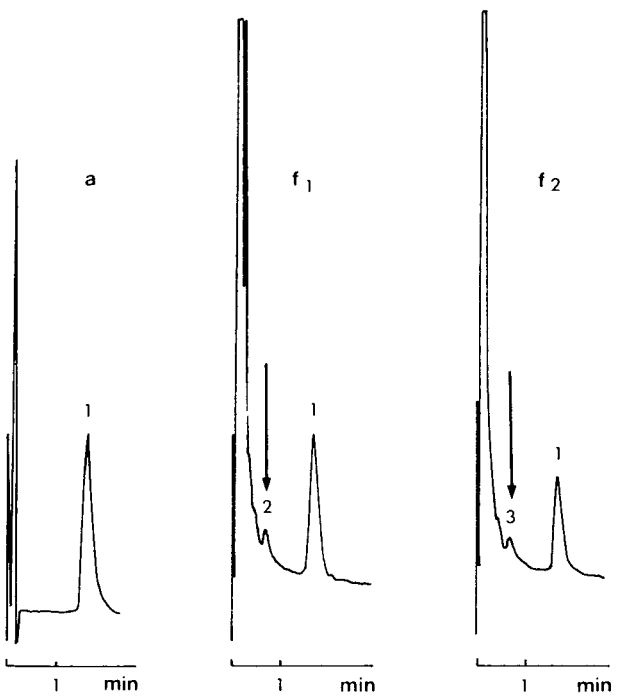


Fig. 7. Chromatograms of the two fractions of II glucuronides (f_1 and f_2) after incomplete hydrolysis with β-glucuronidase as well as the corresponding free benzodiazepine (a). Peaks: 1 = free benzodiazepine (II); 2 = II glucuronide fraction 1; 3 = II glucuronide fraction 2.

chromatographic separation of a mixture of I, II, III and IV glucuronides can be seen. Fig. 5 shows a chromatogram where the optical isomeric glucuronides are not separated together with the corresponding benzodiazepines of I and II.

Separation of II glucuronides before and after fractionation can be seen in Fig. 6. Chromatograms of the fractions of II glucuronides after hydrolysis with β-glucuronidase as well as the reference benzodiazepine are shown in Fig. 7. Results of the mass spectra of the II benzophenones of the two fractions are shown in Table I.

The data suggest that the glucuronides of these benzodiazepines (I, II, III, IV) are mixtures of the enantiomeric glucuronides of these drugs.

TABLE I

m/e VALUES OF THE BENZOPHENONE OF II (BP) AND THE BENZOPHENONES AFTER ACIDIC HYDROLYSIS OF THE TWO FRACTIONS OF THE II GLUCURONIDES (f_1, f_2)

The relative intensity of the mass peaks are indicated by +, ++, +++.

m/e	BP	f_1	f_2
302	+	+	+
262	+++	++	+++
166	+++	++	+++
109	+++	+	+++

DISCUSSION

There are only a few papers published on the determination of glucuronides in vivo (e.g. refs. 1—5). It seems that GC is more difficult to perform than HPLC because quantitative derivatization of the polyhydroxyl groups of the glucuronic acid needs special precautions.

1,4-Benzodiazepines with a hydroxyl group on C-3 undergo rapid glucuronidation. We developed an HPLC method for the direct determination of benzodiazepine glucuronides to circumvent the need for enzymatic hydrolysis. β-Glucuronidase from different sources yielded different results mainly due to the efficiency of hydrolysis of glucuronides. β-Glucuronidase from *Escherichia coli* hydrolysed only 1—5% of the benzodiazepine glucuronides. However, β-glucuronidase from *Helix pomatia*, marine mollusc or beef liver yielded complete hydrolysis. Hydrolysis in acid yields the corresponding benzophenone instead of the parent benzodiazepine. To exclude the formation of benzophenones from other unresolved metabolites, e.g. quinazolines/quinazolone conjugates we compared the retention time of both fractions of the separated glucuronides after enzymatic hydrolysis with the retention time of the aglycone (Fig. 7). Similar results were obtained on different columns.

Benzodiazepine glucuronides under usual separation conditions gave two poorly resolved peaks. Changing the mobile phase such that the glucuronides had a capacity factor of greater than 5, yielded well separated optical isomeric glucuronide peaks. Also at a capacity factor (k') of lower than 5 the ratio of the two diastereoisomeric compounds remains stable; additionally, analysis was performed on columns with different lengths (4, 12.5 and 25 cm; 4—4.6 mm I.D.) with no change in the ratio.

We found that PRP-1 (copolymer of styrene and divinylbenzene) and Li-

Chrosorb NH_2 could not separate the optical isomeric glucuronides, while Partisil ODS 2 gave poorly resolved peaks. However, LiChrosorb RP-8, LiChrosorb RP-18, Polygosil ODS and Sperisorb ODS could be used for complete separation.

To give some explanation of how the separation is effected, we assume that different parts of the molecule, caused by the different steric structure, are getting in contact with the stationary phase. Blaschke and Markgraf [15] showed that the pure enantiomers of I were difficult to handle because of their tendency to racemize. For this reason it was impossible to isolate pure enantiomeric benzodiazepines to verify our results.

Glucuronidation is an important pathway in the biotransformation of 1,4-benzodiazepines and their metabolites [16]. Therefore the advantage of determining the intact glucuronides is obvious. Oxazepam glucuronide is a common excretion product of several marketed 1,4-benzodiazepines, viz. diazepam, medazepam, chlorazepate, prazepam and ketazolam [16].

The proposed method or that of Baker [6] can be used for analyzing unknown benzodiazepine glucuronides. When using Baker's [6] method one has to take in account that there are different k' ratios for each benzodiazepine and its glucuronide under different pH conditions.

Screening of the drugs and their metabolites would be much easier and is independent on the extent of hydrolysis. The only non-benzodiazepine glucuronide we analyzed was codeine glucuronide, and as expected we found only a single peak due to the fact that codeine has no optically active carbon atom.

An interesting observation is the varying degree of optical isomeric benzodiazepine glucuronides in urine. Differences in the concentration of enantiomeric drugs in plasma are known to occur with β-blocking drugs such as propranolol [8], alprenolol and metoprolol [9], or also with naproxen [11].

Human plasma and urine showed a I glucuronide peak area ratio of 19:81 for the optical isomeric glucuronides. Similarly, III glucuronide showed a ratio of 50:50 in human urine, while IV glucuronide showed a ratio of 30:70 in rabbit urine, although presumably a racemic mixture of these benzodiazepines was initially administered. The urine of one man showed a constant ratio during 48 h for II glucuronide of 54.2:45.8 ($\pm$ 3.0; n = 8). In 48-h pooled urines of five men we found II glucuronide ratios of 53.6:46.4 ($\pm$ 3.0). In rabbit urine found in the bladder 24 h after II glucuronide, ratios of 41.1:58.9 ($\pm$ 5.0; n = 9) were noted.

ACKNOWLEDGEMENTS

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QUANTITATION OF CLONAZEPAM AND ITS 7-AMINO AND 7-ACETAMIDO METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is reported for the determination of clonazepam and its metabolites 7-amino- and 7-acetamidoclonazepam. Extraction from buffered plasma is carried out at pH 9.5 with hexane—ethyl acetate (7:3) for clonazepam and with chloroform for the metabolites. Flunitrazepam, 7-aminodemethylflunitrazepam and 7-acetamidoflunitrazepam are used as the internal standards for clonazepam and its 7-amino and 7-acetamido metabolites, respectively. To prevent decomposition of 7-amino-clonazepam a high concentration of 7-aminomethylclonazepam is added to the plasma. Chromatography is carried out on a reversed-phase column with detection at 254 nm for clonazepam and 240 nm for the metabolites. Using the method it was possible to determine 5 ng/ml clonazepam, 7-aminoclonazepam and 7-acetamidoclonazepam in plasma with coefficients of variation of 9.5%, 5.9% and 8.9%, respectively.

This method can be used to measure clonazepam in plasma from patients treated with other antiepileptics. It may also be utilized for in vitro studies on the metabolism of clonazepam in subcellular fractions from the liver.

INTRODUCTION

Clonazepam is a relatively new anticonvulsant drug, preferentially used in the treatment of childhood epileptic disorders such as minor motor and petit mal epilepsia which are refractory to treatment with other drugs [1, 2]. Several analytical methods have been developed in order to improve the selectivity, the sensitivity and the simplicity of clonazepam assays. The most commonly used methods employ gas—liquid and high-performance liquid chromatography

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(HPLC) [3—8]. Analytical methods for 7-aminoclonazepam have employed gas—liquid chromatographic [9] and mass spectrometric techniques [10].

In the present paper we describe a selective and sensitive HPLC method for the determination of clonazepam and its metabolites, 7-amino- and 7-acetamidoclonazepam in plasma samples from patients and in incubates with subcellular fractions from the liver.

Our method combines the advantages that only one extraction step is required and no derivatization or acid hydrolysis [3] is necessary. With ultraviolet (UV) detection of the compounds linearity over a wide concentration range is achieved.

MATERIAL AND METHODS

Apparatus

The liquid chromatograph consisted of a Constametric III pump and a Spectromonitor III variable-wavelength detector (both from Laboratory Data Control, Riviera Beach, FL, U.S.A.) operating at 254 nm (clonazepam) and 240 nm (the metabolites). A Rheodyne Model 7125 was used as injector (Berkeley, CA, U.S.A.) with a 20-μl sample loop. For the absorbance spectra a Beckman 34 spectrophotometer with scanner was employed.

Chemicals and materials

Clonazepam (CLZ), flunitrazepam (FLZ, internal standard), 7-aminoclonazepam (7-ACLZ), 7-aminodemethylflunitrazepam (7-ADFLZ, internal standard), 7-aminomethylclonazepam (7-AMCLZ, protector, see Results and discussion), 7-acetamidoclonazepam (7-AACLZ), 7-acetamidoflunitrazepam (7-AAFLZ, internal standard) were products of Hoffmann-La Roche (Basel, Switzerland). The drugs tested for interference were obtained from the respective manufacturer. Glycine (p.a.), sodium hydroxide, sodium acetate (p.a.), methanol (p.a.), hexane (p.a.), ethyl acetate, acetonitrile (LiChrosolv) and chloroform (p.a.) were purchased from Merck (Darmstadt, F.R.G.). All glass tubes were silanized, washed, and rinsed twice from detergent with distilled water.

The stock solutions were 1 mg/ml of the respective compound in methanol. All working solutions were prepared by dilution with methanol and they contained 1 μg/ml except for the solution of 7-AMCLZ and 7-AAFLZ which contained 100 and 2 μg/ml, respectively. A 2 *M* glycine—sodium hydroxide buffer, pH 9.5, was prepared.

Chromatographic conditions

The column (150 × 4.6 mm I.D.) was prepacked with Supelcosil LC-18 (5-μm octadecylsilane, Supelco, Bellefonte, PA, U.S.A.). The mobile phase for CLZ was a mixture of acetonitrile—0.1 *M* sodium acetate (35:65), pH 7.7, and for the metabolites a mixture of acetonitrile—0.02 *M* sodium acetate (18:82), pH 7.4. The mobile phases were degassed by filtration. Column equilibrium was achieved within 30 min at a flow-rate of 3 ml/min. Distilled water was used to measure the retention time for an unretained solute.

Analytical procedure

CLZ and the metabolites were analysed separately. To 1 ml of plasma were added 1 ml of 2 *M* glycine—sodium hydroxide buffer, pH 9.5, 50 μl of the internal standard and 50 μl of the protector when metabolites were analysed. When CLZ was analysed, the mixture was extracted for 45 min with 7 ml of hexane—ethyl acetate (7:3). When the metabolites were analysed extraction was performed with 7 ml of chloroform for 45 min. The samples were centrifuged for 5 min and the organic phase was transferred to new tubes and evaporated to dryness. The residue was reconstituted in 40 μl of the respective mobile phase (see chromatographic conditions). Thirty microlitres of the sample were injected into the column. An additional extraction step was needed when analysing 7-ACLZ in plasma from patients treated with carbamazepine. After the 45 min extraction the water phase was discarded and 1 ml of 2 *M* glycine—sodium hydroxide buffer, pH 9.5 was added to the chloroform phase, which was extracted for 5 min.

Standards were prepared in duplicate by the addition of known amounts of CLZ (10—100 ng/ml), 7-ACLZ (10—100 ng/ml) and 7-AACLZ (10—100 ng/ml) to drug-free plasma. Quantitations were performed from a standard curve of the ratio of the peak heights of the sample and the internal standard versus the plasma concentration, obtained by analysing the above-mentioned standards.

Test of stability

7-ACLZ and 7-AACLZ were tested for stability in chloroform and ethyl acetate at low concentrations (1.4, 7.1 and 14.3 ng/ml, equal to 10, 50 and 100 ng in 7 ml of the organic solvent) for 1 h at room temperature.

10, 50 and 100 μl of 1 μg/ml 7-ACLZ and 7-AACLZ in methanol were added to empty tubes (used as reference standards) and to tubes with 7 ml of chloroform with and without protector, and with 7 ml of ethyl acetate, respectively. The organic solutions were stored at room temperature for 1 h before evaporation to dryness. The small amount of methanol in the reference standards was added and evaporated to dryness immediately before injection. The residue from each tube was dissolved in 40 μl of mobile phase and 30 μl were injected into the column. The peak heights were measured for each organic solution and concentration and compared with the peak heights of the reference standards (see Fig. 4 and Results and discussion).

RESULTS AND DISCUSSION

The absorbance spectra for 7-ACLZ and 7-AACLZ dissolved in the mobile phase are shown in Fig. 1. In order to monitor both metabolites at the same wavelength we chose 240 nm as the optimum wavelength. The absorbance spectrum for CLZ (not shown) revealed that 254 nm was the optimum wavelength.

Fig. 2 and 3 show the chromatograms of CLZ (Fig. 2) and its metabolites (Fig. 3) in spiked plasma, blank plasma and plasma from treated patients. FLZ was chosen as internal standard because of its similar chemical structure to CLZ. Even though flunitrazepam (Rohypnol) is used as a hypnotic agent, this

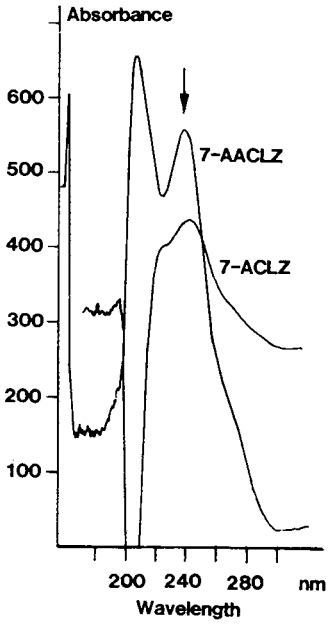

Fig. 1. Absorbance spectrum of 7-aminomethylclonazepam (1.0 μg 7-ACLZ per ml mobile phase) and 7-acetamidoclonazepam (1.0 μg 7-AACLZ per ml mobile phase). Conditions: mobile phase, acetonitrile—0.02 *M* sodium acetate (18:82); scan speed, 50 nm/min; chart speed, 25 mm/min.

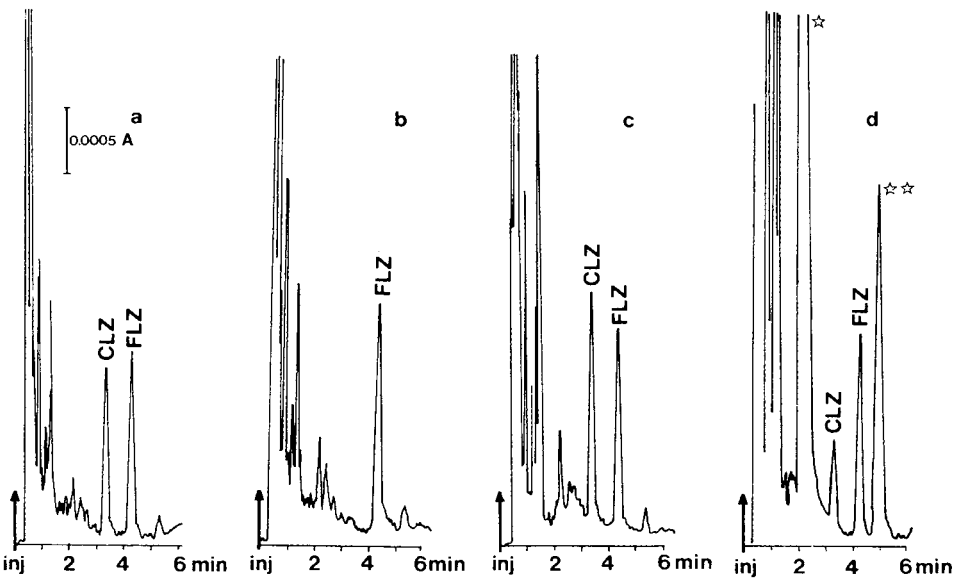

Fig. 2. Chromatograms of clonazepam. Column: Supelcosil LC 18 (5 μm, 150 × 4.6 mm I.D.). Mobile phase: acetonitrile—0.1 *M* sodium acetate (35:65). Flow-rate: 3 ml/min. Detector: Spectromonitor III (Laboratory Data Control) at 254 nm. Peaks: CLZ = clonazepam, FLZ = flunitrazepam (internal standard), ☆ = carbamazepine and phenytoin, ☆☆ = unidentified peak. The figure shows chromatograms from plasma of: (a) a sample spiked with 30 ng/ml CLZ; (b) a blank sample with FLZ; (c) a sample from a patient treated with CLZ (the found CLZ concentration was 46 ng/ml); (d) a sample from a patient treated with CLZ, carbamazepine and phenytoin (the found CLZ concentration was 15 ng/ml).

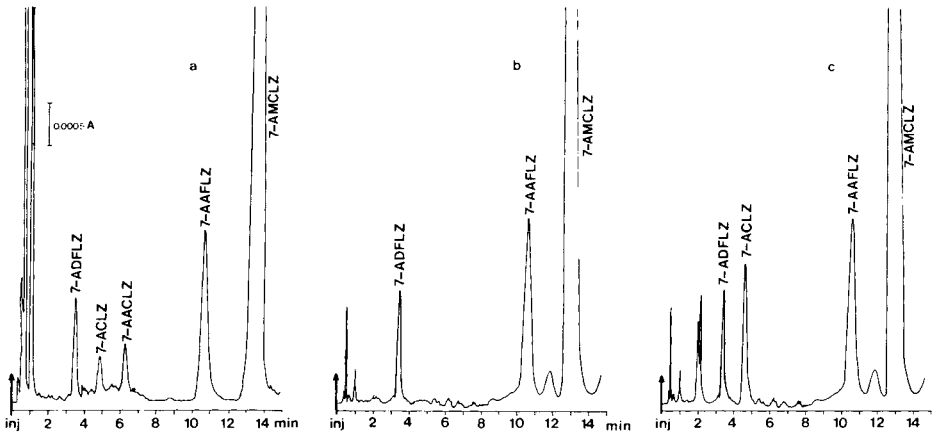

Fig. 3. Chromatograms of clonazepam metabolites. Chromatographic conditions as in Fig. 2 except for the mobile phase: acetonitrile—0.02 *M* sodium acetate (18:82). Peaks: 7-ADFLZ = 7-aminodemethylflunitrazepam (internal standard), 7-ACLZ = 7-amino-clonazepam, 7-AACLZ = 7-acetamidoclonazepam, 7-AAFLZ = 7-acetamidoflunitrazepam (internal standard), 7-AMCLZ = 7-aminomethylclonazepam. The figure shows chromatograms from plasma of: (a) a sample spiked with 40 ng/ml 7-ACLZ and 7-AACLZ; (b) a blank sample with internal standards; (c) a sample from a patient treated with CLZ. The chromatogram shows a peak of 7-ACLZ (110 ng/ml), but no detectable peak of 7-AACLZ.

drug is seldom employed in epileptic patients with clonazepam treatment since this form of epilepsy occurs mainly in children.

Standard curves for CLZ, 7-ACLZ and 7-AACLZ were generated by addition of these compounds to drug-free plasma. The regression equations and the coefficients of correlation in the 10—100 ng/ml range were: for CLZ, $Y = 0.031X + 0.030$, $r = 0.998$; for 7-ACLZ, $Y = 0.012X - 0.009$, $r = 0.995$; and for 7-AACLZ, $Y = 0.0074X + 0.0015$, $r = 0.998$.

Precision

Samples spiked with CLZ, 7-ACLZ and 7-AACLZ at concentrations shown in Table I were analysed within one analysis ($n = 10$) and for CLZ in addition between analyses in duplicate ($n = 10$). The estimated mean concentration and the coefficient of variation (C.V.) are also given in Table I. As seen in Table I, the C.V. did not exceed 11% for any of the compounds at either concentration. Using the method it was possible to determine 5 ng/ml of CLZ, 7-ACLZ and 7-AACLZ in plasma with C.V. of 9.5%, 5.9% and 8.9%, respectively.

Stability

CLZ stability in plasma and in methanol solution was tested and found to be stable over several months. In hexane and ethyl acetate, CLZ was stable for at least two days. These solvents were considered for extraction of CLZ. Chloroform gave too much baseline noise when used for CLZ extraction from plasma and therefore hexane—ethyl acetate (7:3) was chosen.

We found that 7-AACLZ was stable when stored in plasma or methanol for at least one month (—20°C) or in chloroform for at least one week (—20°C, +4°C, +25°C).

TABLE I

PRECISION AND COEFFICIENTS OF VARIATION (C.V., %) FOR ANALYSIS OF PLASMA SAMPLES (n = 10) SPIKED WITH CLONAZEPAM (CLZ), 7-AMINOCLONAZEPAM (7-ACLZ) AND 7-ACETAMIDOCLONAZEPAM (7-AACLZ)

Compound	Concentration (ng/ml)		C.V. (%)
	Added	Estimated mean	
CLZ within analysis	15.6	15.8	7.2
	31.1	28.1	5.5
CLZ between analyses	15.6	16.8	9.1
	31.1	28.7	10.6
7-ACLZ within analysis	10	8.3	7.5
	50	49.1	4.1
7-AACLZ within analysis	10	10.1	10.4
	50	51.0	8.5

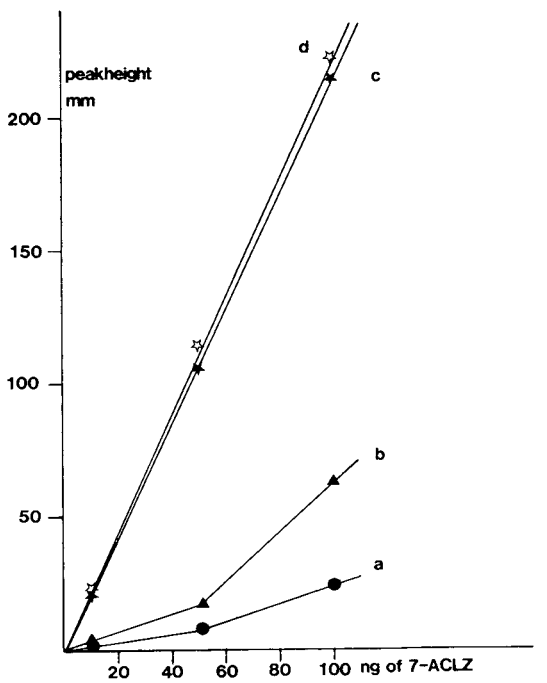

Fig. 4. Decompositon of 7-aminoclonazepam (7-ACLZ) in ethyl acetate and chloroform and the effect of 7-aminomethylclonazepam (7-AMCLZ, 5 μg) in preventing decomposition of 7-ACLZ in 7 ml of chloroform. Procedure according to test of stability: 10, 50 and 100 ng of 7-ACLZ stored for 1 h in (a) 7 ml of ethyl acetate, (b) 7 ml of chloroform, and (c) 7 ml of chloroform with 5 μg of protector (7-AMCLZ), compared with (d) 10, 50, 100 ng reference standards.

In contrast, 7-ACLZ was found to decompose with time when stored in chloroform, ethyl acetate and methanol at low concentrations. It was stable, however, for at least one month when stored in plasma.

At a concentration of 1 μg/ml 7-ACLZ in methanol there was a 10—20% decomposition of the compound when stored at —20°C over 48 h. During extraction of 7-ACLZ from patient plasma to chloroform, concentrations as low as 1 ng/ml in chloroform may occur. Decomposition of 7-ACLZ in chloroform and ethyl acetate at concentrations of 1—20 ng/ml is 80% and 90%, respectively, when stored for 1 h at room temperature (Fig. 4). Despite the decomposition of 7-ACLZ in chloroform this solvent was chosen for extraction of the metabolites instead of ethyl acetate since primary and secondary amines react with esters. 7-AMCLZ, a compound chemically very similar to 7-ACLZ, was tested as a protector and was found to be effective in preventing decomposition of 7-ACLZ (Fig. 4).

Recovery

The recoveries of CLZ and its metabolites were determined by comparison of peak heights of standards without extraction with standards extracted from plasma according to the analytical procedure. The recoveries of CLZ and its metabolites were 90% to 95% when extracting for 45 min.

Interferences

Concomitant treatment with other drugs, particularly antiepileptics, gives rise to a potential risk of interference since clonazepam is present at low concentrations. To test this possibility various drugs were dissolved in the mobile phase and injected into the chromatograph. The retention behaviour of these drugs is listed in Table II. None of the drugs listed in the table interfered with CLZ.

When plasma from patients treated with some of these drugs, notably phenobarbital, carbamazepine, phenytoin and nitrazepam, were processed according to the analytical method, no peaks interfering with CLZ or FLZ were detected.

TABLE II

CAPACITY FACTORS (k') OF VARIOUS COMMONLY USED DRUGS

Column: Supelcosil LC-18 (5 μm, 150 × 4.6 mm I.D.). Mobile phase: acetonitrile—0.1 *M* sodium acetate (35:65). Flow-rate: 3.0 ml/min. Unretained solute: distilled water.

Drug	k'	Drug	k'
Caffein	0.30	Nitrazepam	6.08
Theobromine	0.30	Lorazepam	6.45
Theophylline	0.30	Chlordiazepoxide	6.55
Ethosuximide	0.90	Clonazepam	7.25
Phenobarbital	1.35	Flunitrazepam	9.43
Carbamazepine	4.18	Nordiazepam	11.25
Phenytoin	4.35	Diazepam	19.55
Oxazepam	5.85	Valproic acid	Not detected

However, plasma from carbamazepine-treated patients contained a peak interfering with 7-ACLZ. This peak was identical to the carbamazepine-10,11-dihydrodiol metabolite. This problem can be circumvented by introducing an additional extraction step (see Analytical procedure).

Clinical application

Our HPLC method for CLZ has been used for routine analyses of patient plasma over a period of one year. The dose versus plasma concentration relationship observed is similar to that reported previously [7]. Although the value of clonazepam metabolite monitoring is doubtful we have measured 7-ACLZ and 7-AACLZ in seven patients. The preliminary data indicate that the levels of 7-ACLZ are in the same range as those of CLZ. 7-AACLZ was not measurable in these patient samples. The method can not be applied to plasma from patients treated with FLZ since this drug is used as internal standard. This is the only limitation that we are aware of so far. However, if the metabolites 7-ACLZ and 7-AACLZ are to be analysed, concomitant treatment with carbamazepine (and possibly other antiepileptic drugs) may give interfering peaks.

Conclusion

We have developed an HPLC system for the determination of both CLZ and its metabolites (7-ACLZ and 7-AACLZ) based on a single solvent extraction, reversed-phase column system and UV detection. To obtain good stability for 7-ACLZ it was necessary to add 7-AMCLZ as a protector in high concentration to the extraction solvent (Fig. 4). The selectivity of the column system enables other antiepileptic drugs and benzodiazepines to be well resolved and quantitated (Table II). The method can also be used without modification for the assay of CLZ and its metabolites in studies of CLZ metabolism in vitro in subcellular preparations of, for example, liver.

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CHROMBIO. 1951

SIMULTANEOUS ANALYSIS OF A NEW CARDIOTONIC AGENT, MDL 17,043, AND ITS MAJOR SULFOXIDE METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

MDL 17,043 or 1,3-dihydro-4-methyl-5-[4-(methylthio)benzoyl]-2H-imidazol-2-one, is a new cardiotonic agent being developed for the treatment of congestive heart failure. This communication describes a sensitive and selective analytical procedure for the simultaneous analysis of MDL 17,043 and its major oxidative metabolite in plasma. The method involves addition of internal standard and organic solvent extraction, followed by separation with high-performance liquid chromatography and detection by ultraviolet absorption. The assay has good precision and accuracy. Evidence for the positive identification of the sulfoxide metabolite is also presented.

INTRODUCTION

MDL 17,043, 1,3-dihydro-4-methyl-5-[4-(methylthio)benzoyl]-2H-imidazol-2-one (I) (Fig. 1) is a novel cardiotonic agent which is currently undergoing clinical evaluation in patients with congestive heart failure [1]. In vitro and in vivo studies in laboratory animals indicated that this compound possesses positive inotropic and vasodilatory activities [2, 3]. Its sulfur oxidation product, MDL 19,438 (II), has also been shown to have some qualitatively similar properties [4]. Biochemical studies have been carried out aiming to elucidate the mechanism of action of this new chemical entity on cardiac function. Results from these experiments suggested that the compound might exert its effect through the inhibition of cardiac high-affinity cyclic adenosine 5′-monophosphate (AMP) phosphodiesterase [5]. Previously an analytical method utilizing high-performance liquid chromatography (HPLC) in measuring plasma levels of I was reported [6]. In the course of preliminary

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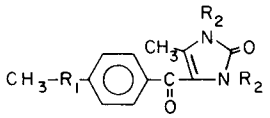


MDL 17,043 (I) $R_1 = S$ $R_2 = H$

MDL 19,438 (II) (sulfoxide metabolite) $R_1 = S{=}O$ $R_2 = H$

MDL 18,763 (III) (internal standard) $R_1 = O$ $R_2 = C_2H_5$

Fig. 1. Chemical structures of MDL 17,043 (I), sulfoxide metabolite (II) and the internal standard (III).

investigation of plasma samples from volunteers receiving the drug, an oxidative metabolite was detected in relatively high quantities. In this present communication, we describe an analytical procedure for the simultaneous measurement of I and its major metabolite in plasma. Evidence for the positive identification of this metabolite in human plasma is also presented.

EXPERIMENTAL

Reagents and chemicals

Ethyl acetate, acetonitrile and chloroform (all glass-distilled) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Methanol (HPLC grade) and ammonium hydroxide (reagent grade) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Glass-distilled water was used for the preparation of aqueous solutions. MDL 17,043 (I), the authentic sulfoxide metabolite (MDL 19,438) (II), and the internal standard (MDL 18,763) (III) (Fig. 1) were obtained from Merrell Dow Pharmaceuticals (Cincinnati, OH, U.S.A.).

Instrumentation

A component HPLC system (Waters Assoc., Milford, MA, U.S.A.) consisted of a Model 6000A pump, a WISP Model 710B auto-injector and a Model 440 UV absorption detector with a 313-nm filter. A pre-packed 25 cm × 4.6 mm I.D. Zorbax CN (6 μm particle size) HPLC column (DuPont, Wilmington, DE, U.S.A.) was operated with a methanol—water (45:55) mobile phase flowing at 1.0 ml/min. Detector output was recorded and chromatograms analyzed by a computer-automated laboratory system (CALS), (Computer Inquiry Systems, Englewood Cliffs, NJ, U.S.A.).

Electron impact mass spectrometry (EI—MS) was carried out using a Finnigan Model 3300 mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) operating at 70 eV. Sample was introduced by direct solid probe into the ionization chamber with gradual heating from ambient temperature to ca. 250° C for vaporization.

Plasma standard curve

A mixed stock solution of I and II was prepared by dissolving both compounds in methanol (300 μg/ml). Appropriate standard solutions were further diluted with methanol. Plasma standards were prepared by adding 0.1-ml aliquots of the standards to 1.9 ml of drug-free human plasma (NaEDTA anticoagulant) resulting in a range of concentrations as shown in Table I.

Internal standard solution was 6.0 μg/ml in methanol and 0.1 ml (600 ng) was added to each sample before extraction.

Validation study

To test the precision and accuracy of the assay, a validation study was carried out. For each run, a duplicate seven-point standard curve was processed. In three of the nine runs, plasma samples were analyzed that had added concentrations of I and II that were unknown to the analysts. In these particular samples, the concentrations of the compounds were not equal as they were in the standards. Rather, the relative concentrations were chosen to reflect the dynamic changes that might be expected during metabolism in vivo. Analyses were carried out by four analysts with three different groups of instruments so as to fully test variability.

Application of the method

To show the feasibility of the assay, oral solution doses of I in three graded single doses (1, 3 and 5 mg/kg) were given to three healthy volunteers. Periodic plasma samples taken from each subject were randomly coded and analyzed by this procedure. The analysts were unaware of the dose or sample time and the code was not broken until all of the samples were analyzed.

Extraction procedure

To each 2 ml of standard or unknown plasma sample were added 600 ng of internal standard in 0.1 ml of methanol. Then 3 ml of acetonitrile were added to each tube and vortexed to precipitate plasma protein. After centrifuging at ca. 900 *g* for 20 min, the supernatant was decanted into another 25-ml extraction tube which contained 9 ml of ethyl acetate. The compounds were extracted into the organic phase by shaking in an Eberbach reciprocating shaker for 20 min. After centrifuging for 5 min, 10 ml of upper organic layer were transferred to a 15-ml conical tube and placed in a heating block which was set at 50—55°C. The solvent was evaporated to dryness with the aid of a slow stream of nitrogen gas. For HPLC analysis, the extracted residue was redissolved in 100 μl of methanol, and 20 μl were injected into the HPLC column.

Calibration and calculations

The general form of the calibration equation was:

$$y^n = a + bx \qquad (1)$$

where y is the peak height ratio (expressed as percent by CALS) of either I or II divided by the internal standard, a is the y-intercept, b is the slope of the curve and x is the concentration of compound. Using an iterative fitting

technique, the value of n was calculated to effectively make $a = 0$ (± 0.00001) when the slope and intercept in eq. 1 were calculated using linear regression.

Isolation and purification of plasma sulfoxide metabolite

A 60-ml volume of pooled plasma obtained from the volunteers in the feasibility study was divided into four equal portions. Plasma protein was precipitated by the addition of 15 ml of acetonitrile to each. After centrifugation, the supernatants were combined and extracted with 80 ml of ethyl acetate twice. The two ethyl acetate phases were combined and evaporated to dryness. The residue was redissolved in 0.1 ml of methanol. This extract residue solution was spotted on a thin-layer chromatographic (TLC) plate (Silica gel 60-F-254, 0.25 mm thickness, G. Merck, Darmstadt, F.R.G.) and developed in a mobile phase which consisted of chloroform—methanol—ammonia (80:20:1). The sulfoxide was located by viewing the plate under UV light at 254 nm and comparing with the R_F of the authentic compound. The band of the TLC plate corresponding to the metabolite was then scraped off and extracted with methanol. The material was further purified by repetitive injections of 15—20 μl of the TLC-isolated component into the HPLC system as described above. The peak corresponding to the sulfoxide was collected and the mobile phase evaporated. The purified metabolite was redissolved in approximately 10 μl of methanol and placed in the solid probe and the methanol solvent evaporated prior to MS analysis.

RESULTS AND DISCUSSION

HPLC conditions

The DuPont Zorbax CN column chosen was ideal for this application. With a relatively non-polar C-8 column used previously [6], the sulfoxide metabolite, being more polar, was eluted almost unretained among several interfering endogenous plasma peaks in the solvent front. The CN column also eliminated the necessity of using a gradient system for the mobile phase. The HPLC conditions for the Zorbax CN column gave retention times for I, II and the internal standard that were about 6.8, 3.9 and 10.5 min, respectively. Figs. 2 and 3 show some typical chromatograms from extracted plasma samples.

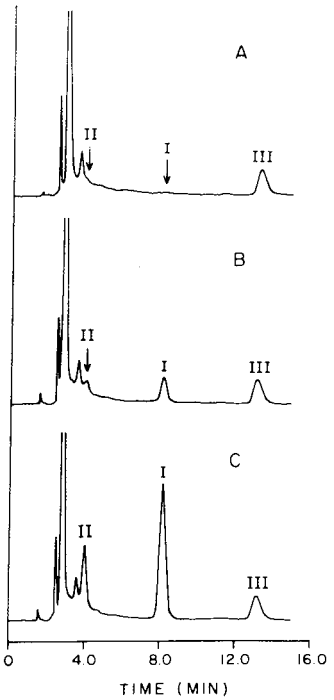
Extraction efficiency

Using the procedure described above, the extraction efficiencies of I and II from plasma at 500 ng/ml were 97.4% and 69.6%, respectively.

Validation study

Composite results for the nine standardization runs are tabulated in Tables I and II. The standardization runs show good linearity and reproducibility. For I, the mean value for the correlation coefficient was 0.99984 ± 0.00011 S.D., the coefficient of variation (C.V.) was 0.01%. The slope of the equation (b) was 0.63818 ± 0.049 S.D. (C.V. 7.8%) and the mean power-fit (n) value was 1.0026 ± 0.01036 S.D. (C.V. 1.0%).

For the sulfoxide metabolite, the mean value for the correlation coefficient was 0.99952 ± 0.0043 S.D. (C.V. 0.4%). The slope (b) was 0.31136 ±



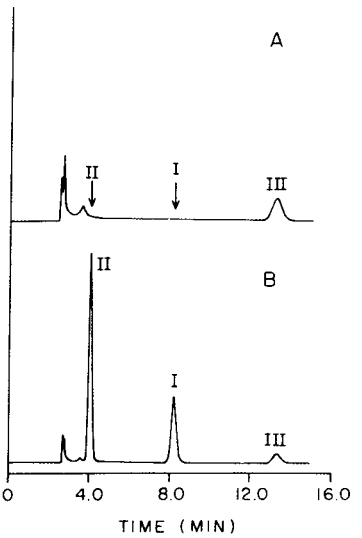


Fig. 2. Chromatograms of extracted plasma standards: (A) blank; (B) 125 ng/ml; (C) 750 ng/ml. Peaks: I = MDL 17,043; II = sulfoxide metabolite; III = internal standard.

Fig. 3. Chromatograms of extracted plasma samples from a patient receiving an oral 3 mg/kg dose of I: (A) pre-dose; (B) 2-h post-dose. Peaks as in Fig. 2.

TABLE I

I (MDL 17,043) STANDARDIZATION RESULTS FOR NINE RUNS

MDL 17,043 plasma concentration (ng/ml)	*n*	Mean percent peak height ratio	S.D.	C.V. (%)
0	18	0	—	—
62.5	18	38.3	1.80	4.7
125	18	78.3	3.53	4.5
375	18	232.9	11.39	4.9
750	16	464.1	18.61	4.0
1500	18	924.9	35.50	3.8
3000	17	1840.0	62.95	3.4

0.01507 S.D. (C.V. 1.5%) and the mean power fit (n) value was 1.01536 ± 0.15965 S.D. (C.V. 15.7%).

The accuracy of the assay was demonstrated by analyzing 58 unknowns in a randomly coded fashion. These results are shown in Tables III and IV. The mean overall accountability for I was 98.8% while that for the sulfoxide was 98.6%. At the extreme of added concentration differences, the presence of I at

TABLE II

II (MDL 19,438) STANDARDIZATION RESULTS FOR NINE RUNS

MDL 19,438 plasma concentration (ng/ml)	*n*	Mean percent peak height ratio	S.D.	C.V. (%)
0	18	0	—	—
62.5	18	18.4	3.92	21.3
125	18	35.8	3.73	10.4
375	18	107.8	8.52	7.9
750	16	215.9	10.75	5.0
1500	18	430.5	22.82	5.3
3000	17	839.4	35.70	4.3

TABLE III

ANALYSIS OF PLASMA SAMPLES CONTAINING UNKNOWN ADDED AMOUNTS OF I

Concentration added (ng/ml)	*n*	Mean concentration found (ng/ml)	S.D.	C.V. (%)	Recovery (%)
0	8	0	0	—	—
29.5	8	30.7	1.87	6.1	104.1
73.7	8	73.4	3.02	4.1	99.6
147	6	144.7	2.52	1.7	98.4
295	8	293.5	13.9	4.7	99.5
737	10	734.7	22.4	3.0	99.7
1473	10	1431.0	49.0	3.4	97.1

TABLE IV

ANALYSIS OF PLASMA SAMPLES CONTAINING UNKNOWN ADDED AMOUNTS OF II

Concentration added (ng/ml)	*n*	Mean concentration found (ng/ml)	S.D.	C.V. (%)	Recovery (%)
0	10	0	0	—	—
48.3	10	46.4	8.1	17.5	96.1
121	8	115.6	4.4	3.8	95.5
242	8	244.4	11.8	4.8	101.0
483	6	472.0	21.2	4.5	97.7
1208	8	1241.0	79.8	6.4	102.7
2415	8	2404.0	75.8	3.2	99.5

1473 ng/ml to plasma in the absence of any II did not show measurable II. Likewise, II added at 2415 ng/ml to plasma did not show measurable I when none was added. These results show that the procedure does not give oxidative or reductive changes that are likely to give artifically high or low values for either compound.

Identification of plasma sulfoxide metabolite

The solid probe EI mass spectra of the purified plasma metabolite and the authentic sulfoxide are shown in Fig. 4. Both mass spectra show an abundant

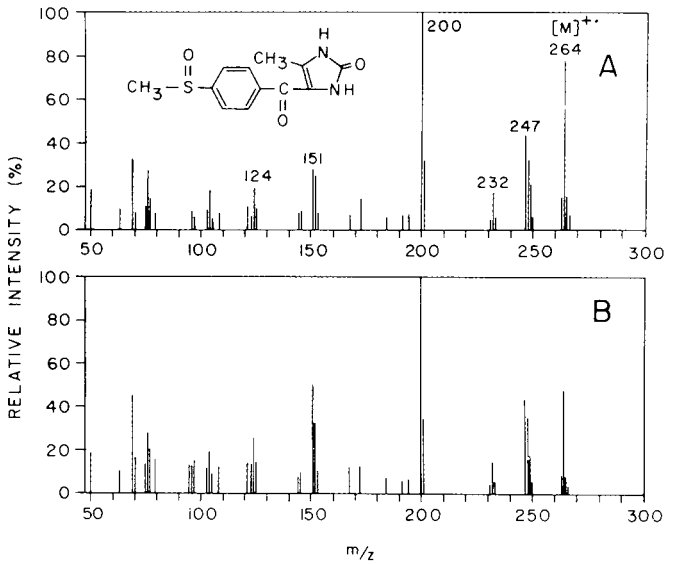


Fig. 4. Solid probe EI mass spectra: (A) authentic sulfoxide; (B) purified plasma metabolite.

molecular ion at *m/z* 264 as well as other similar characteristic fragments. Hence, from the evidence of identical TLC R_F values, HPLC retention times and solid probe mass spectra, it was concluded that the sulfoxide chemical structure was correctly assigned to the plasma metabolite.

Application of the method

Results of the feasibility study are shown in Fig. 5. I was found to be readily absorbed and showed appreciable plasma levels. Its sulfoxide metabolite levels were found to be even higher. The assay was judged to provide adequate sensitivity as the therapeutic dose was estimated to be about 3 mg/kg. The method has been applied successfully in studying single oral and intravenous doses in normal subjects as well as in patients with congestive heart failure.

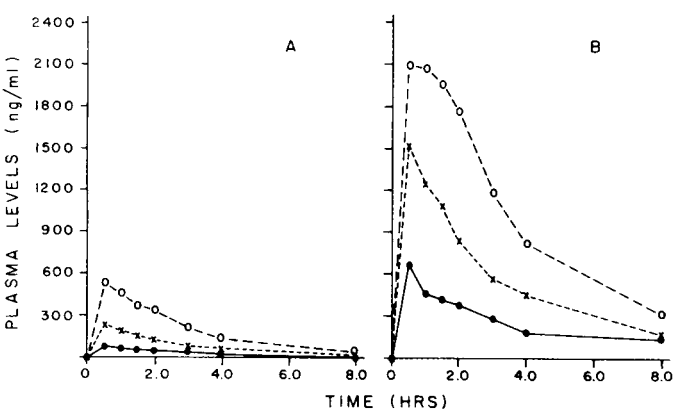


Fig. 5. Mean plasma concentrations of I (A) and the sulfoxide metabolite (B) after single oral doses of (•) 1 mg/kg; (×) 3 mg/kg; and (○) 5 mg/kg (n = 3).

TABLE V

CONCOMITANT MEDICATIONS TAKEN BY HEART PATIENTS THAT SHOW NO INTERFERENCE WITH THE HPLC ASSAY

Chlorthalidone	Isosorbide dinitrate
Clonidine · HCl	Methyldopa
Digitalis	Nitroglycerin
Digoxin	Phenazopyridine
Furosemide	Synthroid
Gantanol	Tolinase
Hydralazine	Zomepirac sodium
Hydrochlorothiazide	

Analysis of plasma from laboratory animals such as dogs and rats also showed the same levels of precision and accuracy.

Stability and interferences

Comparison of freshly prepared plasma standards with those prepared and kept frozen at −20° C showed no significant variations through a period of six months.

Control plasma samples were obtained from heart patients before they received therapy with I. Even though these patients, as a group, were receiving the concomitant medications listed in Table V, there were no substances present in the control samples that showed any potential interference with the analysis of I, II or the internal standard.

CONCLUSION

In conclusion, the validity of the HPLC method to simultaneously measure plasma concentrations of I and its sulfoxide metabolite was demonstrated. The sulfoxide metabolite present in human plasma was also detected in plasma and urine of other species such as rat and dog. Hence, oxidation of the sulfur atom in the molecule appears to be a common and important metabolic pathway of this compound.

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CHROMBIO. 1962

DETERMINATION OF *d*-ISOPROTERENOL SULPHATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

The application of reversed-phase high-performance liquid chromatography (HPLC) to the determination of isoproterenol sulphate in human plasma and urine was investigated. Sulphoconjugation of the inactive isomer of isoproterenol was chosen as an experimental model to study individual variations in the rate of sulphation of phenols in man. This approach allowed the ingestion of relatively large amounts of drug and detection of the conjugated material after acid hydrolysis, using alumina clean-up and HPLC with amperometric detection. This method was found to be rapid, sensitive, precise and suited to pharmacokinetic studies in man.

INTRODUCTION

The involvement of gut sulphoconjugation in the detoxification of phenols and catecholamines has been known for over a century [1]. Work by Richter [2] and Richter and MacIntosh [3] in the 1940s showed that in man 70% of an oral dose of adrenaline was excreted as the pharmacologically inactive suphate conjugate. More recently, the role of sulphate conjugation in pre-systemic elimination, has been recognised as a major pathway for the bio-transformation of phenolic drugs [4]; and this is of particular interest because of the limited capacity and susceptibility to competitive inhibition [5, 6].

Individual differences in the sulphate conjugation of paracetamol, shown by the skewed frequency distribution of the percentage of paracetamol sulphate excreted in the urine following oral dosing [7] and the report of two distinct forms of the sulphoconjugating enzyme, phenolsulphotransferase (EC 2.8.2.1,

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PST), in vitro [8, 9], lead us to speculate the possibility of an underlying polymorphism in gut sulphoconjugation.

Isoproterenol was chosen as a probe for sulphate conjugation of catecholamines in the gut, because it is a synthetic catecholamine available as the *d*-isomer, thus enabling a large oral dose to be given without pharmacological effect (*d*-isoproterenol has only 1% of the pharmacological potency of the *l*-isomer). It has the additional advantage of being both a good substrate for PST [10] and a poor substrate for the potentially competing enzyme monoamine oxidase [11]. Furthermore, earlier work in this department established that oral isoproterenol undergoes extensive pre-systemic metabolism in the gut wall with more than 80% of an oral dose of [^{3}H]isoproterenol being metabolised to isoproterenol sulphate [12—14].

The introduction of isoproterenol as a bronchodilator drug to treat asthma in the 1960s, led to the publication of several fluorimetric methods for its analysis [15, 16]. However, the definitive work on the pharmacology and metabolism of isoproterenol in man was achieved using radiolabelled isoproterenol, because at this time, scintillation counting of the tritium-labelled metabolites offered the most sensitive and specific approach [17]. Ultraviolet spectrophotometry [18] and gas—liquid chromatography with flame ionisation [19] have been applied to pharmaceutical dosage forms, but not to biological samples. More recently Kishimoto et al. [20] described a high-performance liquid chromatographic (HPLC) method involving post-column trihydroxyindole derivatization and fluorescence detection. This method utilised direct injection of urine and deproteinised plasma, but the combination of a 30-μm particle size cation-exchange column and the need for post-column derivatization lead to a long retention time for isoproterenol (24 min).

The reversed-phase ion-pairing HPLC procedure presented here employs N-methyldopamine as internal standard, a compound which is not normally administered or present as a metabolite in patients' plasma or urine. Isoproterenol sulphate is determined as isoproterenol following acid hydrolysis [21] and alumina batch extraction [22]. Following HPLC separation, the catechol groups are oxidised to orthoquinones using an amperometric detector, which also amplifies the current generated and displays this as a function of time on the chart recorder [23].

EXPERIMENTAL

Reagents and standards

N-Methyldopamine, *dl*-isoproterenol hydrochloride, 3′-phosphoadenosine-5′-phosphosulphate (PAPS) and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma (Poole, U.K.). *d*-Isoproterenol-*d*-bitartrate (98% pure by thin-layer chromatography, TLC) was a gift from Sterling Winthrop (Surbiton, U.K.). AnalaR methanol purchased from May and Baker (Dagenham, U.K.) was found satisfactory. [^{35}S]PAPS, 1.9 Ci/mmol (NEG-010) was obtained from New England Nuclear (Boston, MA, U.S.A.) and *dl*-[7-^{3}H]isoproterenol hydrochloride, 12.6 Ci/mmol (TRK 295) from Amersham International (Amersham, U.K.). All other chemicals were of analytical reagent grade and obtained from BDH chemicals (Enfield, U.K.). The activated alumina (Brockmann grade 1,

active, neutral) was prepared as previously described [24]. The internal standard, N-methyldopamine, was prepared as a 1 μg/ml solution in 0.1 *M* hydrochloric acid and stored at 4°C. A stock standard of 6 mg/ml *d*-isoproterenol-*d*-bitartrate was similarly prepared. Standard curves of subdiluted stock standard were prepared in isoproterenol-free urine and plasma.

Synthesis of isoproterenol sulphate

Since isoproterenol sulphate was not commercially available, it was synthesized from isoproterenol and PAPS in a reaction catalysed by partially purified rat liver PST. The isolation of PST was essentially that of Gregory and Lipmann [25] and the preparation of isoproterenol sulphate was based on the method of Foldes and Meek [26]. Initially, [^{35}S]PAPS was used in the reaction to optimise the production and separation of the sulphate conjugate. Once achieved this was replaced by [^{3}H] isoproterenol and unlabelled PAPS, in order to produce [^{3}H] isoproterenol sulphate, needed for hydrolysis recovery studies. The reaction was carried out with 1 m*M* *dl*-isoproterenol hydrochloride (containing 252 mCi of *dl*-[^{3}H] isoproterenol hydrochloride) at pH 8.0 and the product was separated from the starting material by silica gel TLC using butanol—0.880 ammonia—ethyl acetate (60:20:40) as the solvent system. Only two radioactive peaks were detected: the starting material, [^{3}H] isoproterenol with an R_F of 0.71 and the product presumed to be [^{3}H] isoproterenol sulphate, having an R_F of 0.32. This latter TLC band contained 68% of the radioactivity of the starting material. Acidic hydrolysis of the product gave a single radioactive peak with an R_F identical to that of [^{3}H] isoproterenol and all of the radioactivity of the conjugate was recovered. The tritium-labelled conjugate was not extracted by activated alumina, this providing additional evidence that one of the phenolic hydroxyl groups is replaced by sulphate. In combination, these findings provide good evidence for the successful synthesis Downers Grove, IL, U.S.A.).

Equipment

The liquid chromatograph comprised an Altex 100A solvent delivery pump, an Altex 210 injection valve fitted with a 100-μl sample loop and a 150 × 4.6 mm I.D. stainless-steel analytical column packed with 5-μm diameter Altex Ultrasphere octyl particles (Altex Scientific, Berkeley, CA, U.S.A.). The detection system comprised a Model LC4 amperometric detector fitted with a TL5 glassy carbon electrode assembly (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The column, amperometric detector and electrode were enclosed in a Faraday cage of aluminium, itself earthed to the Servoscribe RE 541.20 chart recorder (Smiths Industries, London, U.K.). For TLC work, LK 5 DF silica gel TLC plates (Whatman, Maidstone, U.K.) were used together with a TLC Chromatank (Shandon Southern, Runcorn, U.K.). Radiochromatograms were obtained using a Packard Model 7201 scanner (Packard Instruments, Downers Grove, IL, U.S.A.).

Chromatography

The mobile phase consisted of a citrate—phosphate (McIlvaine) buffer adjusted to pH 6.0, containing 3% v/v methanol and the disodium salt of EDTA

at a final concentration of 2 m*M*. The buffer was composed of 300 ml of 0.1 *M* citric acid and 150 ml of 0.1 *M* disodium hydrogen phosphate per l of glass-distilled water. Solvent was filtered through a 0.5-μm Gf/f glass microfibre filter (Whatman) and helium-degassed (BOC Special Gases, London, U.K.) prior to use. The HPLC system was maintained in continuous use at a flow-rate of 0.1 ml/min and the flow increased to 1.0 ml/min prior to sample work-up and injection. The amperometric detector was typically used at a sensitivity of 10 nA full scale and an applied potential difference of +0.50 V vs. Ag/AgCl reference electrode.

Optimisation of chromatography

In Moyer and Jiang's paper [27] the optimal mobile phase for analysis of catecholamines by HPLC with amperometric detection was established as being in the pH range 5.0—6.5, with an ionic strength in the region of 0.07 *M* (phosphate ion) and with an ion-pair such as heptanesulphonate at 5.0 m*M*. Our previous experiences with reversed-phase ion-pairing HPLC systems for the determination of plasma and urinary catecholamines [28—31] suggested that a reduction in both ion-pair concentration and methanol content of the mobile phase, would be necessary to achieve a practical retention time for the less polar synthetic catecholamines isoproterenol and N-methyldopamine.

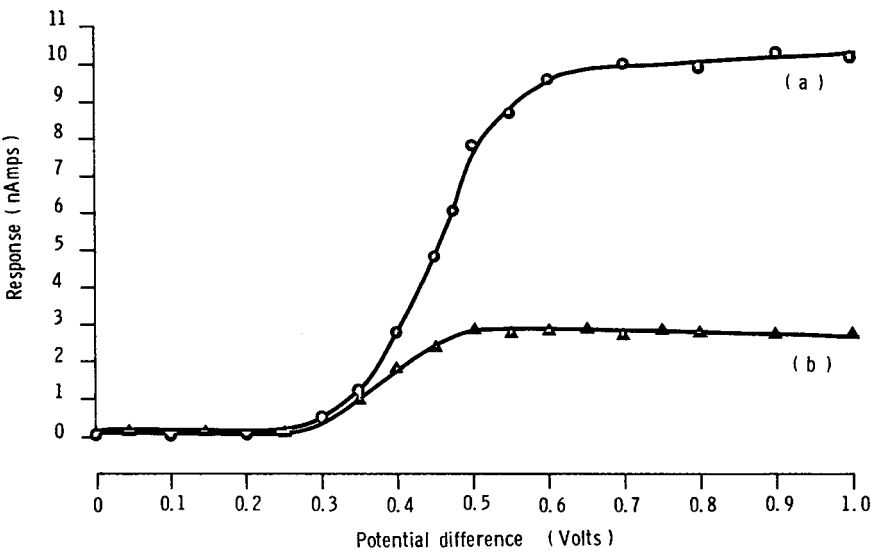

Fig. 1. Hydrodynamic voltammograms for isoproterenol (a) and N-methyldopamine (b) in mobile phase pumped at a flow-rate of 1.0 ml/min.

Hydrodynamic voltammograms for isoproterenol and N-methyldopamine were determined over the range 0—1.0 V vs. Ag/AgCl reference electrode, in the mobile phase described (Fig. 1). An applied electrode potential of +0.50 V was chosen (as in Moyer and Jiang's paper [27]) as this provided sufficient sensitivity for the measurement of plasma and urinary isoproterenol sulphate, but with minimal interference from solvent oxidation and electrical noise.

Sample handling and investigation protocol

Four healthy volunteers (mean age 27.5 years) were asked to avoid headache and cold medicines containing paracetamol or ascorbic acid for 12 h prior to dosing and to avoid eating breakfast, but they were allowed to eat lunch 3 h after taking the dose. On the day of the investigation subjects emptied their bladders at 10 a.m., a butterfly cannula was inserted into a forearm vein and kept patent using heparinised saline. A baseline blood sample (10 ml) was drawn into a lithium heparin tube and the oral dose of *d*-isoproterenol-*d*-bitartrate (1 mg/kg) was given in 330 ml of a proprietary drink, which contained no ascorbic acid [32]. A 24-h urine collection was started and further blood samples were taken at 30-min intervals up to 6 h. A second 24-h urine was started as the first collection finished at 10 a.m. the next day. The protocol for this study was approved by the Research Ethics Committee of the Royal Postgraduate Medical School and Hammersmith Hospital and all subjects gave their informed consent.

The blood samples were centrifuged at 2250 *g* for 15 min at 4°C and the separated plasma stored at −70°C until assayed.

Hydrolysis of isoproterenol sulphate

The unimolecular acid-catalysed hydrolysis of isoproterenol sulphate proved to be simple, quick, inexpensive and quantitative with 98% recovery of [^{3}H]isoproterenol sulphate spiked into plasma and urine. Sulphatase (H-1, from *Helix pomatia*) catalysed hydrolysis of [^{3}H]isoproterenol sulphate was also quantitative from urine but less than 20% efficient with plasma samples. The method of acid hydrolysis involved mixing 2 ml of plasma or urine with 2 ml of 1.0 *M* hydrochloric acid and placing the mixture in a boiling water bath for 30 min. Portions of hydrolysed plasma (2 ml) were transferred into extraction tubes while still warm to avoid gel formation on cooling. Hydrolysed urines were diluted 1:50 and 2-ml aliquots of the dilution taken for extraction.

Validation of sulphoconjugate

Phenolic compounds can be conjugated with glucuronic acid, sulphate or both. Previous work on isoproterenol has indicated very little glucuronide conjugation [17]. Evidence that the compound was a sulphate and not a glucuronide was obtained using sulphatase-catalysed hydrolysis in the presence of the glucuronidase inhibitor saccharic acid 1:4 lactone [33]. This was found to be without effect on the sulphatase-catalysed hydrolysis of the conjugate at concentrations up to 10 mg/ml, indicating that sulphate is the only appreciable conjugate of isoproterenol.

Extraction of isoproterenol

The hydrolysates (2 ml) were placed into conical polystyrene tubes (Sarstedt, Leicester, U.K.) containing 60 mg of activated alumina, 1 ml of 0.1 m*M* Na_2EDTA with 1 m*M* hydrochloric acid and 100 μl of 1 μg/ml N-methyldopamine (internal standard). The pH was adjusted to 8.6 by the addition of 1 ml of 3 *M* Tris—HCl buffer and the tubes were mixed on a Spiramix roller mixer (Denley, Billinghurst, U.K.) for 15 min. The alumina was allowed to settle and the supernatants were aspirated at the sink followed by three washes

with distilled water (pH 7.0). The final aspiration was taken to dryness and the catecholamines were eluted in 200 μl of 0.1 *M* orthophosphoric acid by roller mixing for 2 min. Fines of alumina were centrifuged down (2200 *g*, 4°C, 5 min) and the supernatants finally transferred into Eppendorf tubes (1.5 ml). The supernatants were stored at −20°C for up to one week, if not chromatographed on the same day.

Injection volumes

In order to achieve on-scale peaks for all samples, 50-μl aliquots of hydrolysed urine (diluted 1:100 overall) or hydrolysed plasma (diluted 1:2 overall) were injected into the HPLC system. For unhydrolysed urines 25-μl and for unhydrolysed plasma 100-μl portions of supernatant were injected.

Quantitation

Measurement of *d*-isoproterenol was by comparison of the peak height ratio of isoproterenol to N-methyldopamine in the sample, to that obtained from authentic standards prepared in drug-free plasma or urine, extracted and chromatographed in the same way. At least five such standards were run with each batch of samples.

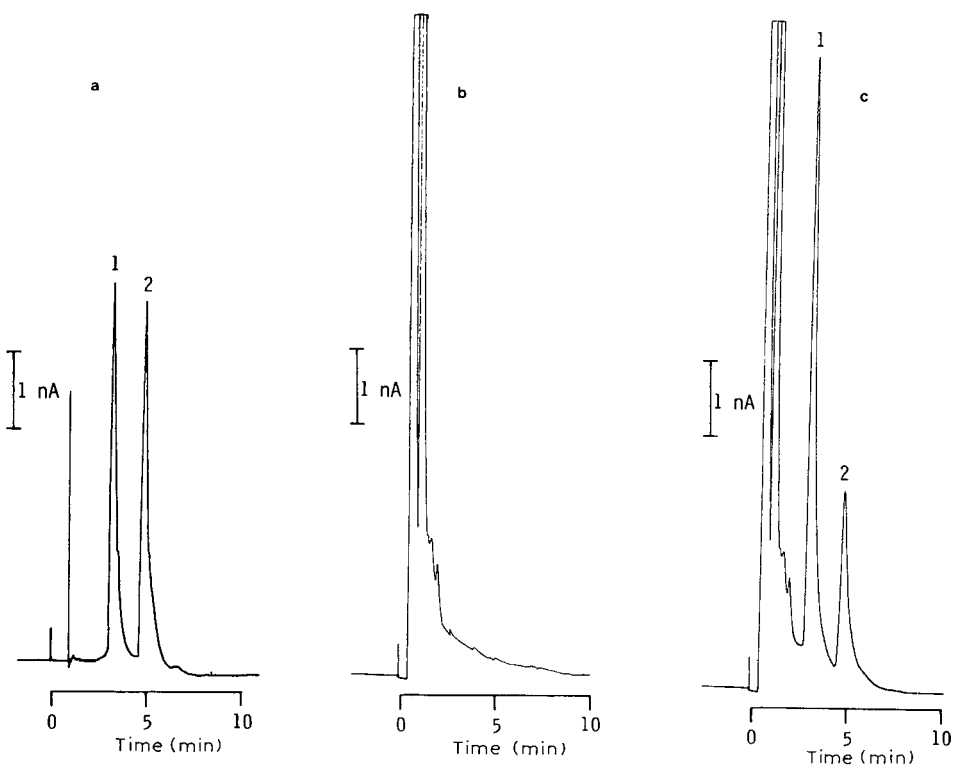

Fig. 2. Chromatograms showing HPLC—amperometric assay of plasma *d*-isoproterenol. (a) Standard mixture containing 100 ng each of N-methyldopamine (1) and *d*-isoproterenol (2); (b) plasma from a drug-free subject; (c) plasma from a subject 30-min post-dose of 1 mg/kg *d*-isoproterenol. Concentration of free isoproterenol is 20 ng/ml.

RESULTS AND DISCUSSION

Chromatography

Resolution and sensitivity of the chromatographic system were determined for each assay by injection of a reference solution containing *d*-isoproterenol and N-methyldopamine. A typical chromatogram obtained from such an injection is shown in Fig. 2a. Chromatograms produced by extracted plasma samples are shown in Fig. 2b and c and Fig. 3, while representative chromatograms obtained from extracted 24-h urine collections are given in Fig. 4.

Validation of the *d*-isoproterenol peak in plasma and urine samples from dosed subjects was obtained by the finding of co-chromatography with authentic *d*-isoproterenol.

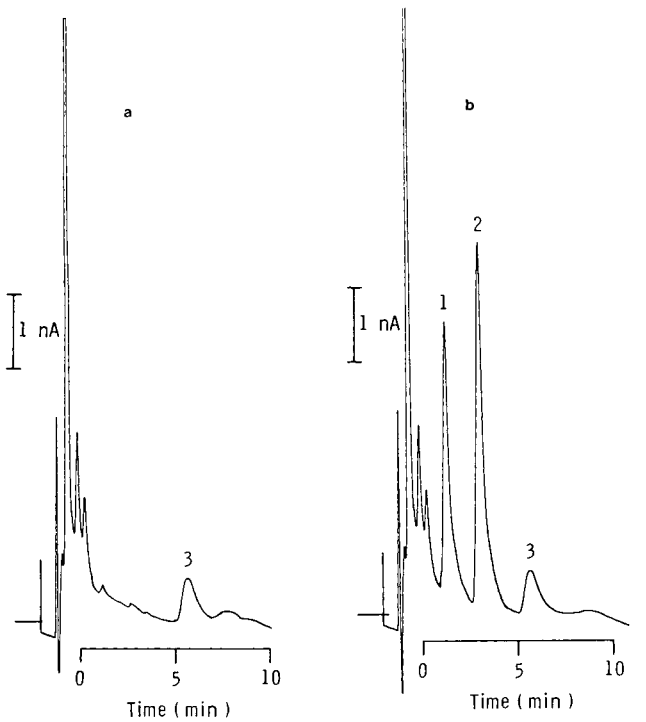

Fig. 3. Chromatograms showing HPLC—amperometric assay of *d*-isoproterenol in hydrolysed plasma. (a) Plasma from a drug-free subject; (b) plasma from a subject 30-min post-dose of 1 mg/kg *d*-isoproterenol. Concentration of isoproterenol sulphate is 196 ng/ml. For peaks 1 and 2 see Fig. 2. Peak 3 is an unassigned peak only present in hydrolysed samples.

Selectivity

The possibility of endogenous interferences in plasma or urine was investigated at the retention times of *d*-isoproterenol and N-methyldopamine (Table I). No interference was seen in the chromatograms of samples extracted without added internal standard or in samples from isoproterenol-free subjects. However, in the chromatograms of acid-hydrolysed samples an extra peak (labelled 3 in the chromatograms) was seen after the isoproterenol peak, with a retention time of 7.6 min. This peak was not related to the dose of iso-

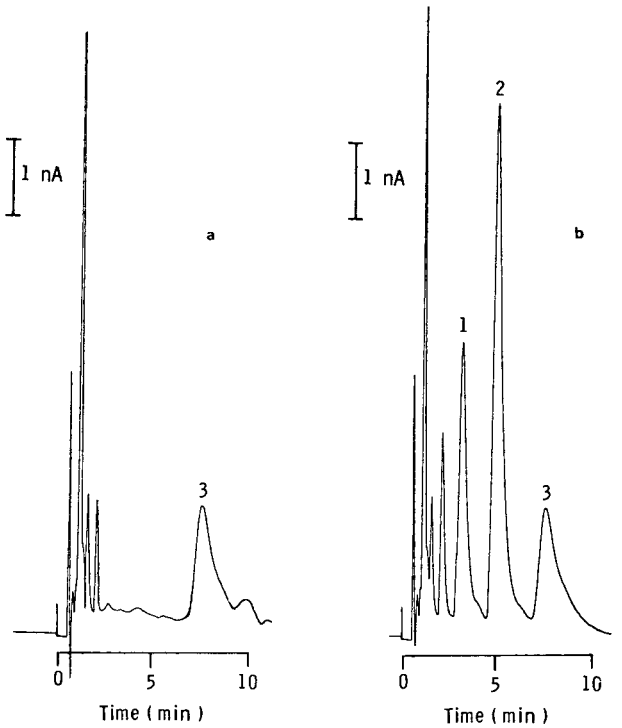

Fig. 4. Chromatograms obtained from extracted hydrolysed urines. (a) 24-h urine from a drug-free subject; (b) 24-h urine from a subject who received 1 mg/kg *d*-isoproterenol at the start of the 24-h period. Peaks: (1) N-methyldopamine; (2) *d*-isoproterenol; (3) unassigned hydrolysis peak.

TABLE I

RETENTION TIMES OF CATECHOL COMPOUNDS

For conditions, see text.

Compound	Retention time (min)
3,4-Dihydroxymandelic acid (DHMA)	0.7
3,4-Dihydroxyphenylacetic acid (DOPAC)	0.9
3,4-Dihydroxyphenylalanine (DOPA)	1.0
3,4-Dihydroxyphenylglycol (DHPG)	1.4
Dopamine	1.8
N-Methyldopamine	3.1
3-Methoxy-4-hydroxyphenylglycol (MHPG)	4.2
Isoproterenol	5.3
Unassigned hydrolysis-related peak	7.6
3-O-Methylisoproterenol	15.8

proterenol given and was present in acid-hydrolysed samples from drug-free subjects. It is therefore concluded that the peak derives from an endogenous acid labile conjugate. Since it it is well resolved from N-methyldopamine and isoproterenol the peak does not interfere with the measurement of

isoproterenol, other than to increase analysis time (in hydrolysed samples) to 10 min, in order to ensure its elution before the next sample is injected.

Precision

The intra-assay precision of the method was determined by replicate analysis of a drug-free plasma and urine pool spiked with authentic *d*-isoproterenol. This gave a coefficient of variation of 2.6% for plasma ($\overline{X}$ = 632.6 ng/ml, S.D. = 16.286, n = 15) and 3.1% for urine ($\overline{X}$ = 50.26 mg per 24 h, S.D. = 1.541, n = 15).

The inter-assay precision was measured by analysis of the same spiked samples, one in each of six separate assays. This gave a coefficient of variation of 4.6% for plasma ($\overline{X}$ = 617.0 ng/ml, S.D. = 28.239, n = 6) and of 7.1% for urine ($\overline{X}$ = 49.48 mg per 24 h, S.D. = 3.54, n = 6).

Calibration

Standard curves were found to be linear over the range 1 ng/ml to 50 μg/ml *d*-isoproterenol, with a correlation coefficient of 0.9996 and linear-regression equation of $y = 7.762x + 0.177$.

Limit of detection

With the amperometric detector at a sensitivity of 10 nA full scale and an applied potential of +0.50 V vs. Ag/AgCl reference electrode, the limit of detection was 0.5 ng/ml at a signal-to-noise ratio of 2.0. This detection limit could be extended into the picogram range by increasing the detector amplification.

Recovery

The absolute analytical recovery of *d*-isoproterenol and N-methyldopamine for human plasma and urine was estimated by comparing the peak heights obtained from the injection of known quantitites of the compounds, with peak heights obtained from the injection of extracts of plasma and urine samples spiked with the analytes. This gave values of 71% for *d*-isoproterenol and 68% for N-methyldopamine (n = 8).

Pharmacokinetics

Practical application of the proposed method was tested in human plasma and urine samples, collected from subjects following the oral ingestion of *d*-isoproterenol. A limited dose/response (d/r) curve was undertaken in one subject (range 0.5—1.5 mg/kg) and plasma isoproterenol sulphate determined by HPLC with amperometric detection (Fig. 5). The peak plasma isoproterenol sulphate concentration occurred between 1.5 and 2.0 h post-dose and increased in a dose-dependent fashion. Plasma isoproterenol sulphate—time curves in four subjects, following the oral dose of 1 mg/kg *d*-isoproterenol-*d*-bitartrate are shown in Fig. 6.

The area under the plasma concentration—time curve (AUC) was calculated assuming monoexponential kinetics and applying a computer program using the trapezoidal rule. Although these kinetics are not proven, the AUC values thus calculated should be a good approximation, even if there is a longer

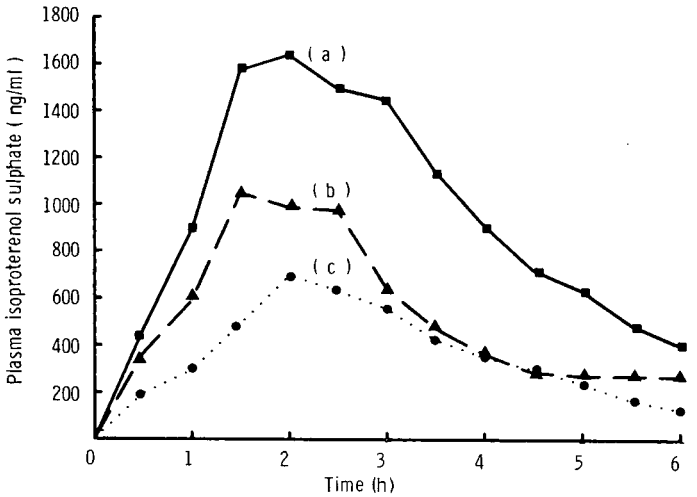

Fig. 5. Dose/response curves of plasma isoproterenol sulphate following (a) 1.5 mg/kg; (b) 1.0 mg/kg; and (c) 0.5 mg/kg *d*-isoproterenol-*d*-bitartrate as an oral dose.

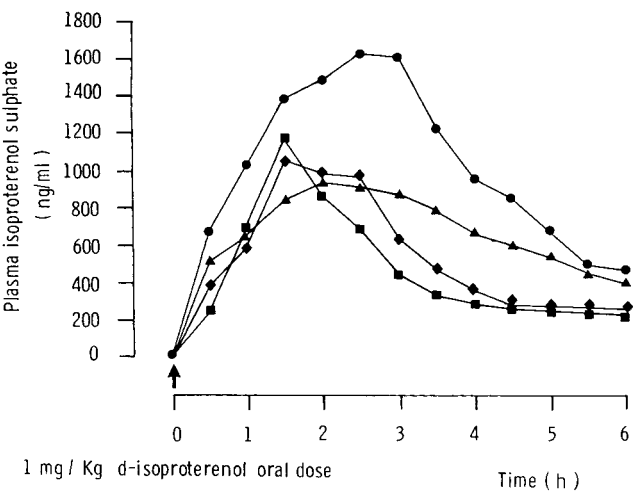

Fig. 6. Plasma isoproterenol sulphate—time curves in four healthy subjects after an oral dose of 1 mg/kg *d*-isoproterenol-*d*-bitartrate.

terminal phase in the elimination. The renal clearances were estimated from the venous plasma AUC values and take no account of possible arterio—venous concentration differences [34]. A results summary showing the urinary values together with derived pharmacokinetic parameters is given in Table II.

CONCLUSIONS

The determination of isoproterenol sulphate as isoproterenol by HPLC with amperometric detection, following acid hydrolysis of the conjugate and alumina batch extraction, gave excellent reproducibility and a short chromatography time of 6 min per sample.

TABLE II

PHARMACOKINETIC PARAMETERS FOLLOWING A 1 mg/kg DOSE OF *d*-ISOPROTERENOL-*d*-BITARTRATE IN HEALTHY HUMAN SUBJECTS

Subject No.	Oral dose excreted as isoproterenol sulphate (%)	Plasma isoproterenol sulphate AUC* (μg/ml min)	Renal clearance of isoproterenol sulphate** (ml/min)
1	57	243	171
2	41	169	154
3	76	242	242
4	99	368	166

*Calculated by the trapezoidal rule.
**Calculated using venous blood samples.

The single subject d/r curve shows that 1 mg/kg *d*-isoproterenol does not saturate the gut PST and is therefore suitable for studies of genetic polymorphism in PST. Plasma isoproterenol sulphate AUC values were proportional to dose and there was no significant change in the percentage of the dose excreted as the sulphoconjugate, as the dose increased from 0.5 to 1.5 mg/kg.

In four healthy volunteers considerable variation in the percentage of the 1 mg/kg dose, excreted as isoproterenol sulphate, was observed. However, very little free isoproterenol was found in the urine and it did not show the same degree of variation as the sulphoconjugate. Some evidence for the renal secretion of *d*-isoproterenol sulphate was obtained from an estimation of its renal plasma clearance, this being greater than the glomerular filtration rate in the period 0—6 h post-dose. The slow decline in plasma levels between 4 and 6 h post-dose may be attributed to a longer terminal phase and this could be investigated by collecting blood samples for longer periods of time. Prolongation of oral absorption may also account for the observed data. Further characterisation of the pharmacokinetics of *d*-isoproterenol sulphate thus requires the collection of more blood samples after 6 h and the investigation of a greater number of volunteers.

These preliminary results confirm that conjugation with sulphate accounts for a major proportion of the pre-systemic elimination of oral *d*-isoproterenol. Excretion of free isoproterenol did not account for a high percentage of the dose. It seems likely that low sulphate conjugation may be accompanied by increased O-methylation, and in future studies we plan to measure the plasma and urinary isoproterenol sulphate/3-O-methylisoproterenol ratio.

The HPLC amperometric method described in this paper has been shown to be suitable for studies on the conjugation of isoproterenol with sulphate. Since isoproterenol is almost exclusively sulphate-conjugated, it should provide a handle on the study of inter-individual variation in sulphation of phenols by man, in combination with the measurement of PAPS and sulphate. These studies will complement those on paracetamol — a drug which is conjugated equally with sulphate and glucuronic acid and which can also be measured by HPLC with amperometric detection [35].

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DETERMINATION OF LIDOCAINE AND ACTIVE METABOLITES IN BLOOD SERUM BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Oxidation of lidocaine and its principal metabolites, monoethylglycine xylidide and glycine xylidide, at glassy carbon electrodes was employed to permit electrochemical detection of these compounds following separation by high-performance liquid chromatography. The absolute detection limits found for these compounds were 2 ng, 5 ng, and 4 ng injected, respectively. The resulting assay was suitable for the routine quantitation of lidocaine and these metabolites in blood serum over the entire therapeutic range, 1—6 μg/ml. Total analysis time is 10—15 min.

INTRODUCTION

Lidocaine (Fig. 1) belongs to a group of aromatic amides widely used as local anesthetics. Lidocaine also possesses antiarrhythmic properties and is frequently used as a therapeutic agent in the treatment of cardiac disorders. For lidocaine to be effective in this latter capacity, it is necessary to achieve blood serum levels of 1—6 μg/ml [1, 2]. Below this range, the drug is ineffective, whereas serum levels greater than 6 μg/ml produce toxic effects. Two of its metabolites, monoethylglycine xylidide (MEGX) and glycine xylidide (GX) (Fig. 1), also possess antiarrhythmic activity and/or central nervous system

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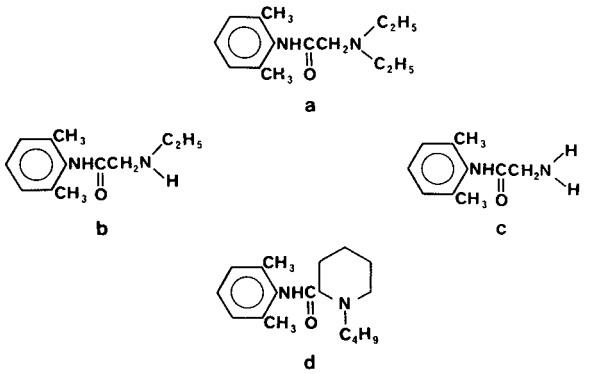


Fig. 1. Structure of (a) lidocaine, (b) MEGX, (c) GX, and (d) bupivacaine.

toxicity [3—6]. Because significant concentrations of these metabolites may be achieved in patients receiving lidocaine treatment, it is desirable to monitor serum levels of all three compounds conveniently and accurately.

Numerous gas—liquid chromatographic approaches have been described for this purpose [7—14]. These methods, employing flame ionization, mass spectroscopic, or nitrogen—phosphorus detection, have been shown to possess excellent sensitivity for lidocaine, generally in the 1—10 ng/ml range. But, in many procedures, the metabolites MEGX and GX either are not determined at all or are not separately distinguished from lidocaine. More importantly, the application of all of these approaches to the routine analysis of real serum samples is limited by the sample preparation time taken up by the lengthy derivatization, extraction, or evaporation/preconcentration procedures required. Recently, several high-performance liquid chromatography (HPLC) methods using ultraviolet (UV) detection have been employed [15—18]. Unfortunately, as these compounds contain no chromophore which absorbs strongly in the visible or near-UV regions, the wavelength employed for detection must be approximately 200 nm. The most sensitive of the HPLC methods reported to date has a detection limit of 20 ng/ml (or 2 ng injected) for lidocaine, an order of magnitude poorer than the most sensitive of the gas chromatographic methods [16]. Sample preparation for HPLC is generally less lengthy and involved than that case for gas chromatography; however, a minimum of two extraction steps is still required even for the simplest HPLC procedure.

In recent years, amperometric electrochemical detection following liquid chromatography (LC—ED) has become increasingly popular for the quantitation of easily oxidizable analytes. Its principal advantages include uniformly high sensitivity and a unique selectivity toward compounds that can be electrolyzed at the applied detector potential [19]. To our knowledge, the electrochemical behavior of lidocaine, MEGX, and GX has not yet been reported. But, since these compounds all possess highly substituted aromatic centers, they might be expected to undergo oxidation readily. In this work, we will describe the anodic electrochemistry of these compounds at graphite electrodes and the development of an LC—ED procedure for their determination. Sufficient selectivity and sensitivity have been attained to permit the

procedure to be useful for routine clinical monitoring of lidocaine and its metabolites in serum with minimal sample preparation.

EXPERIMENTAL

Reagents

Lidocaine, MEGX, and GX were obtained from Astra Pharmaceutical; bupivacaine (Marcaine®), used as an internal standard, was obtained from Sterling-Winthrop Research Institute, a division of Sterling Drug. All compounds were provided as HCl salts. HPLC grade water and acetonitrile were purchased from Burdick & Jackson Labs. Buffer salts, ammonium hydroxide, and phosphoric acid were purchased from Fisher Scientific and were used as received without further purification.

Voltammetry

Cyclic voltammetry was performed with a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model CV-1B potentiostat. Either a glassy carbon or a carbon paste working electrode, a saturated Ag/AgCl reference electrode, and a platinum wire counter electrode were used for all experiments. The supporting electrolyte consisted of 40 m*M* ammonium phosphate adjusted to the desired pH or a binary mixture of this buffer and acetonitrile. Experiments were performed at a scan rate of 30 mV/sec.

Chromatography

The HPLC system consisted of a DuPont (Wilmington, DE, U.S.A.) Model 8800 pump, a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 50-μl sample loop, a Bioanalytical Systems LC-4B amperometric detector equipped with a Model TL-5 thin-layer glassy carbon working electrode and an Ag/AgCl reference electrode, and a Spectra-Physics (Arlington Heights, IL, U.S.A.) Model SP4100 computing integrator.

Chromatographic experiments were performed on a 30-cm, 10-μm silica column (Regis, Morton Grove, IL, U.S.A.). The mobile phase consisted of acetonitrile—40 m*M* ammonium phosphate (pH 7.8) (22—25:75—78). A 3-cm silica cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.) was placed ahead of the analytical column as a guard. The flow-rate used for all experiments was 2.0 ml/min.

Sample preparation

A fresh octadecylsilane Sep-Pak cartridge (Waters Associates, Milford, MA, U.S.A.) was initially prepared by flushing with 6 ml of acetonitrile and then 6 ml of water. A 100-μl volume of bupivacaine internal standard was added to 1 ml serum, followed by 400 μl of water (or lidocaine and metabolites for spiked samples). The tube containing these constituents was vortex-mixed for 30 sec. Three drops of 0.15 *M* ammonium hydroxide were then added to convert the compounds to their unprotonated forms. After the solution was again vortex-mixed, the entire sample was passed through the Sep-Pak cartridge. The Sep-Pak was flushed with 3 ml of water, and the components of interest were eluted with 2 ml of acetonitrile. The eluent was then injected into

the HPLC system through a Swinney filter containing a 13-mm diameter, 0.45-μm Nylon 66 filter.

RESULTS AND DISCUSSION

Electrochemistry

Numerous previous investigations [20] have focused on the anodic electrode reactions of aromatic amines and many derivatives substituted variously at both nitrogen and ring positions. These compounds generally exhibit anodic waves within the potential range between +0.5 and +1.0 V vs. Ag/AgCl. These waves, which presumably involve an initial one-electron oxidation to produce a cation radical with the charge residing primarily on the amine nitrogen, are nearly always irreversible because of rapid follow-up coupling reactions that produce dimeric and polymeric products. To our knowledge, no information has yet been reported for aromatic amides directly related to lidocaine or the other compounds of interest here. However, some general similarities to aromatic amines were expected for these compounds.

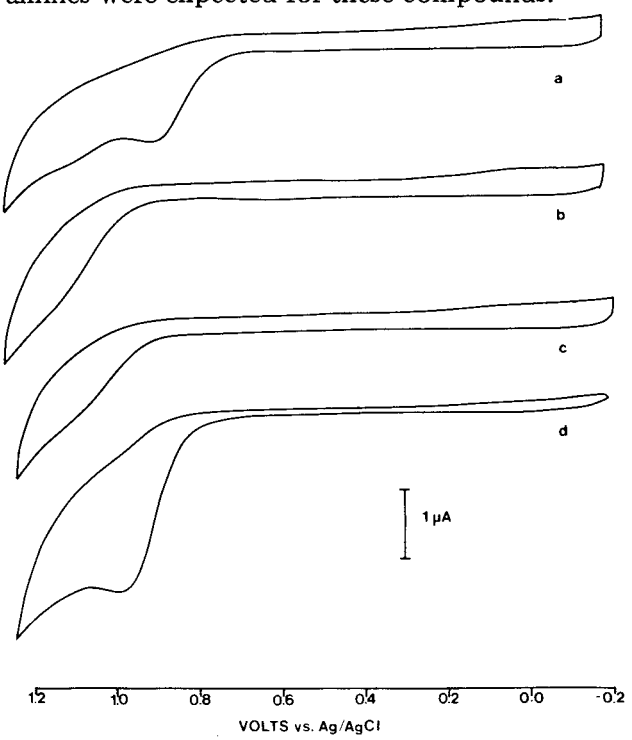


Fig. 2. Cyclic voltammograms of (a) $1.0 \cdot 10^{-4}$ *M* lidocaine, (b) $1.0 \cdot 10^{-4}$ *M* MEGX, (c) $1.0 \cdot 10^{-4}$ *M* GX, and (d) $1.0 \cdot 10^{-4}$ *M* bupivacaine at a glassy carbon electrode. Electrolyte—buffer: 10% acetonitrile—90% ammonium phosphate (pH 7.4).

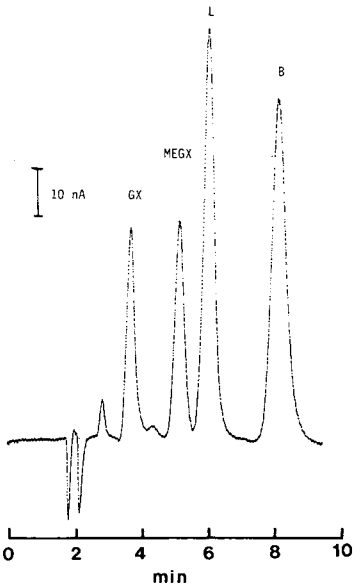
Fig. 2 shows the cyclic voltammograms obtained for lidocaine, MEGX, GX, and bupivacaine, which was used as an internal standard in this assay. (The structure of bupivacaine is also shown in Fig. 1.) All exhibited irreversible

oxidation waves; at pH 7.4 the peak potentials for lidocaine and bupivacaine occurred betweeen +0.90 and +1.00 V vs. Ag/AgCl while those for GX and MEGX occurred only at significantly higher potentials in the vicinity of +1.10 V. In fact, at the analyte concentrations employed, the anodic waves for the latter compounds were only barely observed before the onset of solvent oxidation. In all cases, no reduction waves were evident on the reverse cathodic scan. Subsequent cycles over the same potential range showed no drastically different behavior. All of the oxidation waves were found to shift to higher potentials as the pH was decreased, with the shift for lidocaine amounting to approximately 75 mV per pH unit up to its pK_a, 7.7. Similarly, the waves again shifted anodically and also became noticeably broader when increasing proportions of acetonitrile were added to the electrolysis medium. When the cyclic voltammetric behavior of this series of compounds was measured at a carbon paste electrode, no differences in behavior were noted.

Chromatography

On the basis of the electrochemical information described above for lidocaine and its metabolites, these compounds clearly do not represent ideal candidates for LC—ED. The potential required for their oxidation is considerably higher than that desirable to achieve both ultimate sensitivity and reasonable selectivity. In fact, detection of GX and MEGX by LC—ED could be expected to necessitate detector potentials of the order of +1.20 V vs. Ag/AgCl. However, for routine clinical applications which require the assay of these compounds only at the μg/ml level, high sensitivity comparable to that ordinarily expected of LC—ED is really not essential.

The cyclic voltammetry results indicate that optimum electrochemical conditions in LC—ED would be favored by an alkaline mobile phase of relatively low organic content. Both of these factors will serve to decrease the oxidation potential of the analytes to as low a value as is practical. Unfortunately, in conventional reversed-phase chromatography employing an octadecylsilane column, high pH conditions required that a relatively high acetonitrile (or other organic solvent) content be employed in the mobile phase in order to elute lidocaine and the other compounds within a reasonably short retention time. Preliminary work using this approach (e.g. acetonitrile—pH 7.4 buffer, 1:1) produced chromatograms in which the lidocaine response consisted of a broad, severely tailing peak and the MEGX and GX gave no observable signal at all. An alternate approach, described recently by Bidlingmeyer et al. [21] for the separation of organic amines including lidocaine, consists of the use of a bare silica gel stationary phase in conjunction with an aqueous—acetonitrile eluent. This somewhat unconventional reversed-phase procedure, for which the retention mechanism is thought to consist of a direct interaction of silanol groups on the column material with the amine function of the analyte, was shown not only to produce narrow, symmetrical peaks but also to be compatible with elution by the high pH, low acetonitrile mobile phase required for optimum LC—ED detection. Initial trials employing this latter chromatographic approach immediately produced favorable results for lidocaine, MEGX, and GX. Consequently, the approach was employed in all subsequent work reported here.



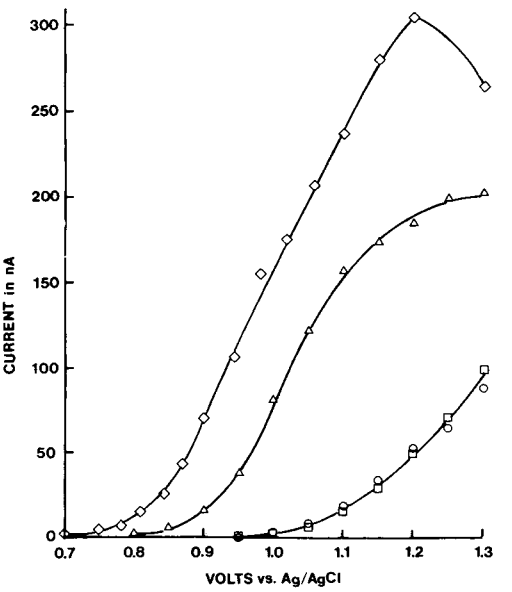


Fig. 3. Chromatogram of standard mixture of lidocaine (L) and metabolites (B = bupivacaine). All concentrations are $1.0 \cdot 10^{-5}$ *M*. Mobile phase:acetonitrile—ammonium phosphate buffer (pH 7.8) (22:78); *E* = +1.20 V vs. Ag/AgCl.

Fig. 4. Hydrodyanmic voltammogram of lidocaine (△), MEGX (□), GX (○), and bupivacaine (◇). All concentrations are $1.0 \cdot 10^{-4}$ *M*; other conditions as for Fig. 3.

The chromatogram shown in Fig. 3 was obtained for a standard solution of lidocaine, MEGX, GX, and bupivacaine at a detector potential of +1.20 V vs. Ag/AgCl. Under the conditions employed, the peaks corresponding to all four compounds were completely resolved. The response of the system as a function of detector potential was demonstrated by obtaining hydrodynamic voltammograms (i.e. profiles of chromatographic peak current vs. applied potential) for each of the analytes. These voltammograms are shown in Fig. 4. It can be seen that a potential of +1.20 V is required if GX and MEGX are to be detected. However, if only lidocaine were of interest, a potential of some 0.2 V less would be equally suitable. The hydrodynamic voltammograms match closely the results anticipated on the basis of the cyclic voltammograms reported above. Detection limits (i.e. signal-to-noise ratio = 2) for standard solutions were approximately 2 ng injected for lidocaine, 5 ng for MEGX, and 4 ng for GX.

Blood serum analysis

The chromatogram of a blood serum blank obtained from a patient prior to intravenous lidocaine infusion and subjected to the Sep-Pak treatment process described in the Experimental section is shown in Fig. 5A. The intense peaks eluting between 1 and 3 min corresponded to serum components weakly retained under the chromatographic conditions employed and occurred consistently for all serum samples examined. The remaining peak at a retention time of 4.5 min was found in varying intensity for all samples — serum,

standards, or even deionized water — eluted through the octadecylsilane Sep-Paks. Numerous Sep-Paks were examined, and various procedures for pretreating the Sep-Paks prior to the serum assay were investigated. Although extensive preliminary flushing of the Sep-Pak generally served to reduce the intensity of the interfering peak considerably, no procedure was successful in removing it consistently and completely. Gas chromatography—mass spectrometry performed on eluent fractions collected from the Sep-Pak indicated the presence of several compounds including bis(2-ethylhexyl)-phthalate and 2,6-di-*tert.*-butyl-4-methylphenol. However, the identity of the compound causing the peak was not determined definitely. Since the unidentified substance did not coelute with any of the compounds of interest, its effect on the lidocaine assay was not significant. No further measures were taken to eliminate it completely.

Calibration curves were prepared from quadruplicate serum samples spiked with lidocaine, MEGX, and GX from 0.20 to 12 μg/ml and subjected to the Sep-Pak treatment. The signal taken for each consisted of the ratio of the peak height obtained for the drug or metabolite to that of the bupivacaine internal standard. In all cases, the calibration curves were linear down to the detection limit of the individual compound. Least-squares analysis of the calibration curves yielded the following characteristics: for lidocaine, slope = 2.21, *y*-inter-

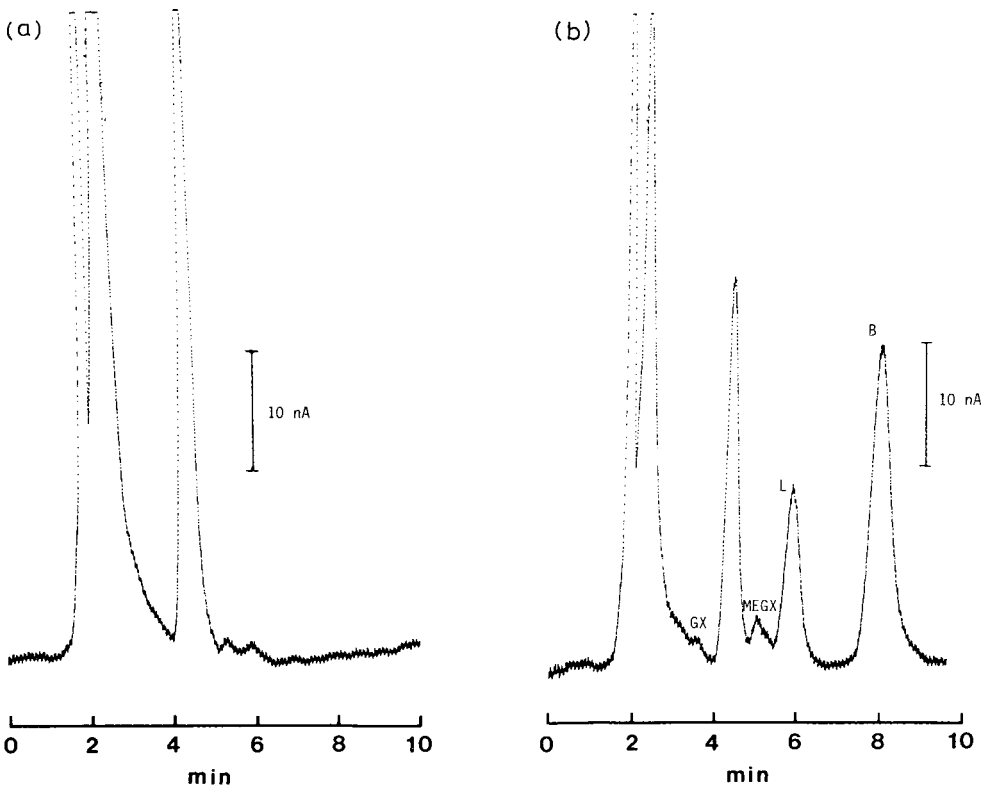


Fig. 5. Chromatograms of blood serum sample obtained (a) prior to lidocaine treatment and (b) 20 min after lidocaine injection. Conditions as for Fig. 3.

TABLE I

DETERMINATION OF LIDOCAINE IN BLOOD SERUM OF PATIENTS FOLLOWING 200-mg INJECTION

Patient	Lidocaine concentration (μg/ml)			
	5 min post injection	10 min post injection	1 h post injection	2 h post injections
A	2.62	0.82	0.64	0.55
B	4.53	2.55	0.89	0.78
C	3.82	2.20	1.45	1.14
D	4.65	2.21	1.50	0.96
E	6.66	3.27	2.62	2.48
F	3.50	0.66	0.48	—
G	2.55	0.95	0.49	0.24

cept = —0.116, correlation coefficient = 0.997; for MEGX, slope = 1.04, y-intercept = 0.056, correlation coefficient = 0.996; and for GX, slope = 0.718, y-intercept = —0.054, correlation coefficient = 0.991. Reproducibility, measured in terms of the relative standard deviation for four trials, was generally 5—10%. Recoveries of the drugs and metabolites and respective detection limits in serum were as follows: for lidocaine, recovery = 76% and detection limit = 0.2 μg/ml of blood serum; for MEGX, 60% and 0.6 μg/ml; for GX, 44% and 0.5 μg/ml. The typical analysis time required for quantitation of all three species was 10 min.

Serum samples were obtained from several patients at intervals of 5 min, 20 min, 1 h, and 2 h following a 200-mg lidocaine intravenous injection. The pharmacokinetic data for seven patients are summarized in Table I, and Fig. 5B shows a typical chromatogram corresponding to a 20-min post-injection serum sample where peaks for MEGX and GX are just beginning to appear. The indicated serum concentrations were all well within the range expected following a 200-mg injection but show the wide inter-patient variability which can be a concern even with identical dosages.

CONCLUSIONS

The method described is sufficiently rapid, selective, and sensitive to be appropriate for quantitating lidocaine, MEGX, and GX levels in patients treated for cardiac arrhythmias. Samples may be prepared quickly and simply enough to permit convenient real-time monitoring in patients undergoing lidocaine treatment.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF AMINEPTINE AND ITS MAIN METABOLITE IN HUMAN PLASMA

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SUMMARY

An isocratic reversed-phase ion-pair liquid chromatographic method for the determination of amineptine and its main metabolite in plasma using an internal standard, is reported. The effects of stationary phase alkyl chain length and the concentration of alkyl sulfonate in the mobile phase were investigated. The drugs were extracted as ion pairs and the influence of various parameters on the extraction efficiency are discussed. Using a heptane—octanol—tetraheptylammonium bromide mixture (98 : 2 : 0.5, v/v/w) as extraction solvent, more than 60% of each drug was recovered with a very good selectivity. UV detection at 220 nm allowed drug determination down to 0.010 μg/ml. Linear standard curves up to 1.00 μg/ml were observed.

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INTRODUCTION

Amineptine* (dihydro-10,11-dibenzo[*a*,*d*]cycloheptenyl-5-amino)-7-heptanoic acid, used as an antidepressant [1, 2], is a molecule characterized by an amino acid side-chain attached to a tricyclic nucleus [3, 4]. Pharmacologically, amineptine appears to act on the dopaminergic system [5].

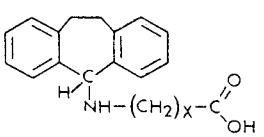
The biotransformation of this drug in man leads to various urinary metabolites which are not well known; in blood, a metabolite with a shortened amino acid side-chain, (dihydro-10,11-dibenzo[*a*,*d*]cycloheptenyl-5-amino)-7-pentanoic acid, has been identified [1].

Up to now, only one method for the determination of amineptine in plasma has been published [6]. This method was later applied to the estimation of amineptine and its main metabolite in human plasma [7]. This gas—liquid chromatographic (GLC) method requires costly equipment, including mass spectrometric detection and use of an external standard. A simple high-performance liquid chromatographic (HPLC) method for the plasma determination of amineptine and its main metabolite is reported here. The drugs are isolated from plasma by ion-pair extraction; the HPLC isocratic method is performed using an internal standard, a reversed-phase ion-pair system, and UV detection, and is found to be suitable for therapeutic determinations and pharmacokinetic investigations.

EXPERIMENTAL

Chemicals and reagents

Amineptine, its metabolite and internal standard, (dihydro-10,11-dibenzo-[*a*,*d*]cycloheptenyl-5-amino)-7-octanoic acid (Fig. 1), were provided by Servier Laboratories (Servier, Suresnes, France). Standard aqueous solutions of 10 μg/ml amineptine and its metabolite, and 10 μg/ml internal standard aqueous solutions were daily obtained from 1 mg/ml stock solutions. The latter were prepared by dissolving the compounds in methanol using an ultrasonic bath; the standard solutions were then stored at +4°C in brown glass flasks and were found to be stable for at least one month. Acetic acid (Rectapur grade), phosphoric acid and *n*-heptane (Normapur grade), and purified octanol were all obtained from Prolabo (Paris, France). Phosphate buffer (pH 7.0), acetonitrile and methanol



	X
amineptine	6
metabolite	4
int. standard	7

Fig. 1. Chemical structure of amineptine, its metabolite and internal standard.

*Trademark: Survector.

of LiChrosolv grade were purchased from Merck (Darmstadt, F.R.G.). The ion-pair reagents, tetraheptylammonium bromide (THABr) and heptanesulfonic acid sodium salt, were purchased from Eastman Kodak (Touzart et Matignon, Vitry-sur-Seine, France).

Chromatography

The chromatographic apparatus consisted of the following components: a Waters Model 6000 A pump (Waters, Paris, France), a Pye Unicam spectrophotometer (Pye Unicam, Paris, France) operated at 220 nm, and a Rheodyne 7125 injection valve (Touzart et Matignon, Vitry-sur-Seine, France) equipped with a 50-μl loop. The detector output was connected either to a Kontron W + W 610 recorder (W.W. Electronic Inc., Basel, Switzerland) or to an HP Model 3390 A integrator (Hewlett-Packard, Paris, France). A stainless-steel column (150 × 4.6 mm I.D.) was packed with a Nucleosil C_{18} (5 μm) stationary phase (Macherey-Nagel, Düren, F.R.G.), using a slurry packing technique [8] with some modifications to the solvents used: the slurry was made with *n*-butanol and the packing solvent was methanol.

The mobile phase consisted of acetonitrile—distilled water mixture (38 : 62, v/v). The aqueous phase contained 1.2 g/l heptanesulfonate and was adjusted to pH 3.0 with phosphoric acid. The mobile phase was filtered using a 0.45-μm Millipore filter and degassed in an ultrasonic bath. The separation was performed isocratically at room temperature.

Sample preparation and plasma extraction

Venous blood samples (5 ml) were collected into a 10-ml Vacutainer green-stoppered tubes (Becton-Dickinson, Missisauga, Canada) and centrifuged at 900 *g*. When the determination was not carried out immediately, the plasma was frozen at −20°C in plastic tubes; under these conditions, no degradation of drugs was noted after one month of storage.

For analysis, 2 ml of plasma were added to 1 ml of 0.05 *M* phosphate buffer (pH 7.0), 100 μl of 10 μg/ml internal standard solution and 10 ml of heptane—octanol—THABr (98 : 2 : 0.5, v/v/w) in 10-ml PTFE-lined screw-capped glass tubes. The tubes were shaken for 10 min on a Laboral oscillating agitator (Prolabo, Paris, France) and then centrifuged at 900 *g* for 10 min. An 8-ml volume of each upper organic phase was collected in a 10-ml conical base glass tube and 200 μl of a 0.17 *M* acetic acid—methanol (90 : 10, v/v) mixture were added. The tubes were capped and shaken on a Breda Scientific rotary agitator (Bioblock, Paris, France) for 5 min at 10 rpm; they were then centrifuged at 900 *g* for 5 min. The upper organic phase was discarded and 50 μl of acetic methanol phase were injected into the chromatograph.

Calibration curves and calculation

Plasma samples (2 ml) were spiked with increasing amounts of amineptine and its metabolite (final concentrations: 0.05, 0.10, 0.25, 0.50, 1.00 μg/ml of each drug in plasma) and with 100 μl of a 10 μg/ml internal standard solution. The samples were extracted as described above and standard curves were generated for each series of determinations by plotting peak height ratios (drug/internal standard) against known drug concentrations.

Plasma concentrations were interpolated from these standard curves. Accurate results in the range 0.01—1.00 μg/ml could also be alternatively obtained using a Hewlett-Packard integrator; in this case, calibration was obtained from a 0.50 μg/ml drug standard solution.

RESULTS AND DISCUSSION

Chromatographic system development

The influence of the counter-ion size on the retention of amineptine and its metabolite was studied. The effects of pentane-, hexane-, heptane- and octane-sulfonate at 1.2 g/l in aqueous mobile phase were successively investigated. As

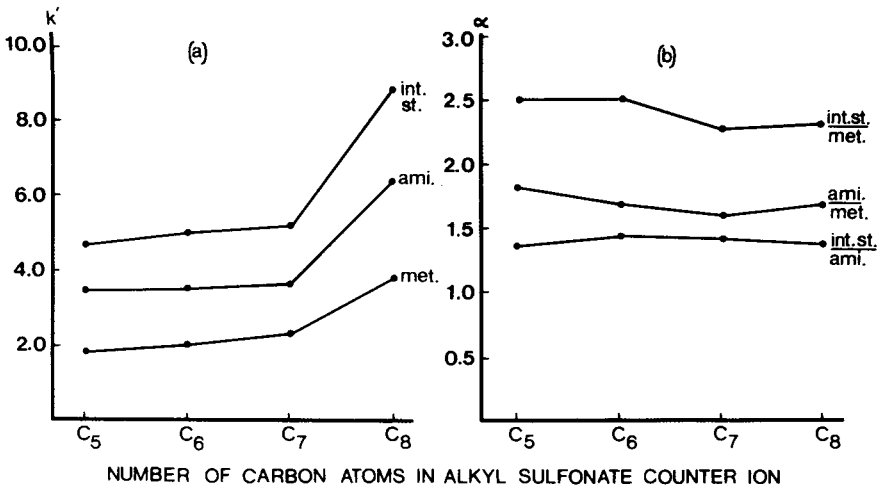


Fig. 2. Effect of the counter-ion size on the k' values (a) and selectivity factor values α (b) for amineptine, its metabolite and internal standard. Chromatographic conditions are as described in Experimental.

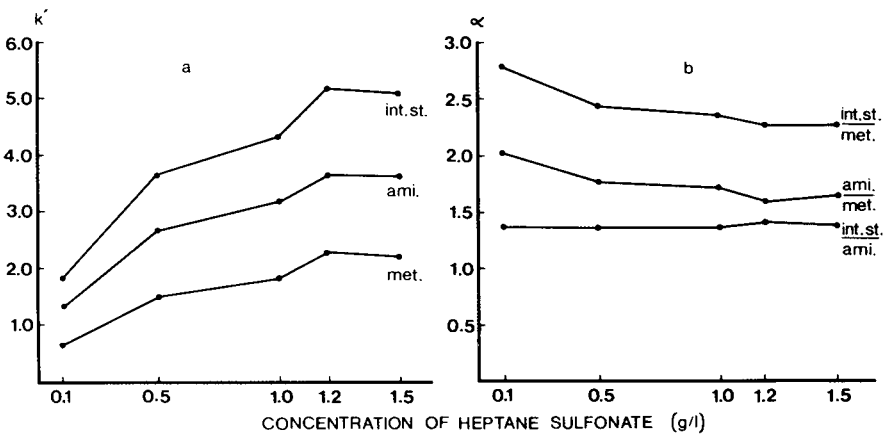


Fig. 3. Effect of the heptane sulfonate concentration on the k' values (a) and selectivity factor values α (b) for amineptine, its metabolite and internal standard. Chromatographic conditions are as described in Experimental.

can be seen from Fig. 2a, the k' values increase with increasing carbon number of the counter-ion alkyl chain, while selectivity factors α (Fig. 2b) show a relative constancy. Heptanesulfonate was chosen as counter-ion since in this condition the k' of the metabolite, the less retained peak, was large enough to be sufficiently resolved from the few endogenous compounds which eluted in less than 3.5 min.

Fig. 3a and b shows the effect of heptanesulfonate concentration in aqueous mobile phase on the capacity and selectivity factors. Concentrations from 0.1 to 1.5 g/l were successively used. According to the results obtained, a good compromise was obtained between retention time and selectivity factors by using 1.2 g/l heptane sulfonate in the aqueous mobile phase. Under these conditions, the metabolite elutes just after the solvent front; on the other hand, the resolution between metabolite and amineptine (6.7) and between amineptine and internal standard (5.4) is very good.

Extraction studies

The use of a polar solvent such as ethyl acetate in the extraction procedure showed that an important amount of endogenous compounds is co-extracted with the drugs of interest and these interfere with the detection of metabolite. It is well known that endogenous substances are co-extracted to a smaller extent by using non-polar solvents; unfortunately, the extraction recovery of polar compounds such as amineptine is very low under these conditions. Schill [9] studied the possibility of isolating drugs using an ion-pair extraction; recently, Smedes et al. [10] described an ion-pair extraction of catecholamines from plasma and urine. In the present study, a similar extraction procedure was used via the ion-pair formation in neutral medium between THABr (the cationic pairing ion) and the negatively charged carboxylate group of the drug. The back-extraction of drugs was then performed in an acetic acid—methanol mixture. The ionized acetate was used as competing ion in order to form an extractable ion-pair with THABr in the organic phase. Acceptable recovery and good selectivity were found using this procedure.

In order to determine the optimal conditions for drug extraction from human plasma, different parameters were investigated: the plasma pH, the nature and the concentration of the counter-ion, the nature of the organic phase, and the acetic acid concentration in the back-extraction step. All experiments were carried out with 2 ml of plasma spiked with the two drugs and the internal standard. The extraction conditions are given in the appropriate legends; throughout the set of experiments, the extraction efficiency was estimated by measuring drug peak heights.

The influence of plasma pH employing THABr as cationic counter-ion is illustrated in Fig. 4; the different pH values were obtained by adding NaOH or HCl. The relative extraction efficiency increases with increasing plasma pH up to pH 7—8 and then decreases. The poor extraction efficiency observed at pH values lower than 7.0 could be due to a simultaneous decrease of the ionized carboxylic group and protonation of the amine group leading to hydrophilic forms of the drugs. The ion-pair formation seems to be complete at pH values near 7—8, resulting from the ionization of the carboxylic group in this pH range. No hypothesis was established which could explain the decrease in extraction we observed for pH higher than 8.0.

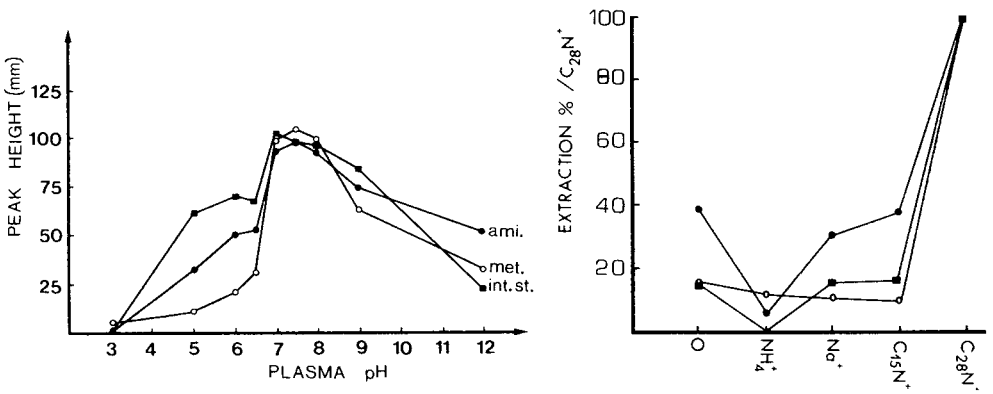


Fig. 4. Effect of the plasma pH on the extraction efficiency. Extraction conditions are as described in Experimental. ami. = amineptine; met. = metabolite; int. st. = internal standard.

Fig. 5. Effect of the counter-ion structure on the extraction efficiency. NH_4^+ = ammonium chloride, Na^+ = sodium sulfate, $C_{15}N^+$ = dodecyltrimethylammonium bromide, $C_{28}N^+$ = tetraheptylammonium bromide. Extraction conditions are as described in Experimental, except for NH_4^+, Na^+ and $C_{15}N^+$ which were dissolved in plasma. (■), amineptine; (○), metabolite; (●), internal standard.

In order to determine the influence of the counter-ion structure, the peak heights of extracted drugs were estimated with various types of inorganic cations or alkyl quaternary ammonium ions. Due to their solubilities, THABr and dodecyltrimethylammonium bromide were respectively dissolved in the organic phase and in plasma; all these pairing ions were used at a concentration of 5 g/l in the organic phase. Fig. 5 shows that the extraction efficiency increases with the carbon number of the quaternary ammonium, while no improvement was noticed using inorganic cations (even for Na_2SO_4 concentrations up to 20 g/l). We thought, in agreement with previous reports [9, 10], that these results can be attributed to solvation effects in both phases; thus, it is possible that the attraction forces between inorganic cations and the aqueous phase are greater than those between ion pairs (formed by these inorganic cations and drugs) and organic phase. The reverse is true when inorganic cations are replaced by THABr.

It must be noted (Table I) that at pH 7.0, when 1 *M* NH_4Cl—NH_4OH buffer [10] was used, the extraction efficiency decreased compared to the results obtained with the phosphate buffer. This decrease is probably due to two competing interactions: interactions between carboxylic group (drug) and tetraheptylammonium ion, and interaction between carboxylic group (drug) and buffer ammonium ions. This decrease is more pronounced in the series metabolite > amineptine > internal standard and could be partly attributed to the more or less polar character of the ion pairs formed.

Extraction and back-extraction steps were investigated by varying the nature of extraction solvent. Solvent polarity, octanol percentage and THABr concentration in the organic phase were successively tested; the percentage of methanol in the aqueous phase and the molarity of the acetic acid solution used for the back-extraction were also studied. The effect of solvent polarity on the ex-

TABLE I

INFLUENCE OF THE NATURE OF THE BUFFER ON THE EXTRACTION EFFICIENCY

Buffer	Peak height (mm)					
	Metabolite $-(CH_2)_4-$		Amineptine		Internal standard	
NH_4Cl-NH_4OH (1 *M*, pH 7.0)	39		50		39	
	39	38*	44	48	35	38
	36		49		41	
Phosphate buffer (Merck, pH 7.0)	84		63		40	
	79	81	62	62	42	41
	80		61		41	

*Mean peak height.

traction yield was studied by using a different organic phase containing 2% of octanol and 1 g/l of THABr. Under these conditions, methylene chloride—octanol and methylene chloride—diethyl ether—octanol mixtures provide a poor extraction yield and should be avoided; a quantitative extraction was observed with toluene—octanol for the metabolite only. Nevertheless, it was found that extraction efficiency increased significantly for the three components when using hexane—octanol or heptane—octanol mixtures. As expected, it could be concluded that the ion pairs behave as lipophilic compounds and are much more soluble in non-polar solvents such as heptane or hexane.

As shown in Fig. 6, an increase in relative extraction efficiency was noticed for amineptine and particularly for its metabolite, with increasing octanol in heptane containing 1 g/l of THABr. Schill [9] described lipophilic alcohols such as octanol as having a moderate polarity and acting both as proton donor and acceptor. Thus, it could be assumed that octanol gave a high solvation for the amino acid side-chain of the studied compounds. In the case of internal standard, a slight increase in extraction efficiency was observed up to 1% octanol, then the extraction efficiency decreased again. The latter result could be attributed to the more non-polar character of this solute, due to the larger alkyl

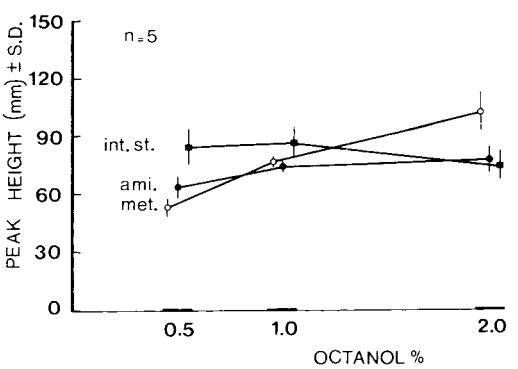


Fig. 6. Effect of the octanol percentage in heptane containing 1 g/l THABr, on the extraction efficiency. Experiments were carried out at plasma pH 7.0.

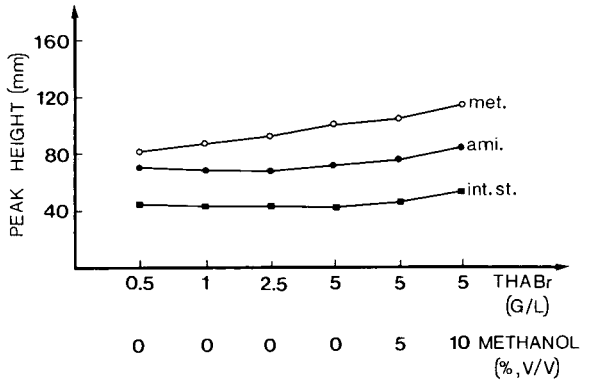


Fig. 7. Effects of the amount of the counter-ion THABr in the extraction phase and methanol percentage in the back-extraction phase, on the extraction efficiency. Other extraction conditions are as described in Experimental.

side-chain. Octanol concentrations higher than 2% were not used in order to avoid the co-extraction of interfering compounds.

The influence of the counter-ion concentration was studied by varying the amount of THABr in the heptane—octanol mixture (98 : 2, v/v) from 0.5 to 5 g/l. As shown in Fig. 7, the relative extraction efficiency was improved when the THABr concentration increased. This improvement was especially observed for amineptine and metabolite, and these findings are in agreement with results obtained by Smedes et al. [10] using an ion-pair extraction system for catecholamines; however, a real improvement was noted when methanol was added to the acetic acid solution used in the back-extraction. According to the limit of counter-ion solubility in organic phase and in order to suppress the co-extraction of undesirable endogenous anions, the following concentrations were chosen: 5 g THABr per litre of organic phase and 10% of methanol in the back-extraction solution.

Although an increase in recovery of amineptine and internal standard was noticed when extracting drugs with acetic acid solution from 0.17 *M* to 3.40 *M*, in these conditions a larger solvent front occurred and no improvement was noted for the metabolite recovery.

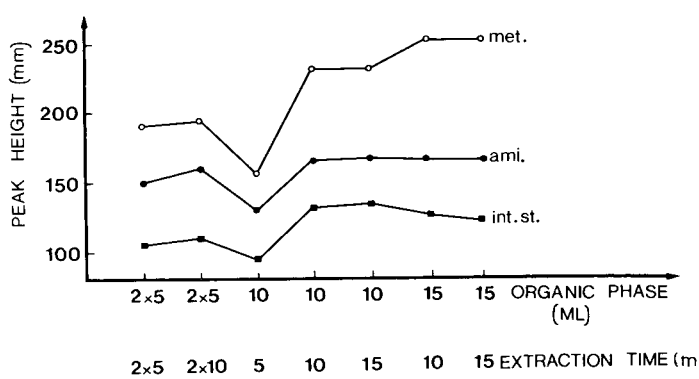


Fig. 8. Influence of the amount of the organic phase and of the extraction time, on the extraction efficiency. Plasma pH and extraction solvent are as described in Experimental.

In order to optimize the extraction procedure for 2-ml plasma samples, the volume of organic phase to be employed and the extraction time were investigated. Results are shown in Fig. 8. Solvent volumes less than 10 ml result in the formation of a precipitate which could adsorb the drugs and decrease the extraction percentage. A 10-min extraction time and a 10-ml volume of organic phase seemed to be in agreement with the quantitative and fast extraction requirements.

Linearity, sensitivity and selectivity

The standard curves were obtained by measuring the peak height ratios (drug/internal standard) on chromatograms obtained from drug-free plasma spiked with amineptine, its metabolite and internal standard. The linear curves were observed when plotting peak height ratios versus concentration (0.01, 0.05, 0.10, 0.50, 1.00 μg/ml of each drug). Each value was the mean of four measurements. The calibration curves for amineptine and its metabolite could be respectively expressed by the following equations: $Y = 0.0049\ X + 0.0228$ ($r = 0.999$), and $Y = 0.0085\ X - 0.0140$ ($r = 0.999$). The detection limit (signal-to-background ratio = 3) was at least 0.01 μg/ml for each drug.

TABLE II

SELECTIVITY OF THE HPLC SYSTEM

Drug (10 μg/ml)*	k'	Interference	Drugs (1 μg/ml)**	k'	Interference
Imipramine	6.09	—	Lorazepam	3.18***	+
Nortriptyline	6.27	—	Nitrazepam	3.36***	+
Maprotiline	6.45	—	Clorazepate	4.27***	+
Amitriptyline	7.00	—	Tianeptine	3.73	+
Protriptyline	7.55	—	Mianserin	3.73	+
Trimipramine	7.91	—	Desipramine	5.36	+
Desmethylclomipramine	9.18	—			
Clomipramine	11.00	—			
Meprobamate	ND	—			
Phenobarbital	1.18	—			
Valproic acid	3.00§	—			
Oxazepam	2.82	—			
Triazolam	1.36	—			
Bromazepam	1.55	—			
Diazepam	8.09	—			
Levomepromazine	7.18	—			

Metabolite ($k' = 2.27$), amineptine ($k' = 3.64$), internal standard ($k' = 5.18$)

*Non-extracted solution (50 μl injected).
**Extracted solution according to the amineptine assay procedure (50 μl injected).
***Peak height is no more than three times the level of the background noise.
§ Very poor signal at this concentration.

The method selectivity was studied and results are given in Table II. Among the drugs tested, tianeptine, mianserin and desipramine at therapeutic range interfere in the analysis described.

Reproducibility and recovery

Within-day reproducibility was determined by carrying out eight determinations from plasma samples spiked with 0.010, 0.250 and 1.000 μg/ml of amineptine and its metabolite. Day-to-day reproducibility was obtained by carrying out eight determinations over one month, no more than one assay per day, from plasma spiked with 0.250 μg/ml of each compound. Coefficients of variation (Table III) for within-day and day-to-day studies were respectively less than 11.93% and 8.15%.

Drug recovery was studied by adding a known amount of amineptine and its metabolite to a drug-free plasma sample at three different concentrations (0.10, 0.50 and 1.00 μg/ml). The organic phase was back-extracted with 200 μl of a 0.17 *M* acetic acid—methanol mixture containing an internal standard amount equivalent to a 100% extraction yield. For each concentration, five extractions were performed; means of peak height ratios were computed and compared to the mean of four height ratios of a drug and internal standard amount equivalent to a 100% extraction yield. Table IV shows that measured recoveries range from 62% to 73% for metabolite and from 64% to 73% for amineptine.

TABLE III

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITY OF THE METHOD

Compound	Concentration (μg/ml)	Within-day (n = 8)		Day-to-day (n = 8)	
		S.D.	C.V.* (%)	S.D.	C.V. (%)
Metabolite	0.010	0.002	11.93		
	0.250	0.006	2.44	0.011	4.43
	1.000	0.021	2.04		
Amineptine	0.010	0.001	7.92		
	0.250	0.008	3.15	0.021	8.15
	1.000	0.019	1.88		

*Coefficient of variation.

TABLE IV

RECOVERY DATA FOR ASSAY OF AMINEPTINE AND ITS METABOLITE

Compound		Peak height after extraction (mm)					Mean of peak height without extraction (mm) (mean of four injections)	Percentage recovery (± 2 S.D.)
Name	Concentration (μg/ml)	1	2	3	4	5		
Metabolite —$(CH_2)_4$—	0.10	112.5	98.8	111	110	97.5	171	62 ± 8
	0.50	137.5	134	146	160	156	229	64 ± 10
	1.00	159	159	159	158	152.5	216	73 ± 2
Amineptine	0.10	63.8	57.5	66.3	62.5	67.5	100	64 ± 7
	0.50	82.5	80	89	99	96	133	67 ± 12
	1.00	94	93	93	91	89	126	73 ± 3

CLINICAL APPLICATIONS

The HPLC system described here was used for routine monitoring of plasma amineptine and its metabolite and for pharmacokinetic studies. Typical chromatograms of extracts from a blank sample, a spiked plasma and a patient plasma sample are shown in Fig. 9. No interfering peak originating from an endogenous compound was formed. Retention times were 4.0 min for metabolite, 6.0 min for amineptine and 8.0 min for internal standard. Samples can be injected at 10-min intervals. The analytical method described above has sufficient sensitivity for pharmacokinetic studies in human subjects. Using this method plasma kinetics were investigated in two adults suffering from depression who received a single 100-mg oral dose of amineptine (Survector). We obtained the following preliminary results: for one patient, peak concentrations of 378 ng/ml for amineptine and 532 ng/ml for metabolite were reached within 120 min and 180 min, respectively, after drug administration; for the other patient, peak concentrations of 1059 ng/ml for amineptine and 685 ng/ml for metabolite, were reached within 45 min and 60 min, respectively, after drug administration. In both cases, the drug could not be detected in blood, at 12 h and at 24 h for amineptine and for metabolite, respectively. Further studies on the pharmacokinetics in depressed and in uremic adults are in progress in our laboratory; the possible relationship between plasma concentration and effect of both amineptine and its metabolite is also being studied.

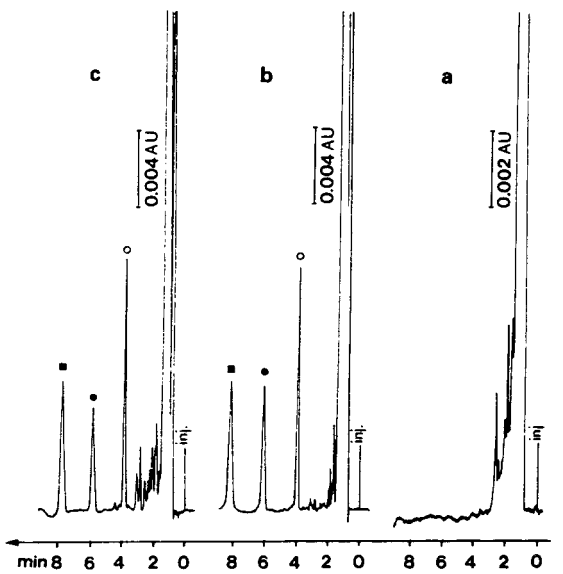


Fig. 9. Typical chromatograms of extracts from: (a) blank plasma; (b) plasma spiked with 250 ng/ml of metabolite (○), 250 ng/ml of amineptine (●) and 500 ng/ml of internal standard (■); (c) a patient plasma sample obtained 45 min after taking a 100-mg oral dose of amineptine, spiked with 500 ng/ml of internal standard (■) and containing 230 ng/ml of metabolite (○), 180 ng/ml of amineptine (●).

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF N-METHYLATED METABOLITES OF NICOTINE

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SUMMARY

An analytical method has been developed, using cation-exchange high-performance liquid chromatography, for the analysis of N-methylated metabolites of nicotine. This method has been used to detect and quantitate seven potential in vivo urinary metabolites of [2′-^{14}C]-nicotine, including four methylated nicotine derivatives, in the guinea pig.

INTRODUCTION

It has been known for many years that aza aromatic ring systems can undergo biological methylation reactions at the heteroatom [1—12]. However, little importance has been given to this route of biotransformation in drug metabolism studies, probably because of the difficulties involved in the isolation, characterization and quantitation of the resulting water-soluble, highly polar quaternary ammonium metabolites. Both nicotine (Fig. 1, 1a) and cotinine (Fig. 1, 2), two of the major pyridino alkaloids in tobacco leaf, have been reported by McKennis et al. [13] to form N-methylated quaternary pyridinium metabolites in the dog. However, no quantitative estimations of these metabolites have yet been carried out. In addition, this communication, which was published twenty years ago, appears to be the only study carried out on the in vivo methylation of nicotine. McKennis et al. [13] have also shown That cotinine (2) can be methylated to the N-methylcotinium (N-methyl-5′-oxonicotinium) ion (Fig. 1, 3) in man. These results indicate that biological methylation of nicotine and related compounds may be a significant route of metabolism in the organisms studied. Since quaternary ammonium compounds are often biologically active and because their formation is accompanied by a

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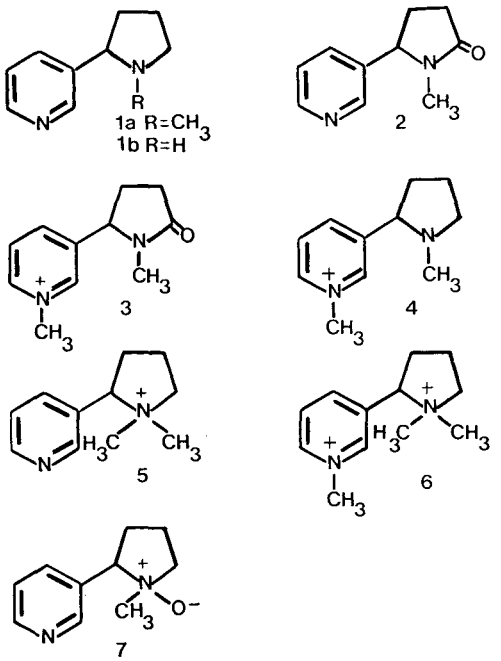

Fig. 1. Structural formulae of (1a) nicotine; (1b) nornicotine; (2) cotinine; (3) N-methyl-5′-oxonicotinium; (4) N-methylnicotinium; (5) N′-methylnicotinium; (6) N,N′-dimethylnicotinium; and (7) nicotine-1′-oxide.

marked change in physico-chemical properties such as charge, solubility and basicity, such metabolites could be of pharmacological and toxicological importance. In this respect it is surprising that relatively little attention has been focused on the in vivo methylation of nicotine and its relevance to the toxicology of tobacco products.

Biological methylation of nicotine could potentially give rise to a number of N-methylated products (Fig. 1) i.e. N-methylnicotinium (4), N-methyl-5′-oxonicotinium (3), N′-methylnicotinium (5) and N,N′-dimethylnicotinium (6) ions; of these, ions 3 and 4 have already been determined as nicotine metabolites in the dog [13]. However, no quaternary ammonium metabolites of nicotine have been isolated bearing a N′,N′-dimethylpyrrolidinium grouping, although a number of endogenous aliphatic tertiary amines are known to be substrates for methyltransferases that convert them into quaternary ammonium compounds [14, 15]. In order to determine the relative importance of N-methylation as a biotransformation route for nicotine, we have carried out in vivo metabolic studies with [2′-^{14}C] nicotine in the guinea pig. Urinary nicotine metabolites have been analyzed by an analytical procedure which allows the simultaneous determination of seven potential in vivo metabolites of nicotine, including four methylated nicotine derivatives, by cation-exchange high-performance liquid chromatography (HPLC). The details of this analytical procedure are described in this paper.

MATERIALS AND METHODS

[2′-^{14}C]Nicotine free base (0.25 mCi, specific activity 60 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Initial metabolic studies were carried out on groups of five male Hartley guinea pigs (412 ± 51 g) which were deprived of food during experimentation, but given water ad lib. Animals were each injected intraperitoneally with approximately 12.5 μCi of [2′-^{14}C]nicotine free base in 1 ml of sterile water, and housed separately in custom-built glass metabolic cages supplied by the Crown Glass Company (Somerville, NJ, U.S.A.). Each unit incorporated an efficient urine and feces separator. Urine samples were collected over 24 h.

HPLC grade methanol was purchased from Fisher Scientific (Pittsburg, PA, U.S.A.), Gold Star triethylamine, analytical-grade sodium acetate and 1-nicotine were purchased from Aldrich (Milwaukee, WI, U.S.A.), cotinine was a gift from Dr. Kostenbauder, University of Kentucky (Lexington, KY, U.S.A.). Nornicotine (Fig. 1, 1b) was synthesized from ethyl nicotinate and N-vinylpyrrolidone via the method of Jacob [16]. Nicotine-1′-oxide (7) was prepared from 1-nicotine via the method of Phillipson and Handa [17], and consists of a mixture of the two possible diastereoisomers. N-methylnicotinium iodide, N′-methylnicotinium iodide and N,N′-dimethylnicotinium diiodide were prepared by the reaction of nicotine with methyl iodide using the conditions previously described by Seeman and Whidby [18]. N-methylcotininium iodide was prepared from cotinine and methyl iodide, using the method of McKennis et al. [13]. All chromatographic standards were prepared as 10 m*M* solutions in methanol.

Analyses were carried out on an Altex programmable HPLC system consisting of two Altex Model 110A pumps, an Altex Model 420 solvent programmer and an Altex Model 153 analytical UV detector operating at 254 nm, and UV output was recorded on an Omniscribe Model 5000 dual-channel recorder (Houston Instruments, Austin, TX, U.S.A.). Samples were introduced via a Rheodyne loop injector. All eluent buffers used were filtered and degassed in vacuo before use. Separations were carried out on a Partisil-10 SCX 10-μm particle cation-exchange column (Whatman), 25 cm × 4.6 mm I.D. to which was attached a CSK-I Whatman pellicular cation-exchange guard column (7 × 0.4 cm). Essential chromatographic operating parameters are to be found in the legends to the tables and figures.

Urine samples (20—50 μl) from animals dosed with [2′-^{14}C]nicotine were centrifuged at 2500 *g* and then injected onto the HPLC column. Radioactivity in column effluents was determined in one of two ways: (a) Directly, by the use of a Model HS Flo-1 radioactive flow-through detector (Radiomatic, Tampa, FL, U.S.A.), equipped with a Radiomatic Model ES stream splitter. In such cases the scintillation cocktail used was Flo-Scint III (Radiomatic), with a mixing ratio of 4:1, v/v, of a 50% split of effluent stream. The output of the detector was recorded simultaneously on the second channel of the recorder. (b) Indirectly, by collecting fractions into scintillation vials at 1-min intervals, using a Superac Fraction Collector (LKB, Baltimore, MD, U.S.A.) and adding 10 ml of 3a70B cocktail (RPI, Elk Grove, IL, U.S.A.) to each fraction. In this case ^{14}C-activity was measured using a Packard TriCarb Scintillation Counter,

and radiochromatograms were constructed by plotting the radioactivity in the fractions against their retention times. To determine the recovery of radioisotope from the analytical column, an identical volume of radioisotopic urine applied to the column was added directly to a scintillation vial containing an appropriate volume of column effluent and treated as described above.

The limit of reliable quantitation for the radiochemical detector under the conditions of the analysis, was reached at a peak height of 80—100 dpm above a background of 20 dpm. The reproducibility of five consecutive injections of a sample affording a single peak of 300 dpm total area, gave an S.D. of 39 dpm and a coefficient of variation of 13.5%.

RESULTS AND DISCUSSION

The determination of in vivo metabolites of nicotine is usually carried out by gas—liquid chromatography [19—20]. Using this technique, various refinements in the methodologies employed have led to the development of sensitive and reliable procedures for the determination of nicotine and its metabolites in biological fluids. However, this method of analysis will only allow the direct determination of volatile components in biological samples. Polar metabolites, such as conjugates of nicotine and its phase I metabolites, and quaternary methyl metabolites, cannot be analyzed using this method unless appropriate derivatization to a more volatile compound is carried out. HPLC analysis has been used in recent years for the determination of nicotine and its metabolites [30—34]. The majority of these analytical systems have utilized a reversed-phase octadecyl silica packing and metabolites have been separated by virtue of their lipophilicity. Unfortunately, this type of analytical system is not suitable for the determination of the charged, highly water-soluble N-methylated quaternary metabolites of nicotine.

The analysis of quaternary ammonium ions by HPLC has received much attention during the last decade [35—37]. Most separations have been carried out using ion-pair reversed-phase chromatography. Our attempts to develop an analytical methodology for the quantitation of methylated metabolites of nicotine using ion-pair reversed-phase chromatography using a variety of sulfonic acid counter-ions were unsuccessful due to the extremely high affinity of the resulting ion-pairs of the methylated nicotine standards for the reversed-phase packing. Since we had previously been successful in developing a method for the analysis of N-methylated metabolites of pyridine by cation-exchange HPLC [38, 39] we decided to investigate the possible utility of this analytical system for the determination of methylated metabolites of nicotine.

Initial attempts to develop an efficient chromatographic system for the separation of nicotine, three oxidative metabolites and four potential methylated metabolites (Fig. 1, 3—6) of nicotine, were carried out on a Partisil-10 SCX 10-μm particle cation-exchange column using a 0.3 *M* ammonium acetate—methanol (70:30) buffer adjusted to pH 4.5 with glacial acetic acid. This system was used previously by us for the determination of methylpyridinium ion. However, poor chromatograms were obtained using this system; the methylated quaternary nicotine standards eluted with extremely long retention times and were observed as broad, tailing peaks. No significant

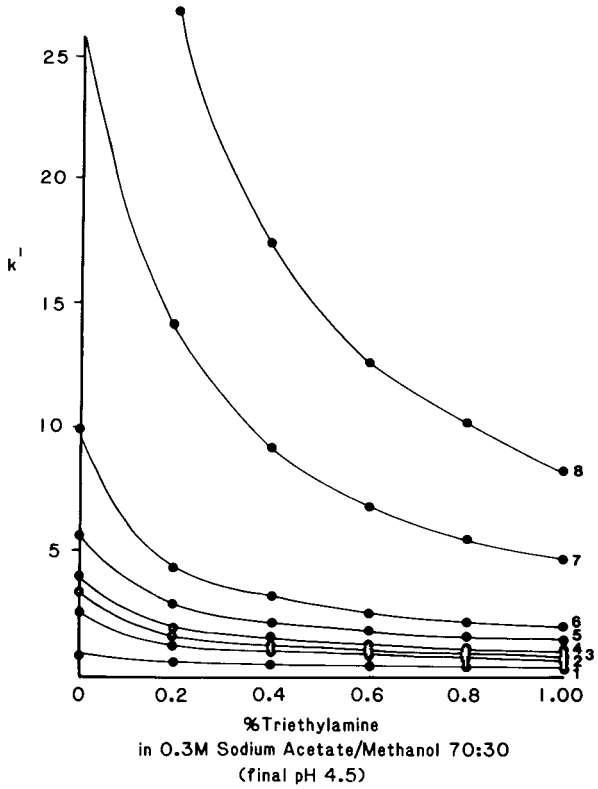

Fig. 2. Changes in k' values with increasing concentration of triethylamine from cation-exchange HPLC analysis of authentic standards of potential nicotine metabolites. Key: 1, cotinine; 2, nornicotine; 3, nicotine-N′-oxide; 4, nicotine; 5, N-methylcotininium iodide; 6, N′-methylnicotinium iodide; 7, N-methylnicotinium iodide; 8, N,N′-dimethylnicotinium diiodide.

improvement could be achieved, even after a variety of modifications to the mobile phase, such as pH variation, change in molarity of ammonium acetate and change in the proportion and/or nature of the organic modifier, were made. The chromatography was improved somewhat when sodium acetate buffer was used in place of ammonium acetate buffer. However, very long retention times were still observed for the methylated nicotine standards (see Fig. 2). To overcome this problem we hypothesized that by inclusion of a water-soluble, non-UV-absorbing protonated base into the buffer system, it might be possible to displace the tightly bound cationic nicotine standards from the sulfonic acid binding sites on the Partisil-10 SCX column, thus decreasing the retention time of these ions, on the column. Fig. 2 shows the effect of gradually increasing the percentage of the tertiary base triethylamine in a 0.3 *M* sodium acetate—methanol (70:30) buffer, pH 4.5, on the retention times of authentic nicotine derivatives. The triethylamine concentration was varied from 0 to 1%, v/v. As can be seen, a drastic decrease in the retention times of the more tenaciously bound quaternary methylated nicotine derivatives is obtained when the percentage of triethylamine in the buffer

increases. The retention times of the other standards are less markedly effected. The triethylamine also had the effect of sharpening up broad, poorly resolved peaks to give a much superior chromatogram. We also investigated the effect of the ionic strength of the sodium acetate buffer on the above chromatographic system (see Fig. 3). Generally, shorter retention times were obtained as the ionic strength of the buffer increased, but this effect was less pronounced than the triethylamine effect. Variation of the proportion of methanol in the buffer afforded some surprising results (see Fig. 4). At low percentages of methanol (0—20%) little effect was seen on the retention times of the standards. However, at methanol percentages above 30%, a marked increase in the retention times of N-methylnicotinium iodide and N,N'-dimethylnicotinium diiodide was seen, while little effect was observed on the retention times of the other standards. We attribute this observation to a solubility phenomenon, the above two compounds probably having poorer solubility in methanol than in water. The effect of buffer pH over the range 4.0—7.0 was also examined. The results are shown in Fig. 5. Generally, longer retention times were observed for all standards as the pH of the buffer increased. N-Methylnicotinium iodide and N,N'-dimethylnicotinium diiodide were again the compounds most affected by pH changes. Taking these data into consideration an optimum system was chosen using an isocratic buffer system consisting of 30% methanol in 0.3 *M* sodium acetate buffer, pH 4.5, to initially elute the less strongly bound standards from the cation-exchange column. This was followed by a gradient of triethylamine rising rapidly from 0 to 1.0% over

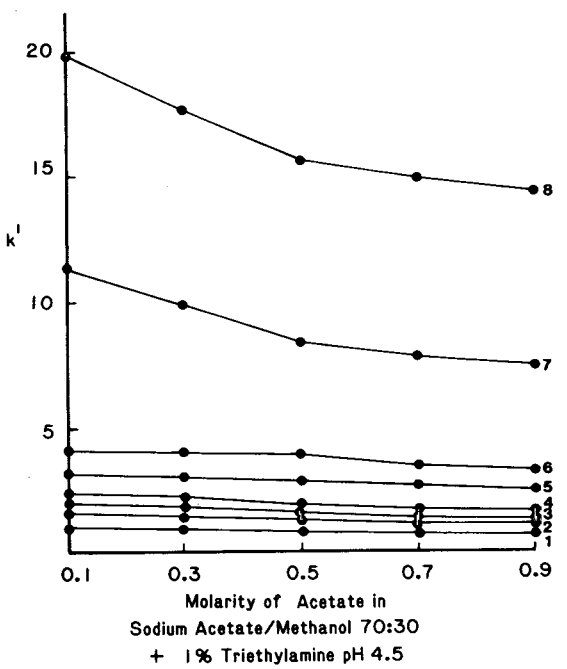

Fig. 3. Changes in k' values with increasing molarity of sodium acetate from cation-exchange HPLC analysis of authentic standards of potential nicotine metabolites. For key see Fig. 2.

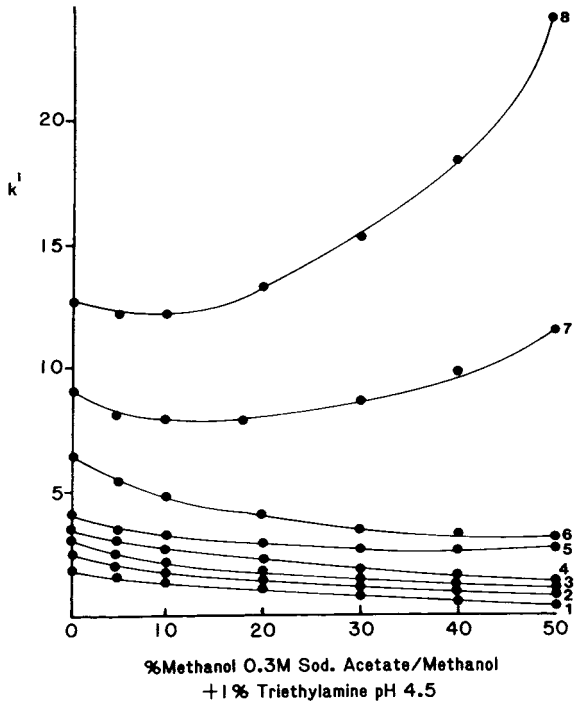

Fig. 4. Changes in k' values with increasing concentration of methanol from cation-exchange HPLC of authentic standards of potential nicotine metabolites. For key see Fig. 2.

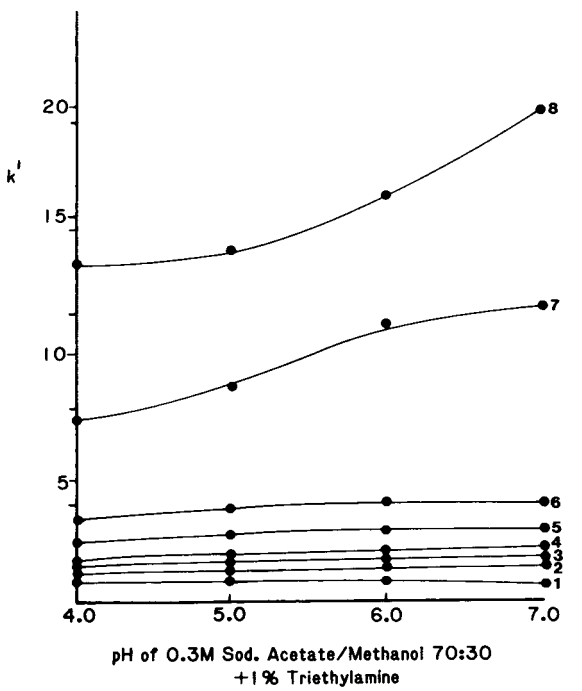

Fig. 5. Changes in k' values with increasing pH from cation-exchange HPLC of authentic standards of potential nicotine metabolites. For key see Fig. 2.

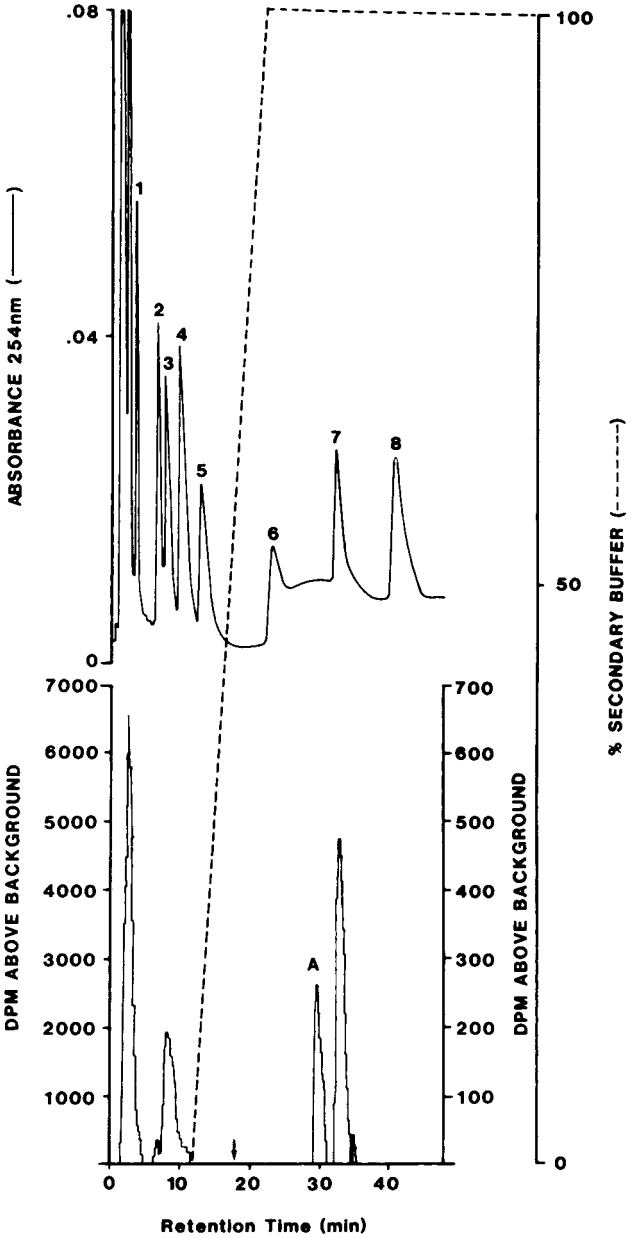

Fig. 6. Radiochromatogram of 6-h guinea pig urine, after intraperitoneal administration of [2′-^{14}C]nicotine, using cation-exchange HPLC. Column, Partisil-10 SCX (25 cm × 4.6 mm I.D.); eluent, primary buffer, methanol—0.3 *M* sodium acetate, pH 4.5 (30:70, v/v), secondary buffer, methanol—0.3 *M* sodium acetate, pH 4.5 (30/70, v/v) containing 1.0% v/v triethylamine (adjusted to pH 4.5); flow-rate 1 ml/min at 141.34 bars; the broken line shows the gradient profile; UV detection at 254 nm; ^{14}C-detection by direct analysis of column effluent, using a Flo-1, Model HS radioactive flow-through detector (see text for details). Peaks: 1 = cotinine; 2 = nornicotine; 3 = nicotine-N′-oxide; 4 = nicotine; 5 = N-methylcotininium ion; 6 = N′-methylnicotinium ion; 7 = N-methylnicotinium ion; 8 = N,N′-dimethylnicotinium ion; A = unidentified metabolite. The left-hand dpm scale refers to the radiochromatogram to the left of the arrow; the right-hand dpm scale refers to the radiochromatogram to the right of the arrow.

a 10-min period, to elute the more strongly bound methylated nicotine derivatives from the column (see Fig. 6). The above analytical system allows the analysis of seven potential metabolites of [^{14}C]nicotine.

Because of the very low dose of carrier-free [^{14}C]nicotine utilized in the metabolic studies (ca. 0.078 mg per kg body weight, ca. 12 μCi), it was not possible to analyze the low levels of metabolites in the urine samples collected, by UV detection in the HPLC effluent. Therefore, the effluent generated after co-injection of urine and authentic standards onto the Partisil 10 SCX column, was monitored for ^{14}C by liquid scintillation counting for the detection and quantitation of the small amounts of metabolites present. A radiochromatogram obtained from the analysis of a 6-h urine sample from a male Hartley guinea pig dosed with [^{14}C]nicotine is illustrated in Fig. 6, and is typical of results obtained from the analyses of each of five animals within the experimental group. Results from the HPLC cation-exchange radiochromatograms of total 24-h urine samples showed that the N-methylnicotinium iodide standard peak generally had about 4% of the ^{14}C-label in the total 24-h urine associated with it and in one animal experiment as much as 8% (see Table I). No radioactivity co-eluted with any of the other methylated nicotine standards. However, an unknown metabolite (metabolite A) is present in the urine of guinea pigs treated with [2′-^{14}C]nicotine which is retained to a greater extent on the cation-exchange column than the N′-methylnicotinium ion. This metabolite accounted for about 2% of the ^{14}C-label excreted in 24-h urine, and was as much as 4% in some cases. This component is most probably a quaternary metabolite in view of its high affinity for the Partisil-10 SCX packing. The identification of this nicotine metabolite is presently being carried out in our laboratories and will be the subject of a further communication. Significant amounts of radioactivity also eluted with the cotinine (69.4%) and nicotine-N′-oxide (21.5%) standards. In the former case, it was not always possible to

TABLE I

ANALYSIS OF URINARY METABOLITES OF [2′-^{14}C]NICOTINE IN THE GUINEA PIG

Compound	Percent in 24-h total urine*	Reproducibility***
Cotinine	69.4 (±3.9)**	61.2 (±0.53)**
Nornicotine	1.6 (±1.10)	1.9 (±0.25)
Nicotine-N′-oxide	21.5 (±3.2)	24.9 (±0.41)
Nicotine	1.3 (±0.65)	0.75 (±0.10)
N-Methylcotininium ion	0.0	0.0
N′-Methylnicotinium ion	0.0	0.0
N-Methylnicotinium ion	4.2 (±0.96)	8.4 (±0.80)
Metabolite A	2.4 (±0.56)	2.5 (±0.13)
N,N′-Dimethylnicotinium ion	0.0	0.0

*Recovery of ^{14}C-label in 24-h total guinea pig urine was 76.9 ± 2.3% of the amount administered, where n = 5.

**S.E.M., n = 5.

***Repeat analyses of a single guinea pig urine sample after intraperitoneal injection of [2′-^{14}C]nicotine.

resolve the radioactivity eluting in the void volume from that associated with the cotinine standard; therefore, quantitation of ^{14}C-label under the cotinine peak may also include radioactivity attributable to very polar, non-basic water-soluble metabolites such as conjugates, which elute from the cation-exchange column with little or no retention. Small amounts of nornicotine (1.6%) and unmetabolized nicotine (1.3%) were also detected in the total 24-h urine (see Table I for details). These results indicate that nicotine is extensively metabolized in vivo in the guinea pig and that in addition to cotinine, the N'-oxide of nicotine is a major urinary metabolite. Average recovery of ^{14}C-label in the urine was about 77% of the total ^{14}C-label administered to animals as [2'-^{14}C]nicotine, and the recovery of radioactivity from analytical HPLC columns was always better than 95% of the ^{14}C-label in the urine sample applied to the column.

In conclusion, an HPLC cation-exchange system has been developed for the identification and quantitation of methylated metabolites of [^{14}C]nicotine in the guinea pig, an animal species known to be a good N-methylater of azaheterocycles [39]. This animal species produces only one identifiable methylation product, N-methylnicotinium ion, which constitutes 4% of the total ^{14}C-label found in total 24-h urine, after intraperitoneal injection of [2'-^{14}C]nicotine.

We are currently utilizing the above analytical procedure to investigate the in vivo methylation of nicotine in other animal species, and studies are also being carried out to determine the role of methylation in nicotine toxicity.

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IMPROVED METHOD FOR THE DETERMINATION OF CHLORHEXIDINE IN URINE

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SUMMARY

A high-performance liquid chromatographic method is described which is suitable for the determination of the common antiseptic chlorhexidine in urine. The method employs Sep-Pak cartridges to remove chlorhexidine from the urine matrix. Chromatographic separation was achieved on a C_{18} reversed-phase column using a mobile phase of methanol—20 m*M* sodium acetate solution (60 : 40) adjusted to pH 5 with glacial acetic acid. An ion-pair agent (pentadecafluorooctanoic acid) was used at a concentration of 100 μg ml^{-1}. 3-Bromobenzophenone was used as chromatographic standard (k' = 4.0). 4-Bromobenzophenone (k' = 3.9) or dibenzal hydrazine (k' = 4.4) may also be used. A series of urine samples was analysed and no interferences were observed. The method is simple and rapid with a total analysis time of ca. 30 min.

INTRODUCTION

Chlorhexidine (1,1′-hexamethylene-bis-5-(4-chlorophenyl)biguanide) was one of a series of polybiguanides first synthesised in the late 1940's. Its antibacterial activity [1] was found to be more potent than other established cationic antiseptics such as cetrimide and hence it was selected for use under the trade name of "Hibitane".

Being a dicationic compound (in neutral or mild acid or alkaline solutions) it is usually formulated as the digluconate to enhance the solubility in water. Its

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mode of action is via adsorption onto bacterial cell walls, a process which is favoured at higher pH values [2].

Methods currently available for the determination of chlorhexidine in pharmaceutical preparations where it is present in high concentrations are based on colorimetry [3] or high-performance liquid chromatography (HPLC) [4, 5]. The colorimetric method lacks sensitivity and is prone to interference. Of the two HPLC methods, one [4] is based on normal phase chromatography employing a silica column with an acetonitrile—0.04 *M* sulphuric acid mobile phase and detection at 254 nm. Bearing in mind that chlorhexidine is dicationic [2, 6] under these conditions it is likely that the separation is based on an ion-exchange process with the silica providing the stationary ionic sites. The other HPLC method [5] is based on a reversed-phase separation employing a C_{18} column and a methanol—water mobile phase containing an ion-pairing agent. Detection was at 238 nm. None of these methods is particularly suited to the study of chlorhexidine in body fluids where relatively low concentrations may be encountered. The HPLC method which employs the silica column suffers from poor analyte peak shape and hence low sensitivity. The reversed-phase HPLC method relies on a solvent extraction step of only moderate efficiency coupled with quantitation by calibration curve.

A sensitive method for the determination of chlorhexidine using gas—liquid chromatography (GLC) has been reported [7]. This method would appear to be very sensitive but, in addition to the extraction procedure from biological samples, a derivatization of the chlorhexidine involving hydrolysis to 4-chloroaniline followed by diazotisation and iodination to 4-chloroiodobenzene is required. Thus this procedure fails to distinguish between 4-chloroaniline resulting from the in vitro or in vivo decomposition of chlorhexidine prior to analysis and that derived from chlorhexidine by the analytical method. Recently [8], 4-chloroaniline has been detected and quantitated in pharmaceutical preparations of chlorhexidine and hence results obtained from this GLC method must be viewed with suspicion.

The physico-chemical properties of chlorhexidine indicate that HPLC with ultraviolet (UV) detection should be the analytical technique of choice. We now report an improved method for the determination of chlorhexidine in urine in which an efficient extraction step and quantitation via a chromatographic standard are combined to yield significant improvements over our previous method [5].

EXPERIMENTAL

Reagents and chemicals

The mobile phase was methanol (Willot Industrial, Bristol, U.K.)—20 m*M* sodium acetate buffer in double distilled water (60:40), adjusted to pH 5 with glacial acetic acid and modified with pentadecafluorooctanoic acid; 100 μg ml^{-1}, (BDH, Poole, U.K.). Degassing of the mobile phase was achieved by ultrasonication for ca. 10 min under reduced pressure (15 mmHg). All mixed solvents were stirred slowly but continuously whilst the chromatography was running.

Chlorhexidine was of analytical quality (97.6%; Bristol Royal Infirmary).

Quantitation was via the use of a chromatographic standard. Several compounds with suitable chromatographic characteristics were found including 4-bromobenzophenone (Aldrich, Dorset, U.K.), 3-bromobenzophenone and dibenzal hydrazine (Aldrich). All sample manipulations were carried out with conventional glassware. Small volume measurements were made with Hamilton 250-μl syringes. For calibration purposes standard solutions of chlorhexidine diacetate, incorporating the internal standard were prepared from methanolic stock solutions (1000 μg ml^{-1} of active ingredient) by serial dilution with the mobile phase.

Samples were collected routinely in screw-top plastic containers by staff at the Bristol Royal Infirmary and were stored at 4°C until analysed. Samples were extracted using C_{18} Sep-Paks (Waters Assoc., Northwich, U.K.) by a process detailed below.

The liquid chromatograph consisted of an LC-XPD Model 100 pump (Pye Unicam, Cambridge, U.K.), a Rheodyne Model 7010 loop injector (PhaseSep, Clwyd, U.K.) with 20-μl sample loop, a μBondapak (C_{18}) column (30 cm × 4 mm I.D., 10 μm particles) (Waters Assoc.) and an LC—UV detector fitted with 8-μl cell (Pye Unicam, Cambridge, U.K.). Output was to a recorder (10 mV f.s.d.) or a suitable integrator (e.g. Minigrator, Erba Science, Swindon, U.K.). Other chromatographic parameters were: flow-rate 1.5 ml min^{-1}; back pressure 90 bar; detector wavelength 260 nm.; sensitivity of the detector 0.02 or 0.04 a.u.f.s. into 10 mV f.s.d.

Sample preparation

A C_{18} Sep-Pak was attached to a 20-ml glass syringe and methanol (2 ml) passed slowly through it to remove any adsorbed material and cleanse the cartridge prior to use. Water (2 ml) was then passed through the cartridge to remove any residual methanol. Urine (10 ml) was placed in the syringe barrel (which was clamped in a vertical position and was forced slowly (ca. 5 ml min^{-1}) through the Sep-Pak using the syringe barrel. After passage of the urine, water (2 ml) was passed through the Sep-Pak to remove unwanted polar materials. Subsequently passage of methanol (2 ml) desorbed the chlorhexidine and this eluate was collected. The chromatographic standard (3-bromobenzophenone in methanol) was added (using a 250-μl syringe) to the methanol eluate to give a final concentration (in 5 ml) of 10 μg ml^{-1}. The volume of eluate was then standardised to 5 ml using mobile phase. From this standardised volume (5 ml) an aliquot (20 μl) was injected onto the chromatograph.

RESULTS AND DISCUSSION

Chromatographic aspects

Chlorhexidine absorbs UV radiation strongly between 215 nm and 270 nm with an absorbance maximum at 260 nm ($\epsilon = 3.1 \cdot 10^4$ l mol^{-1} cm^{-1}). Previously we have selected the detector wavelength as 238 nm because of the restriction imposed by the use of toluene-4-sulphonic acid as the ion-pair agent. The use of an alternative, non-absorbing, ion-pair agent (see below) permits the detector wavelength to be set at 260 nm providing an increase in sensitivity of the order of 40%.

The column used for the determination of chlorhexidine was a μBondapak C_{18} column (Waters Assoc.). Several other reversed-phase materials were also evaluated to ensure general applicability of the method. From this evaluation it was clear that only reversed-phase materials which are fully end-capped are suitable for the elution of chlorhexidine. Columns which are not de-activated in this way should not be used. On such columns chlorhexidine is either completely retained or eluted with very poor peak shape.

Based upon previous results [5] a capacity factor (k') for chlorhexidine of 2 would be adequate to completely resolve the compound of interest from all likely interferences whilst maintaining an acceptable analysis time. However, an ion-pair agent is required to promote the retention of chlorhexidine on ODS columns. Hence the value of k' may be varied by adjusting both the methanol—water ratio and the concentration of the ion-pairing agent. Previously [5] we have used pentadecafluorooctanoic acid (PDFOA) as an ion-pairing agent. An arbitrary concentration of PDFOA was selected (100 μg ml^{-1}) and the water—methanol ratio varied. An 8 μg ml^{-1} standard solution of chlorhexidine was used. From these experiments a mobile phase consisting of methanol—water (60 : 40) containing PDFOA (100 μg ml^{-1}) was selected. This yielded a k' value for chlorhexidine of 1.6.

The control of mobile phase pH by the addition of buffers may be used to encourage (or discourage) the retention of ionic compounds on reversed-phase columns. Chlorhexidine is ionic under the chromatographic conditions used here and thus a complex set of equilibria exist which govern its chromatographic behaviour. These equilibria can be shifted by varying the mobile phase pH. This was done by using 20 m*M* sodium acetate as the aqueous part of the mobile phase and adjusting its pH between 3.0 and 5.0 with glacial acetic acid with a constant concentration of PDFOA (100 μg ml^{-1}). From this set of experiments an optimised pH of 5.0 was selected. At lower pH values (4.5 and 4.0) a second much smaller peak was observed to interfere with chlorhexidine. This peak only occurs when the injected solvent is not closely matched in composition to the mobile phase and presumably is the "system" peak [9] for this particular chromatographic system. It arises because PDFOA has a small absorbance at 260 nm (ϵ = 3.3 l mol^{-1} cm^{-1}) due to part of a very broad, weak band most probably arising from a $n \rightarrow \pi^*$ transition of the carbonyl group. Confirmation of the assignment of this peak to the system was achieved by first injecting water (a positive peak resulted) and then methanol from which a negative peak with an identical k' value was observed. For a detailed explanation of this phenomenon, which only occurs when one of the components of the mobile phase (in this case PDFOA) has a significant absorbance at the wavelength selected for detection, see reference [9].

In order to promote wide applicability alternative ion-pair agents were evaluated. As well as PDFOA, heptafluorobutyric acid (HFBA) and heptanesulphonic acid (HSA) were studied. The variation of k' (chlorhexidine) with concentration is shown graphically in Fig. 1. Clearly the concentration of PDFOA has a marked effect on retention when compared with either HFBA or HSA at concentrations above 100 μg ml^{-1}. There is little point in using more ion-pair agent than is necessary to achieve the desired separation. Quite apart from the cost, excess back pressure may be generated. Although all three ion-

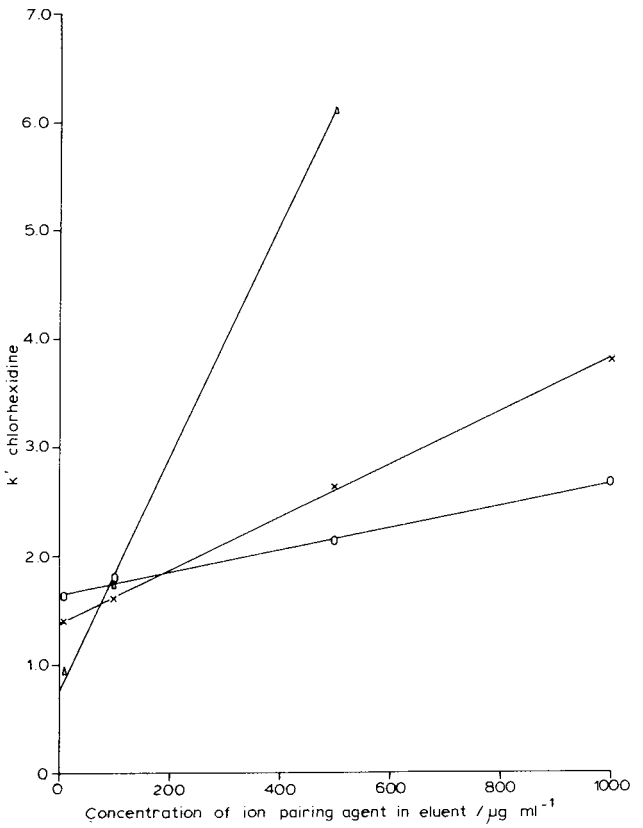

Fig. 1. Plot of k' (chlorhexidine) vs. concentration of various ion-pairing agents in methanol—aqueous acetate buffer (pH 5.0) (60:40) eluent. (○), HFBA; (×) HSA; (△) PDFOA.

pair agents may be used satisfactorily the low cost and wide availability of PDFOA justify its use.

In summary therefore, the optimised chromatographic parameters are a C_{18} reversed-phase column, which is fully end-capped, a mobile phase consisting of methanol—20 m*M* sodium acetate (pH 5.0) (60 : 40) with 100 μg ml^{-1} of PDFOA as ion-pairing agent. The recommended detector wavelength is 260 nm and the recommended flow-rate is 1.5 ml min^{-1}. Using a mobile phase of this complexity requires continual stirring at constant speed to maintain homogeneity and so prevent baseline drift.

Quantitative aspects

An internal standard is desirable in trace analysis of biological samples because it may compensate for some errors likely to occur during isolation and chromatography. Ideally an internal standard should be both chemically similar and chromatographically similar to the analyte species. This latter requirement includes the ability to respond significantly in the detection system. No material is available which fulfils these requirements with respect to chlorhexidine. Benzyl hibitane does fulfil these requirements but is not available

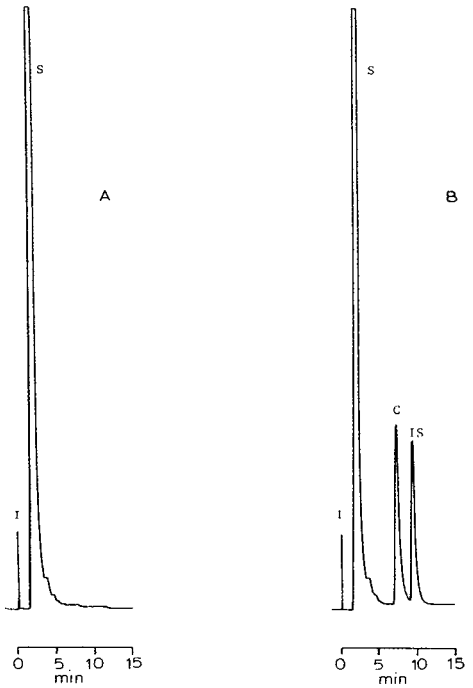

Fig. 2. A, Blank urine; B, urine spiked with chlorhexidine. Peaks: I = point of injection; S = solvent front and co-extracted material; C = chlorhexidine; IS = chromatographic standard. Parameters: column, μBondapak C_{18}; eluent, methanol—sodium acetate buffer (pH 5) (60 : 40) containing 100 μg ml^{-1} PDFOA; flow-rate, 1.5 ml min^{-1}; sensitivity, 0.04 a.u.f.s. into 10 mV f.s.d.; detection, 260 nm.

commercially. Hence a material which is chromatographically similar to chlorhexidine was chosen. Of the many compounds screened as possible chromatographic standards three were found to be suitable. These were 4-bromobenzophenone ($k' = 3.9$), 3-bromobenzophenone ($k' = 4.0$) and dibenzalhydrazine ($k' = 4.4$). 3-Bromobenzophenone was chosen because it was available in this laboratory and because it has a 40% larger extinction coefficient at the analytical wavelength ($\epsilon_{260\ nm} = 1.1 \cdot 10^4$ l mol^{-1} cm^{-1}) than dibenzalhydrazine and is thus similar to chlorhexidine ($\epsilon_{260\ nm} = 3.1 \cdot 10^4$ l mol^{-1} cm^{-1}). Using a mobile phase flow-rate of 1.5 ml min^{-1} both the analyte and the internal standard eluted in ca. 12 min (Fig. 2). 4-Bromobenzophenone ($k' = 3.9$) may also be used and provides a slight reduction in chromatographic analysis time when compared with 3-bromobenzophenone.

Previously [5] we have relied on quantitation via a calibration curve constructed prior to the determination of a set of samples i.e. external calibration. Use of a chromatographic standard permits quantitation via peak height ratios or peak area ratios. The level of chlorhexidine in real samples (urine) was expected to vary from 0—200 μg ml^{-1}. Calibration lines were constructed for the range 0—10 μg ml^{-1} and thus samples of concentration greater than 10 μg ml^{-1} would require dilution prior to analysis. The results of calibration are presented in Fig. 3. (Note: calibration is linear for the range 0—200 μg ml^{-1} and thus dilution is unnecessary if a reporting integrator is used. For peak height

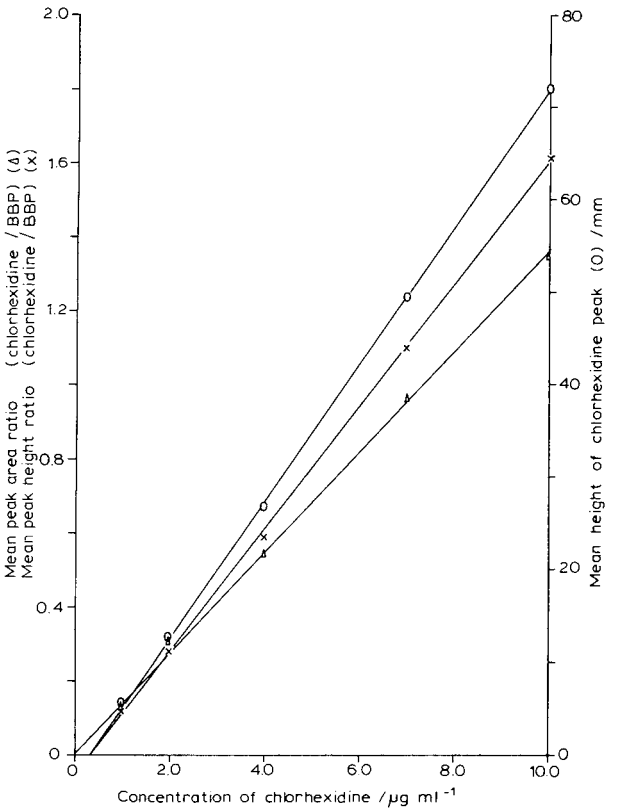

Fig. 3. Calibration graphs of mean peak area ratio (△), mean peak height ratio (×), and mean chlorhexidine peak height (○) vs. concentration for standard solutions of chlorhexidine chromatographed under normal operating conditions.

measurement a narrow range of concentrations should be used to minimise errors of measurement and thus dilution to a particular concentration range is recommended.) Quantitation by peak area ratio measurement resulted in a linear relationship between this parameter and the concentration of chlorhexidine, (regression equation: $y = 0.135x + 0.008$; correlation coefficient 0.9995). Similarly, quantitation by peak height ratio measurement resulted in a linear relationship, (regression equation: $y = 0.166x - 0.056$; correlation coefficient 0.9998). Direct quantitation by chlorhexidine peak height measurement against weight injected (external calibration) also yielded a linear relationship, (regression equation: $y = 7.42x - 2.41$; correlation coefficient 0.9999). Clearly all three methods of measurement are acceptable for quantitative analysis.

Sensitivity and detection limit

For the purposes of this investigation sensitivity was defined as that concentration of chlorhexidine which, when chromatographed, gave rise to a signal-to-noise (S/N) ratio of 20 : 1. Detection limit was defined as that concentration which gave rise to a S/N ratio of 2 : 1. For these experiments the detector sensitivity was set to the maximum, i.e., 0.01 a.u.f.s.: 10 mV f.s.d. The detection limit was determined to be 0.1 $\mu g\ ml^{-1}$ which corresponds to an injected

weight (20-μl sample loop) of 2 ng. Hence the sensitivity was 1.0 μg ml^{-1} or 20 ng weight injected. These values may be improved proportionately if a 50-μl sample loop is used without loss of chromatographic performance. The relative standard deviation (R.S.D.) for ten replicate injections of a 10 μg ml^{-1} chlorhexidine standard solution (with chromatographic standard) chromatographed using a 20-μl sample volume was 3.0%. Huston et al. [5] reported an R.S.D. of 5.0% using a 50-μl sample loop.

Recovery experiments

Chlorhexidine was extracted from urine by use of C_{18} Sep-Pak cartridges (Waters Assoc.). These were used as recommended by the manufacturer. Using a standardised procedure (see Experimental) ten aliquots (10 ml each) of a pooled urine sample spiked to give a concentration of 10 μg ml^{-1} were extracted. A blank urine sample was also extracted. Chromatograms of the blank (A) and spiked (B) samples are shown in Fig. 2. No interference was detected over the k' range of chlorhexidine and the internal standard. (Note: no interference was ever observed from the many extractions of blank urine specimens made during the optimisation of the Sep-Pak procedure.) From these ten extractions the mean recovery was 99.3% with an R.S.D. of 3.0%. Hence, within experimental error, recovery of chlorhexidine is quantitative and no correction need be made for extraction efficiency when assaying clinical samples. This recovery efficiency is significantly better than that obtained by the liquid—liquid extraction procedure [5]. A particular advantage of the use of the Sep-Pak extraction technique is that sample preparation time is reduced to ca. 7—8 min.

Although recovery was quantitative at a concentration of 10 μg ml^{-1} from a 10-ml sample (equivalent to a 100-μg loading of the cartridge) it was deemed necessary to determine whether the efficiency of extraction was constant over the range of concentrations likely to be encountered in real samples. To achieve this another series of samples was prepared by spiking blank urine with aqueous chlorhexidine solution to give concentrations of 1 μg ml^{-1} ($\equiv$ 10 μg loading), 10 μg ml^{-1} ($\equiv$ 100 μg loading), 100 μg ml^{-1} ($\equiv$ 1000 μg loading) and 200 μg ml^{-1} ($\equiv$ 2000 μg loading). These samples (two aliquots were taken), were extracted by the standard procedure. The recoveries for this range of concentrations indicate that chlorhexidine is recovered quantitatively across this range of concentrations. The concentration of chlorhexidine digluconate commonly used for bladder irrigation is 0.02% (w/v) which is equivalent to 160 μg ml^{-1} of chlorhexidine and thus this value is the highest concentration likely to be encountered with real samples. Considerably higher values may be found in urine collection bags and samples where a high chlorhexidine level is anticipated should be diluted (the red dye used to colour the pharmaceutical preparations provides a useful visual indicator of concentration).

Re-usability of Sep-Pak cartridges

Although this type of extraction cartridge is intended for single use the possibility of re-use was investigated. Twelve identical blank urine samples were spiked with an aqueous solution of chlorhexidine to a concentration of 10 μg ml^{-1}. Two Sep-Paks were used. For the first cartridge six samples were extracted sequentially with no washing of the cartridge between samples. The second

cartridge was washed with methanol (5 ml at 5 ml min^{-1}) and water (5 ml at 5 ml min^{-1}) between sample extractions. The two batches of six samples were then chromatographed.

Extraction was essentially quantitative for all the samples. There was no indication of any deterioration of extraction efficiency with successive extractions nor was any cross-contamination apparent.

Determination of chlorhexidine in clinical samples

Eighteen urine specimens were obtained from four patients who had undergone catheterisation — a process whereby a flexible plastic tube is inserted into the urethra. The catheter tube is connected to a plastic urine collection bag via a non-return value. On fitting a new bag, an aliquot (10 ml) of chlorhexidine digluconate (5%, w/v) is dispensed into the bag to sterilise the urine which accumulates. Concern has been expressed recently over the efficiency of the non-return valves particularly when the urine collection bags are subjected to pressure from, for example, the weight of the patient. In order to evaluate the functioning of the non-return valves urine samples were taken from the catheter tube above the non-return valve and from the urine collection bag below the valve. Samples may thus be categorised as either bag or catheter. As is not uncommon with samples collected routinely in hospitals four of the eighteen samples supplied were unlabelled. The optimised analytical procedure

TABLE I

CHLORHEXIDINE CONCENTRATIONS FOUND IN URINE SAMPLES TAKEN FROM CATHETER TUBING AND COLLECTION BAGS

Patient	Sample No.	Sample origin	Dilution factor	Concentration of chlorhexidine in original specimen ($\mu g\ ml^{-1}$)
A	1	Bag	1:10	89
	2	Unknown	1:1	0.15
	3	Catheter	1:1	0.15
	4	Catheter	1:1	<0.1
B	5	Catheter	1:1	<0.1
	6	Catheter	1:1	<0.1
	7	Bag	1:1	6.0
	8	Unknown	1:100	272
	9	Bag*	1:1	0.10
	10	Unknown	1:10	42
	11	Bag	1:100	135
	12	Unknown	0.3:1	0.48
C	13	Catheter	1:1	<0.1
	14	Catheter	1:1	0.28
	15	Bag	1:1000	1400
	16	Catheter	1:1	0.14
D	17	Catheter	1:1	<0.1
	18	Bag	1:1	274

*Sample probably mis-labelled.

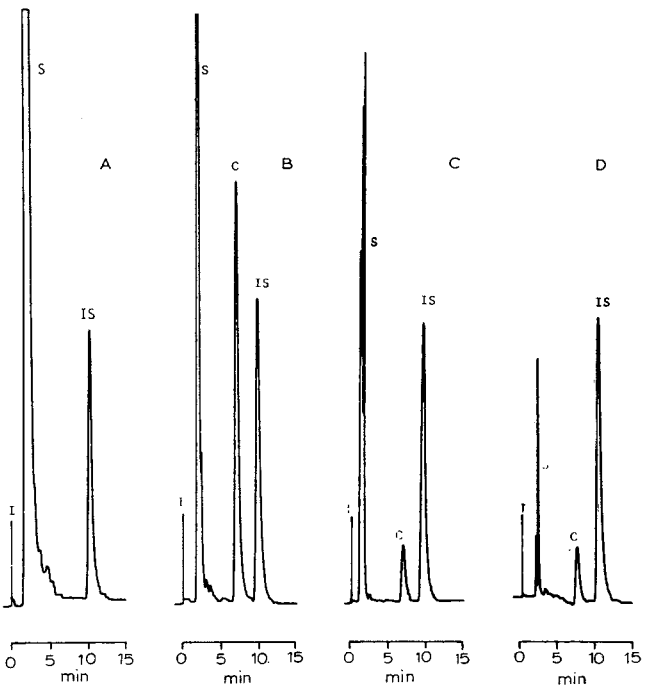

Fig. 4. Some typical chromatograms of urine extracts. A, urine with no chlorhexidine present; B, urine with a large absorbance due to co-extracted material (S) and a high chlorhexidine (C) content; C, urine with a large absorbance due to co-extracted material (S) but a low chlorhexidine (C) content, D, urine containing little co-extracted material (S) and a low chlorhexidine (C) content. For all chromatograms, IS = chromatographic standard and the conditions are as for Fig. 2.

was applied to these samples with appropriate dilution of the bag samples as necessary. Where sample volume was insufficient for a 10-ml aliquot to be extracted a semi-micro extraction procedure was employed. For this case a known volume of urine was passed through the Sep-Pak and the chlorhexidine was then eluted with methanol (2 ml). This methanol eluate was blown to dryness under a stream of nitrogen and then made up to 200 μl with chromatographic standard and mobile phase to give a sample for analysis. The results for the eighteen samples are presented in Table I. Examples of chromatographic traces are shown in Fig. 4. The results of these analyses are quite conclusive. Substantial quantities of chlorhexidine were found in samples taken from the urine collection bags (6—1400 μg ml^{-1}) as was expected, but only negligible amounts of chlorhexidine were detected in samples taken from catheters (< 0.3 μg ml^{-1}). In 63% of the urine samples known to have been taken from catheter tubes no chlorhexidine was detected. The four unlabelled specimens (Nos. 2, 8, 10 and 12) gave chlorhexidine values which fitted well into these two extremes and hence the source of these samples could be assigned with confidence. Specimen 9 was labelled as a sample taken from a urine collection bag but was found to contain only a trace of chlorhexidine. In this case it was suspected that either the specimen was mis-labelled and was actually taken from a catheter tube or that addition of chlorhexidine digluconate to the urine collection bag had been omitted.

The determined chlorhexidine levels in these urine samples indicate that the non-return valves function correctly in these cases. Trace levels of chlorhexidine were found in 37% of the urine samples taken from catheter tubes. This probably arose from detection of the remains of a gel containing chlorhexidine which is used to disinfect the urethra during catheterisation and which may have translocated down the catheter tube.

CONCLUSIONS

Chlorhexidine may be quantitatively extracted from urine using Sep-Paks. The resultant extracts may be chromatographed successfully on a reversed-phase C_{18} column using a methanol—sodium acetate buffer (pH 5.0) (60 : 40) mobile phase and detected by UV absorption at 260 nm. The detection limit is 0.1 $\mu g\ ml^{-1}$. The chlorhexidine present may be quantitated by reference to 3-bromobenzophenone as chromatographic standard with a within-batch precision of 3.0% R.S.D. No between batch precision has been established. No interference from co-eluting compounds has been observed.

The developed method has been used to demonstrate that the non-return valves in urine catheter bags function effectively.

The method is simple, rapid, and suitable for routine hospital use.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF INDOMETHACIN AND ITS TWO PRIMARY METABOLITES IN URINE

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SUMMARY

Quantitation of total amounts (i.e., free compound plus glucuronide conjugate) of indomethacin (INDO) and its deschlorobenzoyl (DBI) and desmethyl (DMI) metabolites in human urine is described. An aliquot (0.4 ml) of urine is incubated with glucuronidase (1000 U, 2 h, 37°C) and extracted with 5 ml of dichloromethane containing the internal standards: the fluoro analogue of INDO, F-INDO, and indole-3-propionic acid (IPA). The organic phase is concentrated, dissolved in mobile phase and aliquots are injected onto the high-performance liquid chromatograph. INDO and DMI are measured with UV detection at 254 nm over a linear range of 0.25—125 μg/ml. Retention times for DMI, F-INDO and INDO are 4.0, 6.8 and 12.1 min, respectively, using a C_8 reversed-phase column with an acetonitrile—0.1 *M* acetate, pH 5.0 (30:70) mobile phase at a 2.5 ml/min flow-rate. DBI is measured using fluorescence detection (excitation = 305 nm, emission = 370 nm) over a linear range of 0.25—12.5 μg/ml. Retention times for DBI and IPA are 4.5 and 7.8 min, respectively, on the same C_8 column with an acetonitrile—0.025 *M* acetate, pH 4.0 (22:78) mobile phase at a 2.0 ml/min flow-rate. Inter- and intra-day precision were smaller than 10% for INDO, DMI and DBI over the concentration ranges indicated.

INTRODUCTION

Total urinary excretion of a drug and its primary metabolites is one measure of bioavailability and/or drug absorption. These measurements are currently recommended to aid in the assessment of indomethacin, [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid, INDO] absorption.

Although there have been numerous reports concerning the determination of INDO in urine, only a few have included an analysis of metabolites other than the glucuronic acid conjugate. INDO exhibits a significant amount of phase I

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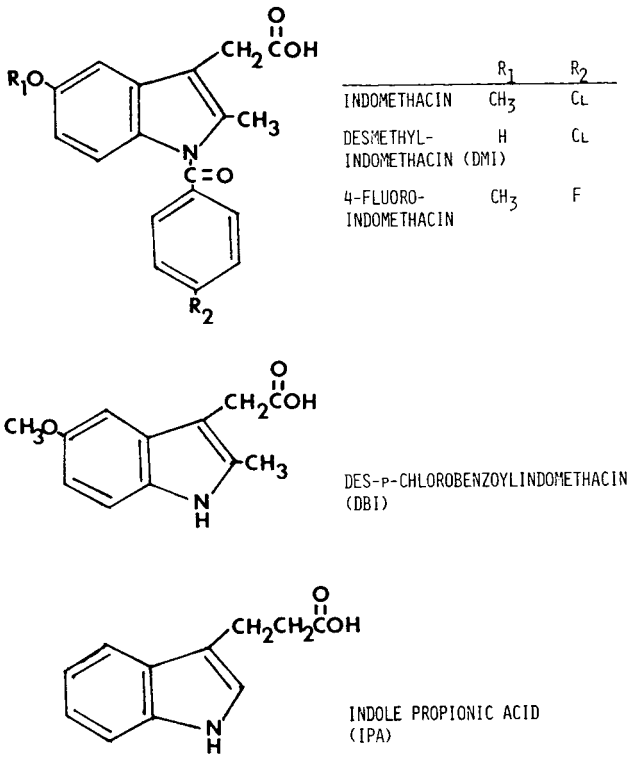

Fig. 1. Structures of indomethacin, its metabolites and the internal standards used in the analysis.

metabolism [1] to deschlorobenzoylindomethacin (DBI) and desmethylindomethacin (DMI). Both of these metabolites can also be subsequently conjugated with glucuronic acid or further metabolized to deschlorobenzoyldesmethylindomethacin (DMBI) which is a minor metabolite. INDO and its metabolites are depicted in Fig. 1. Two earlier reports on methods of analysis for INDO and its metabolites utilized radioactive [^{14}C]INDO with isotope dilution [1, 2]. Lack of labelled drug together with the difficulties of obtaining approval for radioisotope administration to man made this approach prohibitive. Bernstein and Evans [3] recently described a high-performance liquid chromatographic (HPLC) method for the analysis of INDO and its metabolites in both plasma and urine [3]. After alkaline hydrolysis INDO and DMI were measured using a difference method where the fluorescent products of hydrolysis, DBI and DMBI, were determined prior to and after alkaline hydrolysis. Besides the errors inherent in the difference method, the product DMBI is unstable in base [4, 5] which may lead to errors, unless the base hydrolysis is carried out under an inert atmosphere. Our appraoch was to measure the parent and metabolites in urine directly, after cleaving the glucuronic acid conjugates with β-glucuronidase. DMBI was not measured, as this was previously found to be a minor metabolite in the urine of man [1].

MATERIALS AND METHODS

Chemicals

Indomethacin, indole-3-propionic acid (IPA), and bovine liver, β-glucuronidase (Type B-10) were obtained from Sigma (St. Louis, MO, U.S.A.). DBI was commercially available from Aldrich (Milwaukee, WI, U.S.A.). DMI was initially synthesized by demethylation with pyridine hydrochloride as described [6]. A later report [7] mentioned DMI synthesis using boron bromide [4] which proved to be superior to the earlier method. 4-Fluoroindomethacin (F-INDO) (Fig. 1) was generously donated by Merck, Sharp and Dohme (West Point, PA, U.S.A.). Dichloromethane, acetonitrile and acetic acid were HPLC grade, obtained from Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were reagent grade.

Instrumentation

INDO and DMI were analyzed by UV detection. An HPLC pump, Series II, Perkin-Elmer (Norwalk, CT, U.S.A.) was combined with a Perkin-Elmer UV detector, LC-15. The wavelength used was 254 nm with an O.D. of 0.004 and a time constant of 1 sec. Injection of 50—100 μl was by a WISP® autosampler, Waters Assoc. (Milford, MA, U.S.A.). Output was recorded as peak area integration using a 3380A integrator, Hewlett-Packard (Palo Alto, CA, U.S.A.) set at an attenuation of 64 and slope sensitivity of 3. A mobile phase of 30% acetonitrile in 0.1 *M* sodium acetate, pH 5.0 was run at 2.5 ml/min at room temperature through a C_8 reversed-phase, 5 μm particle size, 150 mm × 4 mm I.D., Ultrasphere column, Altex (Berkeley, CA, U.S.A.). For fluorescence analysis of DBI a Perkin-Elmer Model 204A detector was set at an excitation wavelength of 305 nm and a slit of 10 nm, with an emission wavelength of 370 nm and a slit of 20 nm. PM gain was at 2 and sensitivity of 10 with a 10-mV output to a dual-pen recorder (Pedersen, Walnut Creek, CA, U.S.A.). For fluorescence analysis, sample sizes of only 3—5 μl were injected. The mobile phase used was acetonitrile—0.025 *M* sodium acetate, pH 4.0 (22:78) with a flow-rate of 2.0 ml/min at room temperature.

Procedure

The analysis was performed by the extraction of drug, metabolites and internal standard from 0.4 ml of urine after incubation with β-glucuronidase. To a 10-ml screw top tube was added 0.4 ml urine, then 0.4 ml of 0.5 *M* citrate buffer, pH 5.0 containing 1000 U of β-glucuronidase. The solution was mixed, then incubated with shaking at 37°C for 2 h. After incubation the solution was extracted with 5.0 ml of dichloromethane which had been spiked with the internal standards F-INDO and IPA at concentrations of 2.0 and 1.0 μg per 5.0 ml, respectively. The tube was then shaken (60 strokes per min) on a flatbed shaker of 15 min and any emulsion which had formed was broken by centrifugation in a clinical centrifuge (2250 *g*) for 10 min. The lower dichloromethane phase was removed, evaporated to dryness under a stream of nitrogen at 30—40°C, and the residue was dissolved in 0.4 ml of the mobile phase used for the UV assay. The solution was then transferred to vials to be injected by the autosampler. A single incubation and extraction was used for

both the UV analysis of INDO and DMI with F-INDO as the internal standard, and for the fluorescence analysis of DBI with IPA as the internal standard.

Standard curves were prepared by spiking blank urine samples with INDO, DMI and DBI at ten concentrations from 0.25 to 125 μg/ml for INDO and DMI and from 0.25 to 12.5 μg/ml for DBI. These concentrations covered the range found in urine after a 50-mg single oral dose of INDO. Stock solutions of 1.0 mg/ml were prepared for INDO in dichloromethane and for DMI and DBI in methanol. All three compounds were combined in methanol at 50 ng/ml to serve as the dilute solution needed daily for the preparation of the lower concentrations of the standard curve. Spiked urine samples were incubated with β-glucuronidase and extracted as described above.

Peak area ratios for INDO and DMI relative to the UV internal standard were used for quantitation. Because of the large range covered by the UV assay the standard curve was fitted with a weighted (1/concentration) least-squares regression. As the concentration range for DBI was smaller, it was better fitted using unweighted least squares. Since the linearity for the fluorescence assay was limited to a smaller concentration range, samples above 5 μg/ml necessitated injection of smaller sample volumes. Extraction efficiency was determined by comparing extracted spiked samples to unextracted samples using an external standard added prior to injection.

RESULTS

The extraction step produced much cleaner chromatography than was found when direct precipitation of proteins was tried. This allowed quantitation of

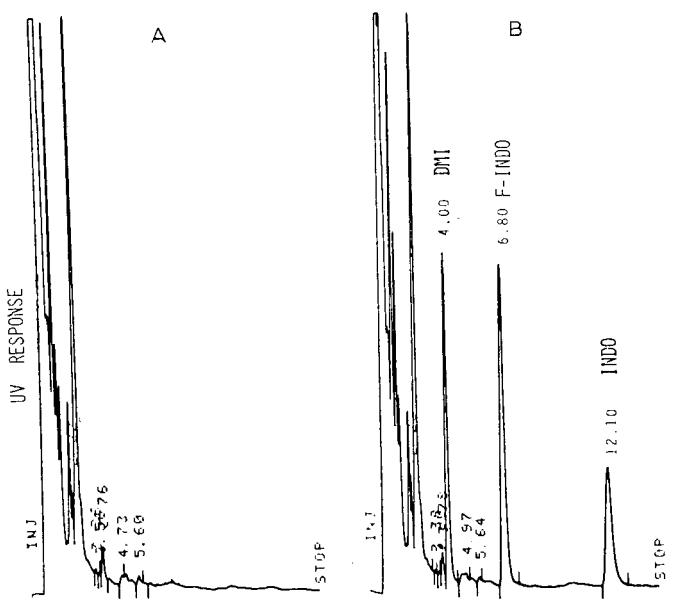

Fig. 2. UV chromatograms of (A) blank urine extract; (B) urine extracted after being spiked with 2.5 μg/ml of DMI and INDO. Conditions are described in the text.

DMI and INDO simultaneously. Retention times were 4.0, 6.8 and 12.1 min for DMI, F-INDO, and INDO, respectively as shown in Fig. 2. The intra-day variability through the range of 0.25 to 50.0 μg/ml, as listed in Table I, exhibit coefficients of variation (C.V.) which are less than 10% at the lowest concentration, 0.25 μg/ml. Between-day variability and extraction efficiencies are listed in Tables II and III, respectively.

Fluorescence provided selectivity for DBI analysis which could not be attained by UV analysis. Chromatograms for a blank sample and spiked urine are shown in Fig. 3. Retention times were 4.5 and 7.8 min for DBI and IPA, respectively. Within-day and between-day variability for the fluorescence assay are listed in Tables I and II.

Hydrolysis with β-glucuronidase was optimized by varying time and enzyme

TABLE I

WITHIN-DAY VARIABILITY FOR INDOMETHACIN AND ITS METABOLITES (n = 10)

Sample concn. (μg/ml)	INDO			DMI			DBI		
	Concn. found (μg/ml)	C.V. (%)	Error (%)	Concn. found (μg/ml)	C.V. (%)	Error (%)	Concn. found (μg/ml)	C.V. (%)	Error (%)
0.250	0.245	5.0	−2.0	0.240	8.2	−4.0	0.281	5.6	12.4
5.00	5.10	1.9	2.0	5.21	2.3	4.2	4.77	4.5	−4.6
50.0	52.3	2.9	4.6	51.8	0.8	3.6	—	—	—

TABLE II

BETWEEN-DAY VARIABILITY FOR INDOMETHACIN AND ITS METABOLITES (n = 10)

	INDO	DMI	DBI
Sample concn. (μg/ml)	5.00	5.00	5.00
Concn. found (μg/ml)	4.91	5.15	4.72
C.V. (%)	5.4	8.6	5.6
Error (%)	−1.8	−3.0	−5.6

TABLE III

EXTRACTION EFFICIENCY FOR INDOMETHACIN AND ITS METABOLITES (n = 7)

Concentration used was 5.0 μg/ml.

Compound	Extraction (%, ± S.D.)
INDO	99 ± 2.1
DMI	83 ± 2.9
F-INDO	99 ± 3.3
DBI	78 ± 6.4
IPA	80 ± 3.5

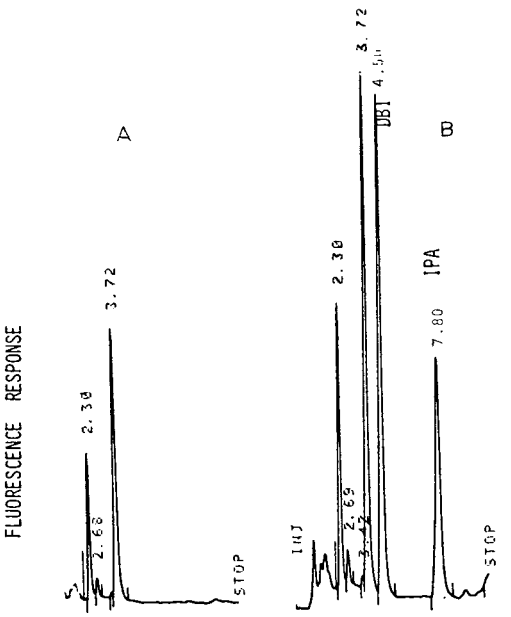

Fig. 3. Fluorescence chromatograms of (A) blank urine extract; (B) urine extracted after being spiked with 1.25 μg/ml DBI and the internal standard, IPA. Conditions are described in the text.

concentrations on several urine samples which had high levels of indomethacin. Incubation for 2 h, at 37°C with 1000 U provided maximum INDO concentrations, so these conditions were used for all subsequent samples. This assay has been applied to urine samples obtained after a single oral dose of 50 mg INDO. The concentration range of the standard curves cover normal urine concentrations found after this single oral dose. The sensitivity of the assay permitted detection of INDO and its metabolites in urine as long as 24—48 h after an oral dose. Chromatograms of patient samples are similar to those of the spiked samples presented in Figs. 2 and 3.

The extraction procedure above was also applicable to plasma samples, however, only INDO was able to be measured by a different reversed-phase HPLC system (data not presented). Interference of endogenous compounds and the lower concentrations in plasma as compared to urine prevented the application of this method to the measurement of DMI or DBI concentrations in plasma.

DISCUSSION

The method described provides an accurate determination of indomethacin and its principle metabolites in urine after glucuronide hydrolysis and has been used successfully for bioavailability studies. Although two separate HPLC systems were employed for the analysis, INDO, DMI and DBI could be extracted simultaneously into dichloromethane which simplified sample preparation. The use of pH 5.0 buffer and dichloromethane for the extraction yielded cleaner blank samples than could be obtained with lower pH solutions.

This was important when analyzing for DMI which, because of its polarity when compared to INDO, was not well separated from endogenous substances.

Because this method does not require radiolabelled drug or measurements of drug and metabolites by difference, it can be more easily implemented when studies on the excretion of INDO in urine need to be done. The advantages of the proposed method are: (a) direct measurement of all compounds, without determination by the difference method where variances are additive; (b) the chromatography has a more improved resolution than that provided by the method of Bernstein and Evans [3]; (c) there are no potential problems of DMBI degradation with base and; (d) glucuronic acid conjugates are cleaved prior to the extraction in our method, whereas Bernstein and Evans [3] suggest measuring unconjugated compounds in urine by extraction into ethyl acetate, but do not consider the likely possibility that conjugates are also extracted.

The availability of the internal standard, F-INDO, can be a problem. We have found, since the development of this method, that the acylation product of DMI with acetic anhydride yields a suitable potential internal standard with retention on the reversed-phase system described above between F-INDO and INDO. This acylation product has been utilized successfully as an internal standard for the analysis of indomethacin in plasma and should work equally well if used with urine.

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RAPID QUANTITATIVE DETERMINATION OF SEVEN ANTHRACYCLINES AND THEIR HYDROXY METABOLITES IN BODY FLUIDS

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described for the determination of seven anthracycline analogues and their hydroxy metabolites. The method is suitable for analysis of 30 plasma samples a day. The detection limit is 0.5 ng/ml for all compounds. Examples of the pharmacokinetics of adriamycin and carminomycin in man are shown.

INTRODUCTION

Since the introduction of the first anthracycline, daunorubicine, in clinical practice for the treatment of cancer [1], a number of analytical methods have been designed to determine concentrations of these compounds and their metabolites in plasma, urine, bile and in the intracellular fluid of different kinds of tissue.

The methods, which have recently been summarized by Arcamone [2], include fluorometry [3, 4], thin-layer chromatography [5, 6], high-performance liquid chromatography [7—13] and methods in which radioactively labeled anthracyclines are used, such as radioimmunoassay (^{3}H) [14] and total body autoradiography (^{14}C) [15].

The analytical procedure described in this paper is a modification of the reversed-phase method of Eksborg et al. [10], permitting the determination of seven anthracyclines: adriamycin (ADM), daunorubicin (DAR), carminomycin (CAM), 4′-epi-adriamycin (4′-epi-ADM), all in clinical use and of the experimental drugs 4-demethoxy-adriamycin (4-dem-ADM), 4′-deoxy-adriamycin (4′-deo-ADM), and 4-demethoxy-daunorubicin (4-dem-DAR) (Fig. 1).

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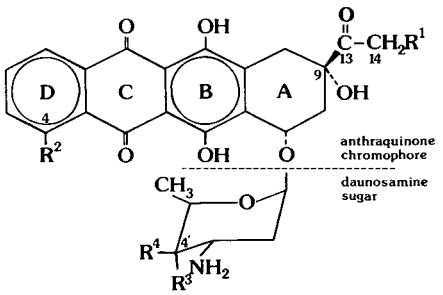

Fig. 1. Structural variations of seven anthracycline analogues.

	R^1	R^2	R^3	R^4
ADM	OH	OCH_3	OH	H
4'-epi-ADM	OH	OCH_3	H	OH
4'-deo-ADM	OH	OCH_3	H	H
4-dem-ADM	OH	H	OH	H
DAR	H	OCH_3	OH	H
CAM	H	OH	OH	H
4-dem-DAR	H	H	OH	H

The active 13-hydroxy metabolites can be determined in the same run. No other active metabolites have yet been reported [5].

The procedure was developed to obtain a determination method which could be easily performed, allowing analysis of 30 plasma samples a day and which would also lower the detection limit. The method is based on an on-column concentration and cleaning procedure [16], which allows injection of large volumes and thus lowers the detection limit. The method has been applied to pharmacokinetic studies performed in humans and dogs. Some examples of the pharmacokinetic behaviour of ADM and CAM and their hydroxy metabolites in man are shown.

MATERIALS AND METHODS

Drugs and chemicals

4-dem-DAR, 4-dem-ADM, 4'-deo-ADM and adriamycinol (ADM-ol) were kindly supplied by Farmitalia (Milan, Italy) and desipramine by Ciba-Geigy (Arnhem, The Netherlands). All other drugs were commercially available for clinical application and were obtained from: Roger Bellon (Neuilly, France) ADM; Specia (Paris, France) DAR; Bristol Meyers (Syracuse, NY, U.S.A.) CAM; Farmitalia (Milan, Italy) 4'-epi-ADM. All chemicals were of analytical grade and were used without further purification.

Chromatography

The chromatographic system consisted of a double-head solvent pump (Orlita, DHP-1515, Bakkers & Co., Zwijndrecht, The Netherlands), two sampling valves (Valco, Houston, TX, U.S.A.) and a sampling loop of 1 ml. Complete pulse quenching was achieved by means of a Silica Si 60 column (25 cm × 4.6 mm I.D.), followed by 2 m of coiled tubing (4 cm I.D.), between

the pump and injection valve. The analytical column (15 cm × 4.6 mm I.D.) was packed with reversed-phase material Cp^{tm}Spher C8, particle size 8 μm (Chrompack, Middelburg, The Netherlands). The concentration column (5 cm × 3.0 mm I.D.) was filled with LiChrosorb RP-8, 10 μm (Chrompack).

Detection

A fluorescence detector (Perkin-Elmer, Model 3000, Delft, The Netherlands), equipped with a red sensitive photomultiplier and a double mirrored flow cuvette was used. The maximum excitation wavelength was 474 nm. Alterations in the electron density of the D-ring (Fig. 1) shift the maximum emission wavelength from 590 nm to 568 nm for 4-dem-DAR and 4-dem-ADM and to 555 nm for CAM. The detector was connected to a 10-mV recorder (BD 40, Kipp & Zonen, Delft, The Netherlands). All experiments were carried out at room temperature.

Cleaning procedure and enrichment method [16]

The sample loop of 1 ml was filled as shown in Fig. 2b. By turning the upper valve, solvent A transports the sample onto the concentration column (Fig. 2a), where the anthracyclines and hydroxy metabolites are retained and concentrated while those products with more hydrophilic properties are wasted. By turning the lower valve, solvent B will flush the concentrated sample onto the analytical column (Fig. 2b). To obtain a sufficient clean up of the sample, 7 ml of solvent A was used. The sample could then be transported with 3 ml of solvent B from the concentration column onto the analytical column.

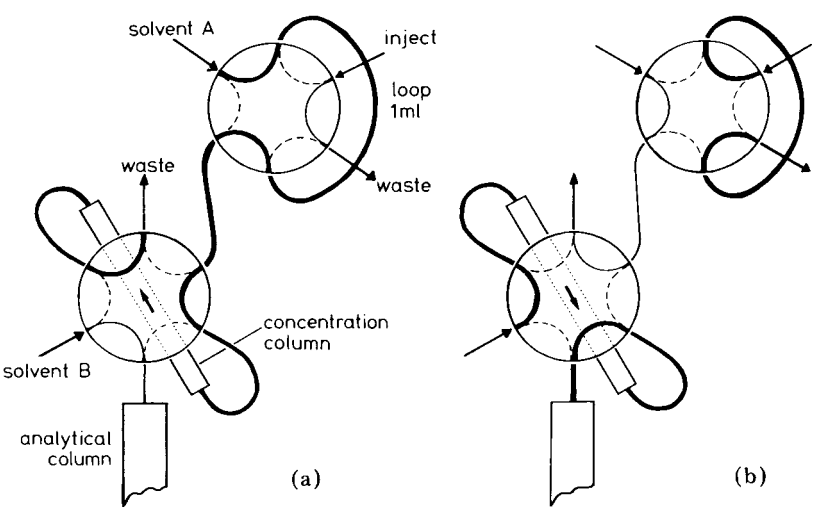

Fig. 2. Diagram showing sample enrichment through the on-column concentration method.

Solvents

Solvent A consisted of a 10 μg/ml solution of desipramine in demineralized water. Desipramine is added to limit the absorption of the anthracyclines

and their hydroxy metabolites on the tubing and column. The liquid flow-rate was kept between 1.5 and 2.0 ml/min. Solvent B consisted of a mixture of acetonitrile—citric acid buffer (9.8 ml of 0.1 *M* citric acid + 0.2 ml of 0.2 *M* Na_2HPO_4, pH 2.2)—demineralized water [x:10:(90—x), v/v]. All anthracyclines could be measured in the same retention time area (± 7 min) by slight alterations in the acetonitrile concentration : ADM and 4′-epi-ADM, 30% v/v; 4-dem-ADM and 4′-deo-ADM, 35% v/v; DAR, 40% v/v; CAM and 4-dem-DAR, 45% v/v. The liquid flow-rate was 1.0 ml/min at a pressure of 5 MPa.

Sample preparation

Plasma. Using a polypropylene tube to avoid absorption, 1.0 ml of boric acid buffer (pH 8.9) and 5.0 ml of a mixture of chloroform—isopropanol (4:1) were added to 1.0 ml of plasma. After mixing for 1 min on a Vortex mixer followed by centrifugation at 1400 *g*, the lower organic layer was transferred into another polypropylene tube. Then 1.0 ml of 0.1 *M* H_3PO_4 was added and the resulting mixture was mixed on a Vortex mixer for 30 sec. After centrifugation, 1.0 ml of the aqueous layer was injected into the chromatographic system.

Urine. Urine samples were diluted with 0.1 *M* H_3PO_4 according to the anticipated concentration and injected without further extractions.

Recovery

The recoveries of parent compounds in plasma were measured in triplicate over a concentration range from 5 to 500 ng/ml and were compared to the direct assay of the standards in 0.1 *M* H_3PO_4. The hydroxy metabolites are not available as pure substances, but can be found in urine after administration of the parent compounds. By dividing the areas under the peaks of the chromatogram for urine, which can be injected without extraction, by those areas obtained from the extracted blank plasma that was spiked with a small predetermined quantity of the same urine (containing a high concentration of the metabolite), the recovery for the extraction procedure of the metabolites could be determined (Table I).

Stability

To study whether the handling and storage of the plasma samples had an influence on the plasma concentration of the anthracyclines, spiked plasma containing 100 ng/ml ADM and ADM-ol was kept at —20°C. During seven consecutive days this plasma was thawed, the concentration of ADM and ADM-ol determined and refrozen again. Also samples which were stored at —20°C during fourteen days were analyzed.

Calibration curve

Using spiked plasma, a calibration curve in the anticipated concentration range (from 5 to 500 ng/ml) was made each morning before analysis. During the day, the analytical equipment was checked by injection of spiked plasma. The correlation coefficient of the calibration curves were always better than 0.98.

Pharmacokinetics

An example of the application of this method was the study of the pharmacokinetics of ADM and CAM. In two patients, admitted to the Sint Radboud Hospital (Nijmegen, The Netherlands) for treatment of their advanced malig-

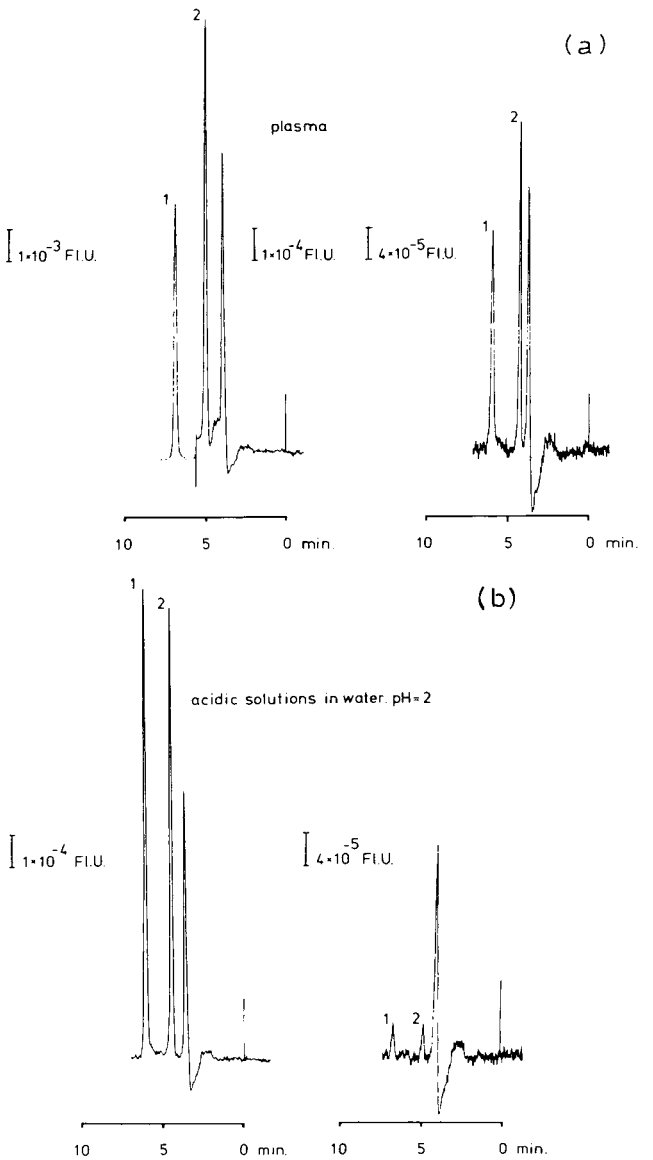

Fig. 3. (a) Chromatograms of plasma samples of a patient receiving 55 mg/m² body surface ADM by i.v. bolus injection. At 0.5 h after the administration, the plasma concentration of ADM (1) was 125 ng/ml and of ADM-ol (2) 19 ng/ml (left). Values of 5.9 ng/ml ADM (1) and 5.3 ng/ml ADM-ol (2) were measured 66 h after administration (right). (b) Chromatogram of an acidic aqueous solution of 20 ng/ml ADM (1) and 13.4 ng/ml ADM-ol (2) (left). The detection limit at a signal-to-noise ratio of 3 was 0.5 ng/ml for ADM (1) and 0.4 ng/ml for ADM-ol (2) (right).

nant disease after an intravenous (i.v.) bolus injection of 1.22 mg/kg ADM and of 0.36 mg/kg CAM. There was evidence of normal renal, hepatic and bone marrow function and no active cardiac disease. The patients gave informed consent. Blood samples were obtained at regular time intervals via an indwelling venous catheter and collected in heparinized tubes. Urine samples were taken from spontaneously voided urine.

The plasma concentration data were fitted according to a three-compartment open model, and the pharmacokinetic parameters were calculated according to conventional procedures [17].

RESULTS

Chromatography

Chromatograms of plasma samples obtained after 0.5 and 66 h from a patient, treated with an i.v. bolus dose of adriamycin of 55 mg/m^2 body surface are shown in Fig. 3a. Blank plasma showed only the injection peak. In Fig. 3b, the chromatograms of the acidic aqueous solutions in water are given, showing the detection limit of 0.5 ng/ml for ADM and of 0.4 ng/ml for ADM-ol at a signal-to-noise ratio of 3. The other anthracyclines all show the same pattern with different acetonitrile concentrations. The capacity ratio's (k') measured with an acetonitrile concentration of 35% v/v of the seven anthracyclines and their hydroxy metabolites are listed in Table I. The covariance in the concentration determinations of the anthracyclines and their hydroxy metabolites calculated from five spiked plasma samples were better than 5% in the concentration range of 5 to 500 ng/ml. The recovery rates after the extraction procedure of the seven analogues and their hydroxy metabolites are listed in Table I.

TABLE I

CAPACITY RATIOS (k') AND RECOVERY PERCENTAGES

For description of analytical procedures, see text.

Compound	k' (35% acetonitrile)	Recovery (%) (mean ± S.D.)
ADM	0.55	89 ± 2
ADM-ol	0.15	81 ± 3
4'-epi-ADM	0.74	87 ± 2
4'-epi-ADM-ol	0.29	79 ± 4
4-dem-ADM	1.02	90 ± 2
4-dem-ADM-ol	0.46	81 ± 3
4'-deo-ADM	1.28	93 ± 2
4'-deo-ADM-ol	0.53	83 ± 3
DAR	1.77	82 ± 2
DAR-ol	0.76	90 ± 3
CAM	2.98	74 ± 2
CAM-ol	1.50	83 ± 1
4-dem-DAR	2.98	80 ± 3
4-dem-DAR-ol	1.50	90 ± 1

Stability

During the seven consecutive days of thawing and refreezing, no decrease in the plasma concentrations of ADM and ADM-ol was observed. Also the concentrations of the samples after a storage period of fourteen days were found to be unchanged.

Clinical pharmacokinetics

Figs. 4 and 5 show the plasma concentration—time and renal excretion rate—time profiles of the parent compounds and their hydroxy metabolites in two patients after an i.v. bolus injection of ADM and CAM, respectively. Only 10% of the dosage administered is recovered in the urine as unchanged drug. A large difference exists between the percentage of the metabolites recovered in the urine (2.2% ADM-ol; 66% CAM-ol).

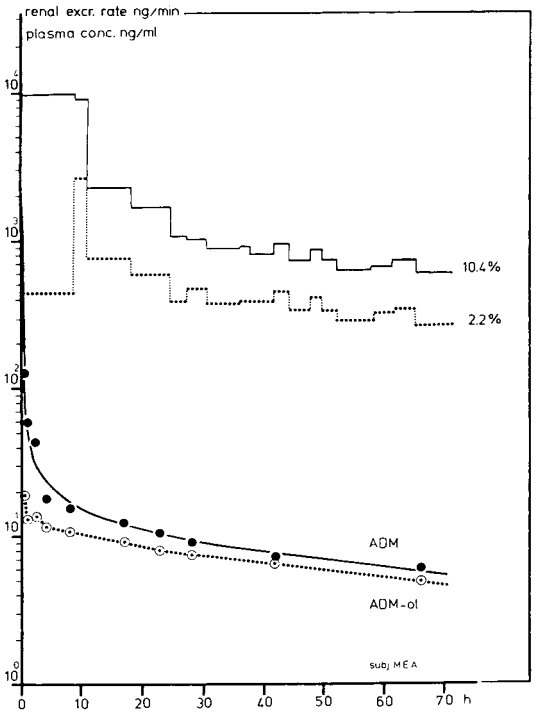

Fig. 4. The plasma concentration—time profiles of ADM (•) and of ADM-ol (○), measured over a period of 66 h, from a patient given 1.22 mg/kg by i.v. bolus injection. The renal excretion rate—time profiles of ADM (——) and of ADM-ol (• • •) are also shown.

A linear relationship between the renal excretion rates (ng/min) and the plasma concentrations (ng/ml) 10 h after administration were found for all four compounds. Two typical examples for ADM and ADM-ol are given in Fig. 6. The average renal clearance values (ml/min) as well as some pharmacokinetic parameters are listed in Table II.

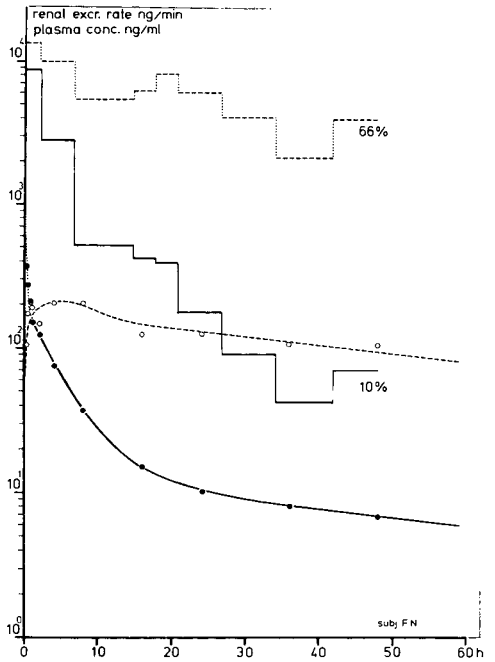

Fig. 5. The plasma concentration—time profiles of CAM (•) and of CAM-ol (○), measured over a period of 50 h, from a patient given 0.36 mg/kg by i.v. bolus injection. The renal excretion rate—time profiles of CAM (——) and of CAM-ol (• • •) are also shown.

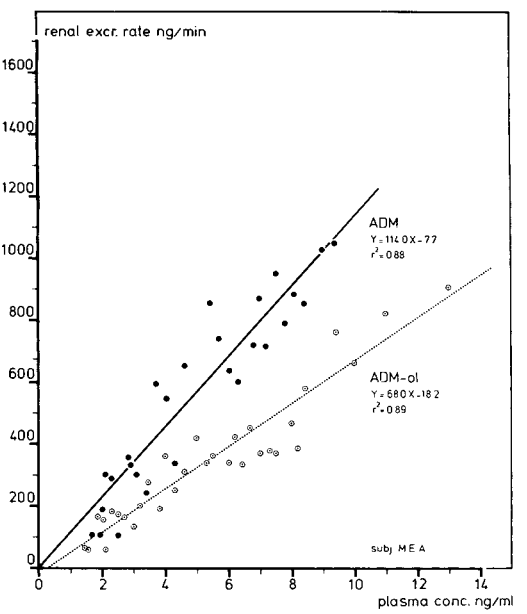

Fig. 6. The renal excretion rates versus plasma concentrations of ADM and of ADM-ol of a patient. The renal clearance of ADM was 114 ml/min, that of ADM-ol 68 ml/min.

TABLE II

PHARMACOKINETIC PARAMETERS OF ADRIAMYCIN AND CARMINOMYCIN IN MAN AFTER i.v. BOLUS ADMINISTRATION

Abbreviations: $t_{½}$ = half life time of terminal phase; V_f = apparent distribution volume; Cl_p = total body clearance; AUC_p = area under the curve of parent compound; AUC_m = area under the curve of hydroxy metabolite; Cl_{rp} = renal clearance of parent compound; Cl_{rm} = renal clearance of hydroxy metabolite; $rec._p$ = recovery of parent compound from urine; $rec._m$ = recovery of hydroxy metabolite from urine.

Drug	$t_{½}$ (h)	V_f (l)	Cl_p (ml/min)	AUC_p^* (ug.h/l)	AUC_m^* (ug.h/l)	Cl_{rp} (ml/min)	Cl_{rm} (ml/min)	$rec._p^{**}$ (%)	$rec._m^{**}$ (%)
ADM	54	1380	640	2760	1100	114	68	10	2.2
CAM	47	500	230	1800	14100	23	46	10	66

*The AUC values are extrapolated to $t = \infty$.
**The recoveries are calculated from the measured period of time.

DISCUSSION

From Fig. 4, it can be seen that the plasma concentrations of ADM and ADM-ol decline below 10 ng/ml within 10 h after i.v. bolus administration. Thereafter the plasma concentrations of ADM and ADM-ol decline with a half-life time ($t_{½}$) of 50 h. With the described method, it is possible to determine plasma concentrations of ADM after administration of conventional dosages for several days. The same holds true for CAM (Fig. 5). As the concentration of the anthracyclines in urine are higher than in plasma, there will be no problems using this method in determining urine concentrations during the week following administration, since the detection limit is 0.5 ng/ml (Eksborg et al. [10], 2 ng/ml).

In a recent article by Eksborg et al. [18], a decreased ADM plasma concentration was measured after ten thawings and refreezings. The concentration declined from 1000 to 50 ng/ml. After a storage time of six months at −20°C, the concentration had declined from 250 to 150 ng/ml.

In our experiment, however, no decrease in plasma concentration could be observed after a storage time fourteen days and after seven thawings and refreezings.

The structural difference between ADM and CAM results in an increased plasma concentration of the hydroxy metabolite of CAM, CAM-ol. At 20 h after administration, the ratio of the plasma concentrations ADM/ADM-ol was 1.11, that of CAM/CAM-ol 0.073. The renal clearances of ADM-ol and CAM-ol are in the same range: ADM-ol, 68 ml/min; CAM-ol, 46 ml/min. The high plasma concentration of CAM-ol explains the high recovery in urine (66%). Whether an increased affinity for the reducing enzymes of CAM or a lower volume of distribution of CAM-ol are the cause for its higher plasma concentration still needs to be investigated.

No glucuronides of the parent compounds or of the hydroxy metabolites have been found in plasma or urine. In case of ADM, excretion occurs mainly along the biliary route. This was confirmed in a dog experiment, where ± 50%

appeared to be excreted via this way measured over a period of three days (unpublished results).

The described method has been applied to study pharmacokinetics of ADM in man [19] and to study structure related pharmacokinetics of the anthracyclines in dogs [20].

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Note

Improved method for quantitative analysis of vitamin K_1 and vitamin K_1 2,3-epoxide in human plasma by electron-capture gas—liquid capillary chromatography

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Application of capillary columns in gas chromatography results in higher resolution, shorter analysis time and higher sensitivity compared to packed columns [1]. In recent years high-temperature-resistant stationary phases for capillaries have been developed and the introduction of fused-silica capillaries simplified installation and handling of such columns. We therefore tested if the use of a fused-silica capillary column instead of a packed column results in an improvement of our recently published gas-chromatographic method for the quantitative determination of vitamin K_1 and vitamin K_1 2,3-epoxide in plasma of man [2].

EXPERIMENTAL

Reagents

All solvents were pro analysi grade (Merck, Darmstadt, F.R.G.) and were used without further purification. *Cis*- and *trans*-vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), racemic vitamin $K_{2(20)}$ (menaquinone-4), *cis*- and *trans*-vitamin K_1 2,3-epoxide (2-methyl-3-phytyl-1,4-naphthoquinone 2,3-oxide), commercially available Konakion® solution (1 ml ampoules containing 10 mg of vitamin K_1) were kind gifts from Dr. Weber and Dr. Gloor (Hoffmann-La Roche, Basel, Switzerland).

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Gas—liquid chromatography

A Dani gas chromatograph (Model 3900) equipped with an electron-capture detector (radioactive source ^{63}Ni, 10 mCi, operated in the pulse mode with modulated frequency) was used. The column was a 25 m × 0.32 mm I.D. fused-silica capillary column with chemically bonded stationary phase (CP^{tm} Sil 5 CB, film thickness 0.12 μm; Chrompack Nederland B.V., Middelburg, The Netherlands). A Dani splitter introduction device was used. Injections of diluted samples (0.8—1.5 μl of *n*-heptane) were made by the Grob [3] splitless injection technique into the closed injection port at 280°C while the column was maintained at 60—80°C. After 40 s the injector was flushed with carrier gas (split ratio 1:50) and the column oven was quickly heated up to its final temperature of 285°C (heating rate about 40°C/min). The detector was set at 330°C. Oxygen-free helium was used as carrier gas (inlet pressure 1.2 bar) and nitrogen as make-up gas for the detector (flow-rate 30 ml/min).

Calibration

Calibration curves were constructed by adding known amounts of vitamin K_1 (Konakion ampoules) and *trans*-vitamin K_1 2,3-epoxide (12.5, 25, 50, 100, 150, 200 ng/ml) to pooled human plasma. The peak height ratios of vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide to the internal standard (vitamin $K_{2(20)}$) were plotted against the concentrations of vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide. Corrections had been performed which accounted for the fact that *trans*-vitamin K_1 2,3-epoxide contained about 10% of *cis*-vitamin K_1 2,3-epoxide and the Konakion (vitamin K_1) about 5% of *trans*-vitamin K_1 2,3-epoxide as impurities (as checked by gas chromatography).

The least-squares regression lines for vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide were fitted through the data points. The vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide concentrations of unknown plasma samples were determined by using the regression equations of the calibration curves which were run with each set of determinations of unknown plasma samples. Samples containing concentrations higher than 200 ng/ml for vitamin K_1 or *trans*-vitamin K_1 2,3-epoxide were suitably diluted with blank plasma.

Preparation of samples

Plasma samples of 0.4 ml were placed into 15-ml glass tubes. A volume of 50 μl of an ethanolic solution of vitamin $K_{2(20)}$ (2.0 μg/ml, internal standard), 2 ml of double-distilled water, and 10 ml of *n*-hexane—absolute ethanol (1:1) were added. The tubes were fitted with PTFE-lined screw-caps and extracted for 30 min on a rotary mixer at 25 rpm. After centrifugation the upper *n*-hexane layer was removed, placed into a pointed glass tube, and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 40 μl of *n*-heptane and 0.8—1.5 μl were injected into the gas chromatography by the Grob [3] splitless injection technique.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram obtained after splitless injection of 2 ng of *cis*- and *trans*-vitamin K_1 2,3-epoxide, vitamin K_1 (Konakion) and vitamin

$K_{2(20)}$. There was a baseline separation between all four compounds. *Cis*- and *trans*-isomers of vitamin K_1 could not be separated under our experimental conditions. The retention time of the vitamin $K_{2(20)}$ peak, which was eluted last, was about 12 min. Since cooling down of the oven from 285°C to injection temperature of 80°C takes about 6—8 min, repeated injections could be performed at 20—30-min intervals. The vitamin $K_{2(20)}$ peak showed a small "shoulder" at the end of the peak (Fig. 1,★). This was probably due to an unknown impurity or to an incomplete separation of a minor amount of the *cis*- or *trans*-isomer of vitamin $K_{2(20)}$. Since the isomers of vitamin $K_{2(20)}$ were not available this phenomenon could not be checked. When the *trans*-vitamin K_1 2,3-epoxide was injected it contained about 10% of its *cis*-isomer. The commercial available Konakion solution consisted of about 95% of vitamin K_1 and 5% of *trans*-vitamin K_1 2,3-epoxide.

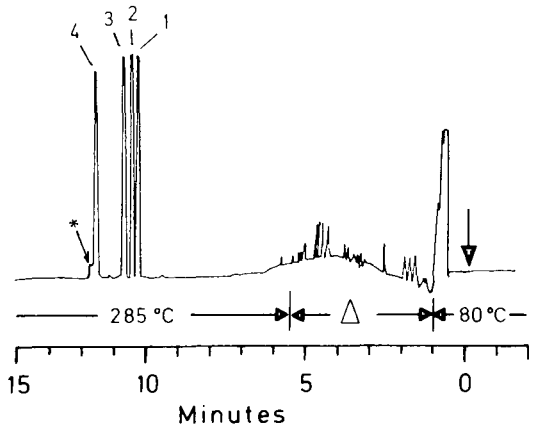

Fig. 1. Chromatograms after splitless injection of 1 μl of *n*-heptane containing *trans*- and *cis*-vitamin K_1 2,3-epoxide, racemic vitamin K_1 and racemic vitamin $K_{2(20)}$ (menaquinone 4), 2 ng of each. Peak 1 = *trans*-vitamin K_1 2,3-epoxide; peak 2 = *cis*-vitamin K_1 2,3-epoxide; peak 3 = racemic vitamin K_1; peak 4 = racemic vitamin $K_{2(20)}$ (* = resolution of an impurity which could be the *cis*- or *trans*-isomer of vitamin $K_{2(20)}$. Injection (↓) was done at an oven temperature of 80°C with the closed splitter. The splitter was opened after 40 s (split ratio 1:50) and the oven was heated up at maximal heating rate (△) to final temperature of 285°C. This temperature was reached at about 5—6 min after injection.

Examples of chromatograms from plasma extracts of blank plasma and plasma to which 15 ng/ml vitamin K_1 and 15 ng/ml *trans*-vitamin K_1 2,3-epoxide were added are shown in Fig. 2A. The *cis*-vitamin K_1 epoxide peak which resulted from an impurity of the added *trans*-vitamin K_1 2,3-epoxide appeared even visible between the K_1 and K_1 epoxide peak in the chromatogram (★ in Fig. 2A). No endogenous material in the blank plasma of ten healthy subjects and patients interfered with either vitamin K_1, *cis*- or *trans*-vitamin K_1 epoxide or vitamin $K_{2(20)}$ signals (Fig. 2B).

The calibration curves for vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide were linear up to concentrations of 200 ng/ml when 0.4-ml plasma samples were extracted, and passed through the origin ($r > 0.98$; intercept at the y-axis < 0.01). The lower limit of quantitative detection of vitamin K_1 and vitamin K_1 2,3-epoxide was about 5 ng/ml in plasma. This limit of detection in plasma was due to the fact that plasma extracts must be diluted by a factor of 1:40

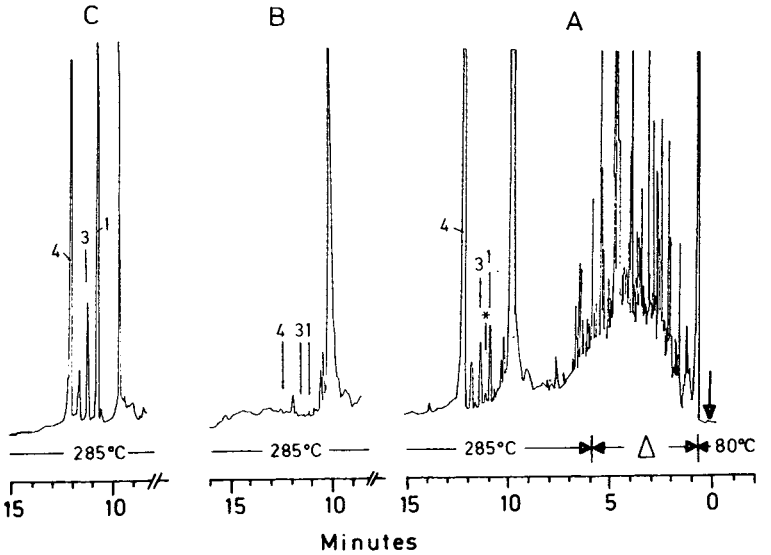

Fig. 2. Chromatograms of vitamin K_1 (peak 3), *trans*-vitamin K_1 2,3-epoxide (peak 1) and vitamin $K_{2(20)}$ (peak 4) from plasma extracts. (A) Extract of 0.4 ml of human plasma to which vitamin K_1 (15 ng/ml) and *trans*-vitamin K_1 2,3-epoxide (15 ng/ml) and vitamin $K_{2(20)}$ (250 ng/ml) were added. (B) Blank extract of 0.4 ml of human plasma. (C) Extract of 0.4 ml of plasma from a patient who was under treatment with the oral anticoagulant drug phenprocoumon (plasma concentration of 0.64 μg/ml). This patient had received a single intravenous dose of 10 mg of vitamin K_1; 4 h later a plasma sample was drawn, spiked with vitamin $K_{2(20)}$ as internal standard and analyzed. ⋆ indicates the even visible *cis*-vitamin K_1 2,3-epoxide peak (about 1.5 ng/ml) since the administered *trans*-vitamin K_1 2,3-epoxide contained about 10% of its *cis*-isomer (A). △ = heating period, heating rate of about 40°C/min.

to avoid an overload of the column by other coextracted lipids. For pure substances the lower limit of detection was about 10—20 pg.

The accuracy and reproducibility of the method is given in Table I. There was a good agreement between added and found vitamin K_1 and vitamin K_1 epoxide at the two plasma concentrations studied. Moreover, day-to-day variations in the slopes of the calibration curves were small (coefficient of variation below 10% within a time period of 3 months).

An example of the application of the method is shown in Fig. 2C. From a subject treated with the anticoagulant drug phenprocoumon (phenprocoumon plasma concentration of 0.64 μg/ml) and who received in addition 10 mg of vitamin K_1 intravenously, a plasma sample was drawn 4 h after injection of the vitamin. The concentration of vitamin K_1 in this sample was 78 ng/ml and that of vitamin K_1 2,3-epoxide 211 ng/ml. In former studies it was shown that in subjects treated with oral anticoagulant drugs the plasma levels of vitamin K_1 2,3-epoxide were maximal at 3—4 h after administration of vitamin K_1 [4]. Interestingly, the endogenously formed vitamin K_1 2,3-epoxide was the *trans*-isomer as shown in Fig. 2C.

Thus, the described capillary gas chromatographic method is suitable to study the kinetics and the metabolism of vitamin K_1 and vitamin K_1 2,3-epoxide in plasma of man following therapeutic doses of these vitamins. The lower limit of detection for vitamin K_1 and vitamin K_1 2,3-epoxide in plasma

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE ANALYTICAL METHOD

	Concentration (ng/ml)							
	Vitamin K_1 added to plasma		Vitamin K_1 found		Vitamin K_1 epoxide added to plasma		Vitamin K_1 epoxide found	
	1	2	1	2	1	2	1	2
	25.0	150.0	27.3	143.8	25.0	150.0	27.4	152.4
	25.0	150.0	22.3	165.3	25.0	150.0	19.7	151.2
	25.0	150.0	29.8	154.9	25.0	150.0	30.6	151.1
	25.0	150.0	25.6	154.5	25.0	150.0	23.2	153.5
	25.0	—	25.4	—	25.0	—	24.0	—
	25.0	—	30.4	—	25.0	—	28.2	—
	25.0	—	22.5	—	25.0	—	29.5	—
Mean ± S.D.	25.0	150.0	26.2 ±3.2	154.6 ±8.7	25.0	150.0	26.1 ±3.9	152.1 ±1.2
Coefficient of variation (%)			12	6			15	1

was about the same as that obtained with packed columns [2]. However, there are some profound advantages using the silica capillary column over the packed column:

(1) There was a complete baseline separation between vitamin K_1 and the isomers of vitamin K_1 2,3-epoxide. Using the packed column isomers of vitamin K_1 2,3-epoxide could not be separated and separation between vitamin K_1 and vitamin K_1 epoxide was incomplete.

(2) Efficiency and resolution remained constant over months even after repeated daily injections of plasma extracts. Using the packed column peak broadening and loss of efficiency often occurred when plasma extracts were injected repeatedly.

(3) Replacing the capillary column by a new one from the same manufacturer gives identical resolution and retention times. Using the packed column efficiency was influenced by minor differences in manufacturing of the glass column, and by packing and conditioning procedures.

(4) The high sensitivity of the method for pure substances should allow the detection of endogenous vitamin K_1 plasma levels, following adequate prepurification of plasma samples.

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CHROMBIO. 1952

Note

Comparison of high-performance liquid chromatography and gas chromatography—mass spectrometry for the analysis of indole-3-acetic acid in brain tissue

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Tryptamine, a presumably neuroactive metabolite of tryptophan, is one of the so-called trace amines [1] whose exact role in the mammalian central nervous system has not been elucidated to date. Existing experimental data suggest that it plays a role in the behavioural syndrome produced by administration of tryptophan with an inhibitor of monoamine oxidase [2] and that its effects resemble those of lysergic acid when infused in man [3]. Also, it has been shown that its actions in cerebral cortex [4] and hypothalamus [5] are opposite to those exerted by serotonin (5-hydroxytryptamine). Lately, a high-affinity binding site for [^{3}H]tryptamine has been described [6]. Thus, all experimental evidence supports a neuroregulatory role for tryptamine in the central nervous system. However, the concentration of this amine in rat brain is far below the levels of the classical neurotransmitter amines, such as serotonin and catecholamines [7]. Indole-3-acetic acid (IAA), the main metabolic product of tryptamine, could be taken as an index of the functional activity of tryptamine in the central nervous system [8] since routine measurements of this amine have proved to be extremely difficult due to its exceedingly low levels (down to 0.5 ng/g in rat brain [9, 10]). The levels of IAA are also low (10—15 ng/g in adult rat brain [9, 11]), but its determination does not pose the same difficulties as in the case of its parent amine. Two selected ion monitoring (SIM) methods have been developed recently for its quantification in brain tissue [9, 11]. Also, a high-performance liquid chromatographic (HPLC) procedure, involving a modified fluorimeter cell, has been described [12] and successfully applied [8].

Although SIM (isotope dilution technique) is considered to be a reference

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analytical technique, it has a number of disadvantages for routine work: the need of obtaining suitable chromatographic derivatives with high enough yield, the problem of adsorption and/or degradation of labile compounds [13], and the high cost of the equipment could be mentioned as the most important ones. In contrast, HPLC coupled to fluorimetric detection (HPLC—F) offers a remarkable degree of specificity (capitalizing on the natural fluorescence of indolic compounds) and less sample handling, although in the absence of modified equipment [12] the sensitivity of SIM is several times better than that achieved by HPLC—F.

This work describes a comparison of the SIM and HPLC—F techniques for the assay of IAA in rat brain tissue, using standard equipment for both methods.

MATERIAL AND METHODS

Chemicals

Standard indole-3-acetic acid was purchased from Sigma (St. Louis, MO, U.S.A.). Side-chain deuterated IAA (IAA-d_2) was from Merck, Sharp and Dohme (Montreal, Canada). Isotopic purity of IAA-d_2 was checked by SIM and was found to be better than 98%.

Pentafluoropropionic (PFP) anhydride (Regis, Morton Grove, IL, U.S.A.) was used to obtain the PFP derivatives.

All solvents and reagents were analytical grade and were used without further purification, except ethyl acetate and methanol which were distilled in an all-glass apparatus. Water used for the preparation of the homogenization media was distilled over potassium permanganate.

Stock solutions of IAA were prepared in distilled methanol, and stored at −30° C. Working solutions (approx. 1 ng/μl) were prepared by dilution for each experiment.

HPLC instrumentation

The HPLC system consisted of a solvent delivery pump (Model 6000) and a U6K injector from Waters Associates (Milford, MA, U.S.A.). The column was a μBondapak C_{18} (particle size 10 μm). Column effluents were monitored with a 650-10S fluorimetric detector (Perkin-Elmer, Norwalk, CT, U.S.A.). Fluorescence excitation and emission wavelengths were set at 280 and 340 nm, respectively [14].

Gas chromatography—mass spectrometry (SIM) instrumentation

A Hewlett-Packard 5995 gas chromatography—mass spectrometry (GC—MS) system was used, equipped with an open-split interface and a standard 9825A microprocessor. Samples were run on a 25 m × 0.3 mm I.D., bonded phase OV-101 capillary column, also from Hewlett-Packard (Palo Alto, CA, U.S.A.).

Experimental procedure

Homogenization and extraction. Adult male Sprague-Dawley rats weighing 180—200 g were killed by decapitation, their brains removed and rapidly taken up in a plate on ice. The brain tissue was blotted with filter paper to eliminate

the contribution of blood IAA, weighed and homogenized. Homogenization was carried out in 10 volumes of 1 mol/l potassium chloride solution at pH 2.0 (adjusted with hydrochloric acid) containing 0.1% of ascorbic acid.

The homogenate was divided into sixteen aliquots taking eight of them for GC—MS and the other eight left for HPLC, as described below.

The corresponding homogenates were centrifuged in a J-21 Beckman high-speed centrifuge (50 000 *g*, 30 min) and the supernates were processed as follows. To each of them (in a capped glass test tube), 6 ml of freshly distilled ethyl acetate were added. After vigorous shaking for 5 min, they were centrifuged at 1000 *g* for 5 min. This procedure was repeated once and the pooled organic extracts were evaporated in a rotary film evaporator at 37°C using heart-shaped flasks, to a final volume of 200—400 μl. The extracts were next transferred to 1 ml Regis conical microvials, and pooled with the 2 × 200 μl of distilled methanol used to wash the walls of the heart-shaped flasks. The extracts were evaporated to dryness under a helium flow.

Addition of standards for quantitative purposes. The internal standard (IAA-d_2) used for the GC—MS (SIM) determinations and the supplements of reference IAA added for quantification in HPLC by the method of "spiking" were added to aliquots of the same homogenate in order to insure that both were subjected to the same experimental conditions as the endogenous brain IAA. Thus for GC—MS studies a total of eight aliquots (6 ml each, pipetted under gentle stirring) were all supplemented with 41 ng of IAA-d_2 and either 0, 11.5, 34.5 or 57.5 ng of unlabelled IAA (concentration corresponding to 0, 20.4, 61.2 and 102.0 ng/g, respectively) obtaining duplicate samples A, B, C and D. The same sample preparation procedure was used for the HPLC analysis, except that no labelled IAA was added, resulting in an equivalent "spiked" duplicate sample series for the quantitative HPLC determinations. All this is summarized in Table I.

TABLE I

SCHEME OF SAMPLE PREPARATION

GC—MS			HPLC		
Sample	ng IAA-d_2	ng IAA	Sample	ng IAA-d_2	ng IAA
A_{GC}	41	0	A_{LC}	0	0
B_{GC}	41	11.5	B_{LC}	0	11.5
C_{GC}	41	34.5	C_{LC}	0	34.5
D_{GC}	41	57.5	D_{LC}	0	57.5

HPLC procedure. The HPLC identification and quantitative fluorimetric detection of IAA was carried out isocratically with 0.01 mol/l sodium acetate at pH 4.0 and 40% methanol. Eluents were filtered and degassed before use. The dry extracts were dissolved in 300 μl of the chromatographic eluent and aliquots of 30 μl were injected.

The content of IAA in each sample was calculated by the method of the added standard (sample supplemented with known amounts of IAA), which has proved to be useful in the determination of IAA in human plasma samples [15].

The precision of the HPLC method was evaluated by processing and analysing in duplicate six non-supplemented homogenates, corresponding to 0.52 g of brain tissue.

GC—MS procedure. Samples were chromatographed on an OV-101 bonded phase capillary column (25 m × 0.3 mm I.D.). Injection was carried out in the splitless mode. Helium was used as carrier gas. Injection port temperature was set at 250° C and injection column temperature at 60° C. Injection time was 0.7 min. Column temperature was programmed at 10° C between 170 and 250° C. The IAA-Me-1PFP derivative eluted from the column at 185° C approximately.

The dried samples for SIM analysis were treated according to described procedures [9] in order to obtain the methylpentafluoropropionyl derivatives of IAA and IAA-d_2 (IAA-Me-1PFP). For the methylation of the free carboxyl group, 20 μl of HCl—methanol (10%, v/v) were used instead of the BCl_3—methanol. The final solution was in 20 μl of isooctane and aliquots of 2—4 μl were injected into the GC—MS system.

The content of IAA in each sample was calculated by comparing the peak heights of the corresponding deuterated and non-deuterated compound.

The *m/z* values used to monitor the elution of IAA-Me-1PFP and its corresponding deuterated internal standard were 276 and 335 (M^+) for IAA and 278 and 337 (M^+) for IAA-d_2. Routine determinations were performed by comparing peak heights of either 278 vs. 276 or 337 vs. 335, the latter providing somewhat cleaner SIM profiles, even though their corresponding abundances in the mass spectra of IAA and IAA-d_2 are lower than those of 276 and 278.

The precision of the GC—MS method was evaluated by processing and analysing five identical samples containing 4.1 ng of IAA-d_2 (equivalent to 7.9 ng of IAA-d_2 per g of tissue).

RESULTS AND DISCUSSION

The GC—MS technique is the most sensitive for the determination of low levels of IAA. Using capillary columns, 5 pg of authentic IAA can be unequivocally detected at a signal-to-noise ratio of 2. In contrast, the detection limit by HPLC can be set at 150 pg.

Fig. 1 shows the GC—MS (SIM) and HPLC profiles corresponding to a brain extract. In both cases, these profiles are free from interference. The identity of IAA in GC—MS is confirmed by monitoring also the ion at *m/z* 335 (molecular ion) and by the practical coincidence of the retention times corresponding to the peak of IAA (*m/z* 276 and 335) and the internal standard IAA-d_2 (*m/z* 278 and 337). In HPLC the identity is assigned by the increased response when the sample is supplemented with IAA as well as by the coincidence of retention times between standard IAA and brain IAA.

Fig. 2 shows the least-square roots correlation of quantitative data for the two methods in the range of 18—120 ng/g. Points represent the mean of duplicate (HPLC runs) or triplicate (SIM runs) determinations carried out on all of the duplicate homogenates. The corresponding parameters are [SIM value = $a + b$ (HPLC value)] a = 0.8 ng/g, b = 0.995, and r^2 = 0.991. Both parameters a and b indicate that the accuracy of each method is almost identical.

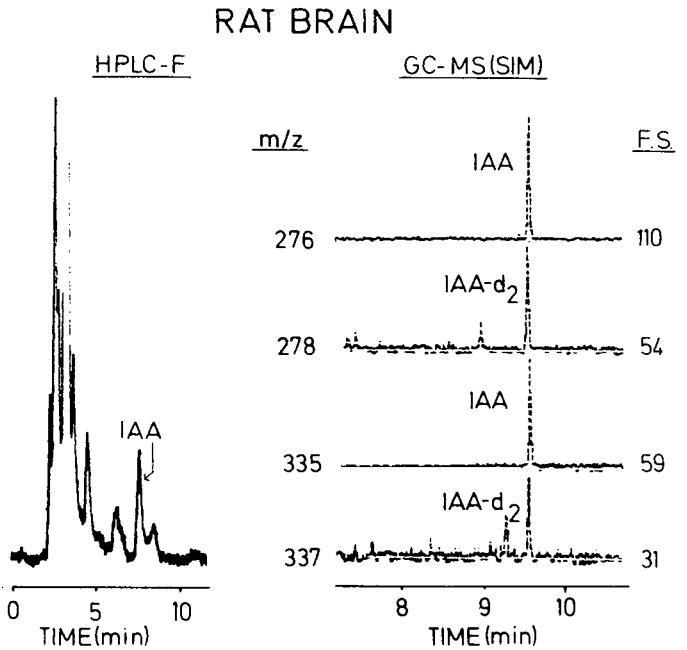

Fig. 1. HPLC—F and GC—MS (SIM) profiles corresponding to a brain extract. The figures on the right (F.S.) refer to the full-scale values of each of the GC—MS (SIM) traces.

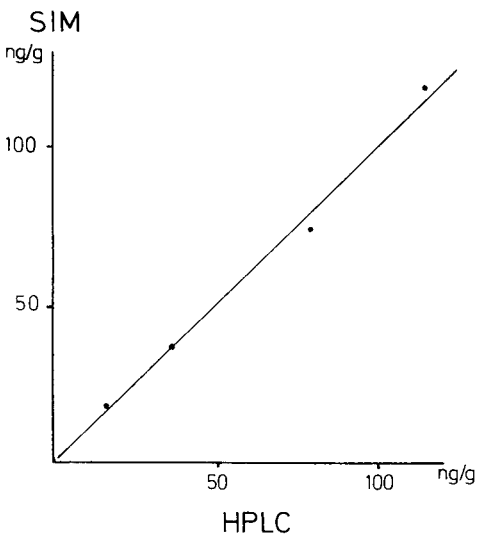

Fig. 2. Correlation of quantitative data for GC—MS (SIM) and HPLC methods. SIM value = $a + b$ (HPLC value), where $a = 0.8$, $b = 0.995$, $r^2 = 0.991$.

The coefficients of variation were 7.1% for HPLC and 8.5% for SIM. Such a result could be considered somewhat surprising and unexpected in view of the high precision usually claimed for the selected ion monitoring methods in GC—MS. However, it could be readily accounted for by the following practical facts. When the GC column was programmed at a rate of 10°C/min, the

coefficient of variation was as high as 17%. Under these conditions, and using the minimum dwell time for the two monitored *m/z* values (335 and 337), the peak profile of IAA-Me-1PFP consisted of only 5—6 sampling points. The entire peak width was less than 3 sec, so that the reliability of any measurement of peak maxima could be relatively poor. In contrast, it must be noted that the reported SIM precision has been obtained using a low program rate (2°C/min), so that, under these conditions, the chromatographic peak profile of IAA-Me-1PFP consists of at least ten steps, thus achieving a better definition of the true peak maxima. Consequently, a reduced peak width, as obtained with capillary columns, is not always an advantage for quantitative precision work in the GC—MS (SIM) mode. It must be taken into account that the coefficient of variation in GC—MS (SIM) is free from errors due to injection, derivatization and overall sample loss since it is calculated relative to deuterated analog used as internal standard, which does not apply in the case of HPLC runs. The fact that the precision in the GC—MS (SIM) mode is somewhat poorer must reflect the practical limitation of this and similar instrument software for fast ion switching within the short time domain of a peak eluted from a capillary column. In accordance, to increase the reliability of peak height measurements, we have found it advisable to degrade the GC performance by going to longer elution times, thus obtaining wider peaks.

In summary, we have shown that the two methods herein described, based on a classic extraction and further analysis by HPLC or GC—MS (SIM), give practically identical accuracy and precision for the assay of IAA in brain tissue. However, HPLC would be considered in this case as the method of choice for routine purposes, while GC—MS (SIM) would be very useful in cases requiring a higher sensitivity or for confirmatory purposes.

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Note

Simultaneous analysis of cortexolone and cortisol by high-performance liquid chromatography for use in the metyrapone test

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Analysis of cortexolone, cortisol, and other endogenous steroids has become important in assessing the integrity of the hypothalamic-pituitary-adrenal (HPA) axis in Addison's disease, Cushing's syndrome and depression [1—3]. Under physiological conditions, low concentrations of most endogenous steroids have necessitated the use of sensitive assay methods such as the radioimmunoassay (RIA). The higher physiological concentrations of cortisol have allowed its analysis by fluorometry [4], competitive protein binding [5], liquid chromatography [6], as well as RIA techniques [7]. Many of these methods lack specificity in separation or specific antibodies to distinguish various endogenous steroids. This may be particularly important with the metyrapone test. The administration of metyrapone results in elevated cortexolone concentrations to a range which can be determined by current high-performance liquid chromatography (HPLC) techniques. The concomitant analysis of cortisol has been suggested as a measure of sufficient adrenal hydroxylase inhibition [3]; however, the supra-physiological cortexolone concentrations may cause falsely elevated cortisol values due to lack of antibody specificity with the RIA technique [6, 8]. Previous liquid chromatography methods for cortexolone and cortisol lack the ability to assay both steroids simultaneously and therefore require two separate internal standards and chromatographic conditions [9]. Other techniques do not utilize an internal standard and fail to selectively extract the steroids without extracting metyrapone [6]. In the single dose version of the test, metyrapone is present in μg/ml quantities and may interfere with the quantitation of ng/ml quantities of the steroids especially under reversed-phase chromatographic conditions.

This report describes an HPLC method which was modified from our previ-

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ous exogenous steroid assays [10—12] to allow selective and simultaneous quantitation of cortexolone and cortisol with a single internal standard in human plasma for use in the metyrapone test.

EXPERIMENTAL

Materials

The HPLC system consisted of a continuous flow, constant volume Model 6000A solvent delivery system and a Model 441 UV absorbance detector (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 universal loop injector (Rheodyne, Berkeley, CA, U.S.A.), and an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). A Zorbax Sil (DuPont, Wilmington, DE, U.S.A.) column (25 cm × 4.6 mm I.D., 5—6 μm particle size) and a 70 × 6 mm stainless-steel precolumn packed with HC-Pellocil (37—53 μm particles) (Whatman, Clifton, NJ, U.S.A.) were used to separate the compounds.

Methylene chloride, used in the extraction procedure, and hexane, used in the mobile phase, were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Pure ethanol (U.S.P.), obtained from U.S. International Chemicals (New York, NY, U.S.A.) and glacial acetic acid, purchased from J.T. Baker (Philipsburg, NJ, U.S.A.), were employed in the mobile phase. The cortisol, dexamethasone, and cortexolone analytical standards were obtained from Sigma (St. Louis, MO, U.S.A.). Pharmaceutical grade decolorizing carbon, neutral, was purchased from Amend Drug and Chemical Co. (Irving, NJ, U.S.A.). Anhydrous sodium sulfate, sodium hydroxide solution (1 *M*) and hydrochloric acid solution (1 *M*) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Standard preparation

Decolorizing carbon (12.5 g) was added to 240 ml of pooled human plasma and stirred for 2 h at room temperature. Carbon was then removed by centrifugation at 17,000 *g* for 6 h at 4°C and by filtration through 5-μm and 0.45-μm Millipore filters (Millipore, Bedford, MA, U.S.A.). To this cortisol-stripped plasma, standards of cortisol and cortexolone in acetonitrile—methanol (1:1) were added to provide concentrations of 5 to 500 ng/ml.

Extraction procedure

Plasma samples (1 ml) were placed into acid-washed glass extraction tubes with PTFE-lined screw caps (20 × 150 mm) and 30 μl of a 5 μg/ml solution of dexamethasone in acetonitrile—methanol (1:1) were added as the internal standard. Then 0.3 ml of 1 *M* hydrochloric acid and 15 ml of methylene chloride were added. The tubes were capped, shaken for 15 min, and then centrifuged for 10 min. The aqueous layer and creamy interface were aspirated. The remaining organic phase was then washed with 1 ml of 0.1 *M* sodium hydroxide, with 1 ml of distilled water, and dried by adding 1 g of anhydrous sodium sulfate to each tube. After decanting, the organic phase was evaporated to dryness at 45°C under a nitrogen gas stream.

Chromatography

The residue was reconstituted with approximately 250 μl of mobile phase for injection. All chromatography was carried out at ambient temperature. The mobile phase, composed of a hexane—methylene chloride—ethanol—acetic acid (26:69:3.4:1) mixture, was pumped through the column at a constant flow-rate of 2 ml/min (85 bars). Concentrations of cortexolone and cortisol were determined by comparison of the peak height ratio of drug to internal standard with the peak height ratio of known standard concentrations of the drugs.

Steroid recovery

The assay recovery of each steroid was assessed at 50 ng/ml and 300 ng/ml. The peak heights from ten extracted plasma samples (1 ml) and from ten direct injections of the same amount of steroid (e.g. 50 and 300 ng) in mobile phase were compared. The assay recovery of each steroid was computed using the following equation:

$$\text{Percent recovery} = \frac{\text{peak height, extracted drug}}{\text{mean peak height, direct injection}} \times 100$$

Assay comparison

Plasma samples from depressed-psychiatric patients receiving the metyrapone test to assess the hypo-activity of the HPA axis were measured by the HPLC method and a radioimmunoassay (RIA) method. The commercial RIA kits for cortexolone and cortisol were purchased from Radioassay Systems Labs. (Carson, CA, U.S.A.).

RESULTS

The chromatogram shown in Fig. 1a illustrates the response to steroid concentrations of approximately 25 ng/ml in plasma from which endogenous steroids were removed by charcoal. Each steroid eluted with a sharp peak and distinct separation at baseline. Cortexolone eluted before cortisol, and also showed a relatively greater detector response. Good separation is maintained in chromatograms of plasma samples taken with and without elevated cortexolone from the metyrapone test (Fig. 1b and c). The mean assay recoveries were 59% for cortexolone and 50% for cortisol. Similar recoveries were seen with the internal standard. The apparent minimum quantification limit for both cortexolone and cortisol is 5 ng/ml. Calibration plots of peak height ratio versus steroid concentration were linear over the range of 5 to 1000 ng/ml.

The within-day and between-day precision of the assay were determined by analysis of ten plasma samples containing high (300 ng/ml) and low (50 ng/ml) concentrations. These data are presented in Table I. Assay specificity was determined by comparing retention times of standards to those of samples (Table II) and by analysis of samples from patients receiving other drugs. These drugs and their metabolites which had no apparent assay interference included: imipramine, amitriptyline, doxepin, oxazepam, flurazepam, chlorpromazine,

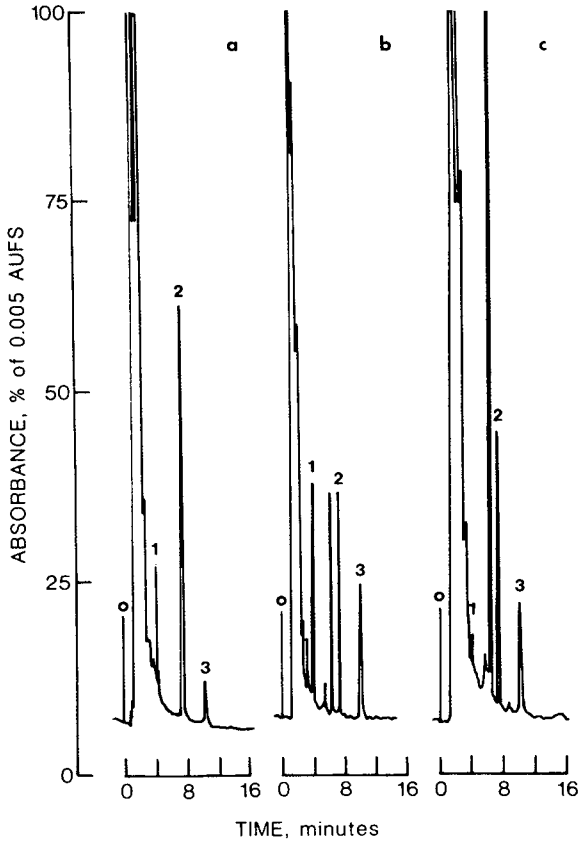

Fig. 1. Chromatograms of (a) charcoal-stripped plasma extract spiked with 25 ng/ml of cortexolone (1) and cortisol (3); (b) plasma extract from a patient with metyrapone and, (c) plasma extract from a patient without metyrapone. Dexamethasone, 150 ng (2) is the internal standard. The symbol (o) designates the injection point.

TABLE I

WITHIN-DAY AND BETWEEN-DAY VARIABILITY OF ASSAYS

Steroid	Within-day				Between-day			
	Mean concn. (μg/l)	S.D.	C.V. (%)	*n*	Mean concn. (μg/l)	S.D.	C.V. (%)	*n*
Cortexolone	50.9	6.04	11.9	10	47.2	6.19	13.1	11
	276	14.2	5.16	10	290	31.7	10.9	10
Cortisol	48.8	2.47	5.06	10	48.6	7.83	16.1	11
	277	5.33	1.92	10	291	22.6	7.76	11

haloperidol, fluphenazine, thiothixene, acetaminophen, various bronchodilators, anticonvulsants, and antibiotics as has been previously reported [12].

Twelve plasma samples were measured by the HPLC method and the commercial RIA method. The two assays showed excellent correlation for cortexolone with a regression line slope of 1.05 (Fig. 2a). Cortisol was also assayed in five of these samples as well as in eight other samples from patients who had not received metyrapone. The RIA method yielded similar results as

TABLE II

RELATIVE CHROMATOGRAPHIC DRUG RETENTION TIMES

Drug	Relative retention time
Deoxycorticosterone	0.31
Cortexolone	0.45
Fluocinonide	0.51
Cortisone	0.56
Corticosterone	0.57
Methylprednisone	0.58
Prednisone	0.66
Beclomethasone	0.73
Aldosterone	0.84
Betamethasone	0.86
Dexamethasone	0.93
Cortisol	1.00
Methylprednisolone	1.2
Prednisolone	1.3

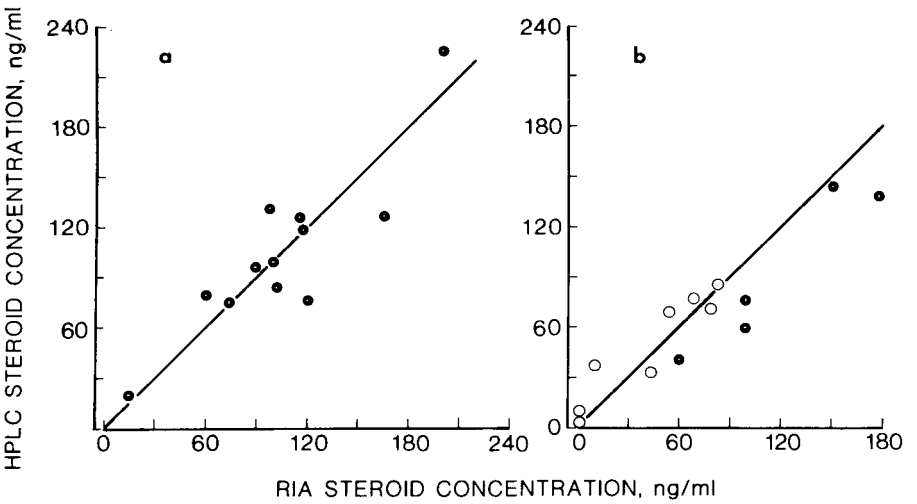

Fig. 2. Correlation between plasma cortexolone (a) and cortisol (b) concentrations, as measured by HPLC and RIA methods. Plotted lines denote slopes of unity. For cortexolone, the calculated slope = 1.05, the intercept = —4.65, and the correlation coefficient = 0.881. For cortisol without metyrapone testing (○), the slope = 1.01, the intercept = 9.77, and the correlation coefficient = 0.935. For cortisol with metyrapone testing (●), the slope = 1.00, the intercept = —25.6, and the correlation coefficient = 0.960.

the HPLC method for cortisol in the absence of the metyrapone test; however, in the presence of metyrapone and elevated cortexolone concentration, the RIA assay yielded higher values than the HPLC method (Fig. 2b). The mean cortisol concentration (117 ng/ml) measured by RIA in the presence of metyrapone was significantly higher (p = 0.017, non-paired Student's t test) than the mean cortisol concentration (92 ng/ml) without metyrapone.

DISCUSSION

The simultaneous measurement of cortexolone and cortisol by this HPLC

method is efficient, precise, sensitive and selective. To date, over 150 cortexolone and over 2000 cortisol and exogenous steroid samples have been analyzed by this chromatographic procedure in studies of the disposition kinetics of exogenous steroids, cortisol, and in psychoendocrinologic studies.

Variability between assay methods in population mean cortisol concentrations has been described following the single-dose metyrapone test [6, 8, 9]. This may be a function of variability in both the assay method and in adrenal hydroxylase inhibition. The latter may be caused by interpatient differences in metyrapone pharmacokinetics or perhaps variability in sampling and handling methods. To further assess this discrepancy and to avoid interpatient variability, the same serum samples were analyzed by both HPLC and RIA methods. Therefore, the increased variability of cortisol measurements following the metyrapone test can be attributed solely to differences in the assays. In our small patient sample, the RIA values were 4 to 66% higher than the HPLC values. This is similar to the 50 to 90% difference between HPLC and RIA results reported by Reardon et al. [6] between reversed-phase HPLC with UV detection and a commonly available RIA method (Beckman Instruments, Irvine, CA, U.S.A.). The more pronounced difference reported by Reardon et al. [6] may be due to differences in antibody specificity between our two RIA assays. On the contrary, Schoneshofer et al. [13] describe a gradient HPLC method with UV detection following a solid phase extraction method which produced interferences in 21% of 195 serum samples analyzed for cortisol in comparison with a commonly available RIA method. The intermediary washing steps usually exclude such interferences in our liquid–liquid extraction method.

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Note

Determination of 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 in urine by high-performance liquid chromatography and radioimmunoassay

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6-Keto-prostaglandin $F_{1\alpha}$ (6ketoPGF$_{1\alpha}$) and thromboxane B_2 (TXB_2) are the non-enzymatic hydrolysis products of prostacyclin (PGI_2) and thromboxane A_2 respectively [1]. Both these compounds have been found in urine [2,3] and their excretion rate may reflect renal synthesis [3,4]. The parent compounds are formed both in the kidney and elsewhere in the body [1] and hydrolyze rapidly to 6ketoPGF$_{1\alpha}$ and TXB_2. If formed in extra-renal sites they then can be metabolised to several other compounds. When PGI_2, 6ketoPGF$_{1\alpha}$ or TXB_2 are injected intravenously, little of each appears in urine but several metabolites of each are excreted [5,6]. These metabolites and other substances could conceivably interfere with the determination of 6ketoPGF$_{1\alpha}$ and TXB_2 by radioimmunoassay. By subjecting urine to complex extraction and purification procedures it is hoped that potentially interfering substances will be separated from the compounds of interest, but this hypothesis is difficult to test because of the lack of availability of all possible metabolites. Because reversed-phase high-performance liquid chromatography (HPLC) is a powerful separation technique for lipids we applied it to validate our extraction, purification and radioimmunoassay procedure.

EXPERIMENTAL

Materials and solvents

Pure standards of 6ketoPGF$_{1\alpha}$ and TXB_2 were donated by the Upjohn Company (Kalamazoo, MI, U.S.A.). [^{3}H]6ketoPGF$_{1\alpha}$ (150 Ci/m*M*) and [^{3}H]TXB_2 (100 Ci/m*M*) were purchased from New England Nuclear (Montreal, Canada) and used without further purification. All glass columns for Sephadex LH-20 were purchased from Johns Scientific (Toronto, Canada). Liquid scintil-

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lation counting was performed on a Nuclear Chicago Mark II instrument using 9 ml Aquasol (New England Nuclear). All other solvents were of the highest available purity.

Extraction of 6ketoPGF$_{1\alpha}$ from urine

Urine (10 ml) to which a tracer amount (1000 cpm) of [^{3}H]6ketoPGF$_{1\alpha}$ had been added was brought to pH 3.0 with 1.0 *M* hydrochloric acid and applied to a 6-ml prepacked, disposable octadecyl (C_{18}) column (J.T. Baker, Phillipsburgh, NJ, U.S.A.). The column was successively eluted with 20 ml of 15% methanol, 20 ml hexane and 15 ml methyl formate. The methyl formate fraction, containing the bulk of the 6ketoPGF$_{1\alpha}$, was evaporated to dryness under nitrogen, the residue taken up in 1 ml of a solvent system composed of chloroform—heptane—methanol—acetic acid (100:100:10:2) and applied to a 130 × 10 mm glass Sephadex LH-20 column. This column had previously been equilibrated with chloroform—heptane—methanol—acetic acid (100:100:30:2). After the sample was applied, the column was successively eluted with 10 ml of the original solvent, 9 ml of chloroform—heptane—methanol—acetic acid (100:100:20:2), and 20 ml of chloroform—heptane—methanol—acetic acid (100:100:25:2). In this latter fraction 78±3% (S.E.M.) (n=13) of the added [^{3}H]6ketoPGF$_{1\alpha}$ was contained. The sample was evaporated to dryness under nitrogen in a 40-ml conical centrifuge tube and stored for up to three days at −20°C before assay.

Extraction of TXB$_2$ from urine

Ten ml of urine, to which 1000 cpm of [^{3}H]TXB$_2$ had been added was titrated to pH 7.0 with 1.0 *M* potassium hydroxide and washed with 15 ml hexane to remove neutral lipids. The aqueous layer was brought to pH 3.5 with concentrated formic acid and extracted with 30 ml chloroform. The extract was evaporated under nitrogen, taken up in 0.4 ml of chloroform—heptane—ethanol—acetic acid (100:100:10:2) then applied to a 130 × 10 mm Sephadex LH-20 column. The column was eluted with the following solvent systems in sequence (chloroform—heptane—ethanol—acetic acid) 10 ml (100:100:10:2), 12 ml (100:100:20:2) and 20 ml (100:100:25:2). Recovery of added [^{3}H]TXB$_2$ was 64±3% (S.E.M.) (n=13). The final fraction, containing the TXB$_2$ was evaporated under nitrogen in a 40-ml conical centrifuge tube and stored at −20°C for up to three days prior to assay.

Radioimmunoassay procedures

Radioimmunoassays were performed in 75 × 10 mm polystyrene tubes. All reagents were diluted in Tris—PVP buffer (6.08 g Tris base, 9.0 g sodium chloride, 1.0 g polyvinylpyrrolidone (MW = 40,000), 3.4 ml of 0.5 *M* magnesium sulfate and 1.6 ml of 0.1 *M* calcium chloride, 2.0 ml of 10 *M* hydrochloric acid, per l water, pH 7.4). All assays were performed in duplicate. For 6ketoPGF$_{1\alpha}$ 5000 cpm [^{3}H]6ketoPGF$_{1\alpha}$ were added to tubes containing either buffer alone, known amounts of 6ketoPGF$_{1\alpha}$ (5—250 pg) or aliquots of urine extracts. Rabbit antiserum to 6ketoPGF$_{1\alpha}$ (supplied by Dr. P.V. Halushka, Medical University of South Carolina, Charleston, SC, U.S.A.) was added in a final dilution (assay volume 450 μl) of 1:25,600. The tubes were incubated

12—18 h at 4°C, and separation of bound and free [^{3}H]6ketoPGF$_{1\alpha}$ was achieved with 1.0 ml dextran–charcoal (1:10, w/w). Standard curves were constructed using Scatchard plots from which unknowns were calculated after subtracting the mass of [^{3}H]6ketoPGF$_{1\alpha}$.

A similar procedure was used for radioimmunoassay of TXB_2. To tubes containing either buffer, known amounts of TXB_2 (10—750 pg) or aliquots of urine extracts were added 5000 cpm [^{3}H]TXB_2. Rabbit antiserum to TXB_2 (again supplied by Dr. Halushka) was added to each tube in a final dilution of 1:20,000 for the total assay volume of 200 μl. Tubes were incubated at 37°C for 3 h. Separation of bound and free [^{3}H]TXB_2, using dextran—charcoal, and analysis were identical to the 6ketoPGF$_{1\alpha}$ radioimmunoassay.

HPLC experiments

Known amounts of 6ketoPGF$_{1\alpha}$ or TXB_2 were added to 10-ml aliquots of urine from a normal volunteer and extracted as detailed above. For these extractions, recovery calculation was based only on the amount assayed by HPLC. In some experiments, the amount of tritiated standard was increased 10—20 fold to allow measurement of radioactivity in fractions collected after HPLC.

The HPLC hardware consisted of a M-6000 pump, a U6K universal injector, a Model 480 UV detector, a Model 720 system controller and a Model 730 data module (Waters Assoc., Milford, MA, U.S.A.). Fractions were collected after the solvent had passed through the detector. A Waters Fatty Acid Analysis steel jacketed column (300 × 3.9 mm, particle size 10 μm) was used. Prostanoids were isocratically eluted at 1.5 ml/min with a mobile phase consisting of 2 m*M* potassium dihydrogen phosphate—acetonitrile (70:30), pH 3.5. The detector was adjusted to 194 nm with mobile phase in the reference cell. Fractions (0.75 ml) were collected after HPLC and subjected to radioimmunoassay as above. Where larger amounts of tritiated standards were added to the original urine specimens, the fractions were counted for tritium.

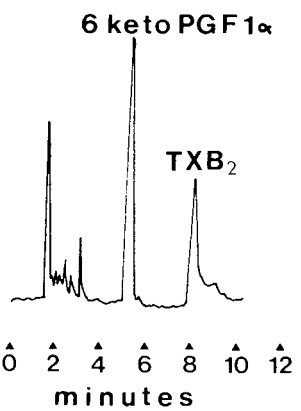

Fig. 1. Chromatogram of 6ketoPGF$_{1\alpha}$ and TXB_2 (100 ng each) in mobile phase. Retention times: 6ketoPGF$_{1\alpha}$ = 5.05 min; TXB_2 = 7.90 min.

RESULTS

Standard curves

Fig. 1 is a HPLC chromatogram of 6ketoPGF$_{1\alpha}$ and TXB_2, 100 ng of each injected in mobile phase. Retention times are 5.05 and 7.90 min, respectively. Standard curves, were linear over the range 25—100 ng. Regression lines relating area of the peak in mm^2 (Y) to ng of prostanoid added (X) were: 6ketoPGF$_{1\alpha}$, $Y=-3.5+5.29X$ ($r=0.998$); TXB_2, $Y=3.3+3.2X$ ($r=0.997$).

Radioassay and radioimmunoassay of HPLC fractions

Figs. 2 and 3 show chromatograms of urine extracts. The upper and lower

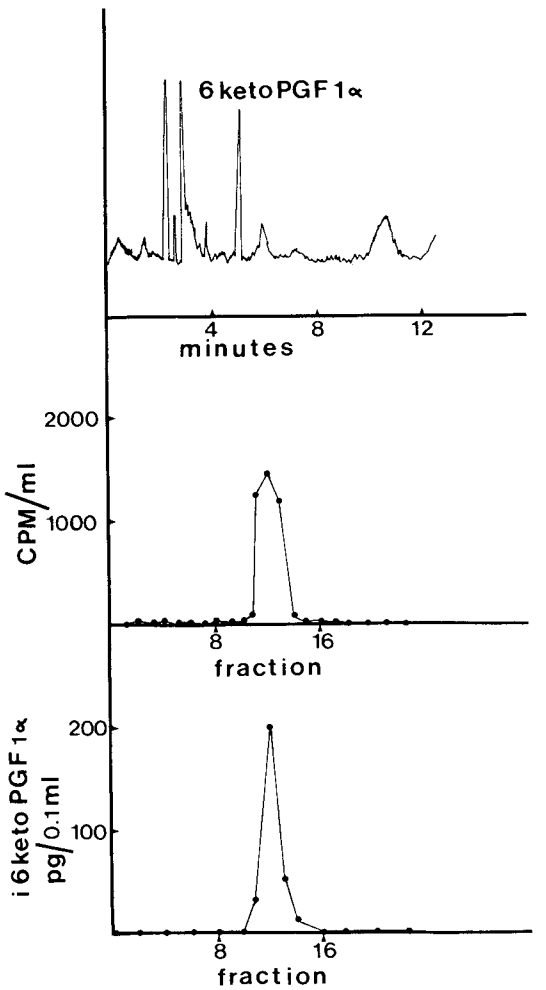

Fig. 2. Chromatograms of urine extracts; 100 μg authentic 6ketoPGF$_{1\alpha}$ or 10,000 cpm [^{3}H]6ketoPGF$_{1\alpha}$ added to 10 ml urine. Upper panel: HPLC chromatogram showing peaks representing more polar materials with early retention times. Middle panel: radioactivity per fraction. Lower panel: immunoreactive (i) 6ketoPGF$_{1\alpha}$ per fraction. The early peaks seen in the upper panel do not contain radioactivity or immunoreactive 6ketoPGF$_{1\alpha}$. The peaks in the lower two panels are delayed 1—2 min due to the volume from the UV detector to the fraction collector.

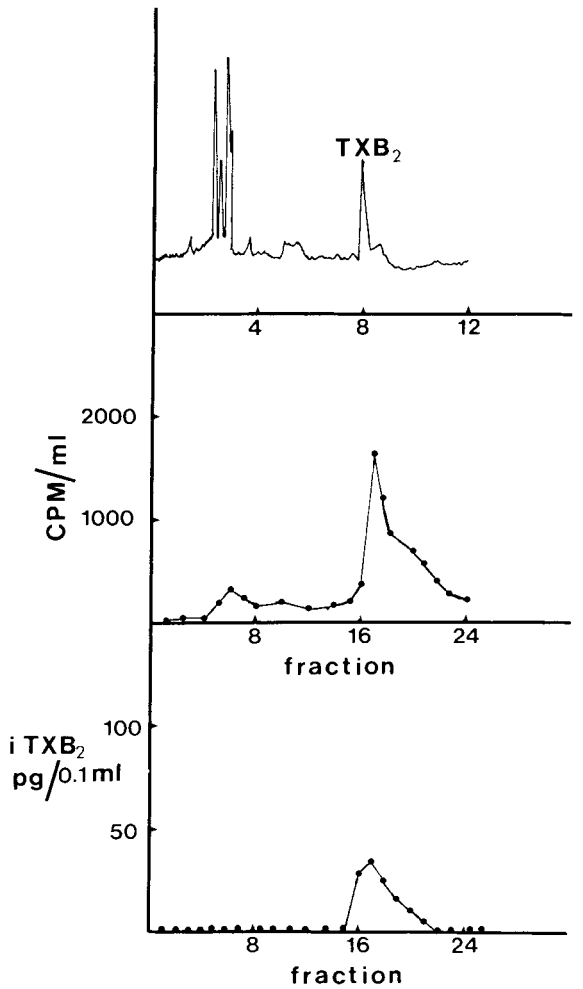

Fig. 3. Chromatograms of urine extracts; 50 μg authentic TXB_2 or 10,000 cpm [^{3}H]TXB_2 added to 10 ml urine. Upper panel HPLC chromatogram. Middle panel: radioactivity per fraction. Lower panel: immunoreactive (i) TXB_2 per fraction.

panels of each figure represent a 10-ml urine specimen to which 100 μg of each cold standard was added before extraction and purification. The middle panels represent similar urine aliquots to which tritiated compounds were added. It can be seen that the only HPLC peaks containing significant tritium or immunoreactive 6ketoPGF$_{1\alpha}$ and TXB_2 correspond to those with the retention times of authentic compounds. We take this result to mean that our extraction and purification procedures removed the bulk of potentially immunoreactive interfering substances.

The peaks drawn for tritium quantitation and for immunoassayable substance are somewhat more broad than those representing the HPLC chromatograms. This is probably due to mixing in the tubing and the fraction collector of material after its passage through the UV detector.

Recovery of added compounds

Table I shows the recovery of added 6ketoPGF$_{1\alpha}$ and TXB_2 to urine samples by HPLC. The values in the table represent the absolute mass of each prostanoid recovered at each concentration of prostanoid added. Three urine aliquots were measured at each concentration and the results averaged. It can be seen that the recovery is linear and averages 82% for 6ketoPGF$_{1\alpha}$ and 69% for TXB_2. These values are very similar to the recoveries obtained when tritiated standards were extracted from urine (78±3% and 64±3%, respectively). No HPLC peaks corresponding to either compound were seen when normal urine was extracted and assayed with the a.u.f.s. setting at 0.02. Given the recoveries (80 and 70%) and the minimal detectable level (25 ng) measurable by HPLC, we conclude that normal urine contains less than 4 ng/ml of each.

TABLE I

RECOVERY OF AUTHENTIC STANDARDS ADDED TO URINE BY HPLC

	Added (μg/10 ml)				
	0	25	50	100	200
	Recovery (μg/10 ml)				
6ketoPGF$_{1\alpha}$	0	20.3	39.5	85	not done
TXB_2	0	not done	38.5	65	130

Normal values

Thirteen normal volunteers (9 males, 4 females) aged 19–47 years who had received a 20 ml/kg oral water load excreted 551±63 ml (S.E.M.) of urine in a 4-h period. They excreted 0.11±0.03 (S.E.M.) ng/ml TXB_2 and 0.27±0.06 ng/ml 6ketoPGF$_{1\alpha}$. There was no correlation between the excretion rate of either compound and urine volume: for volume vs. 6ketoPGF$_{1\alpha}$ excretion rate, $r=-0.032$, for volume vs. TXB_2 excretion rate $r=0.379$.

DISCUSSION

The determination of prostanoids in biological specimens by radioimmunoassay suffers from uncertainty due to undetermined effects of potentially cross-reacting substances. While antisera used for radioimmunoassays (including our own; [7,8]) are commonly tested for cross-reactivity with other prostanoids and metabolites, the concentration of these compounds in biologic samples is usually unknown. Thus, if a compound is in 1000-fold excess, a cross-reactivity of 0.1% becomes very significant. Extraction and purification techniques are used to reduce the concentration of interfering substances but they should be validated. By subjecting our extracts to HPLC we showed a number of extra peaks which could represent potentially interfering substances. However, these peaks did not contain cross-reacting material when tested by radioimmunoassay. Furthermore, when urine was enriched with a large amount of tritiated 6ketoPGF$_{1\alpha}$ or TXB_2 and subjected to extraction, purification and HPLC, tritium appeared only in the fractions corresponding to authentic com-

pounds. While these results do not constitute absolute proof of the molecular specificity of our extraction and radioimmunoassay procedures it seems very unlikely that potentially interfering substances would have identical retention times on both the Sephadex LH-20 column and the HPLC column plus be immunoreactive. We are therefore, lead to the conclusion that our assay procedures are specific for urine 6ketoPGF$_{1\alpha}$ and TXB$_2$.

The HPLC procedure described in this paper can be used to measure reproducably as little as 25 ng of either 6ketoPGF$_{1\alpha}$ or TXB$_2$. This equals the reported sensitivity of other reported HPLC methods [9,10]. Such sensitivity results from performing the assay at a UV wavelength of 194 nm, which is near the maximal absorbance for most prostanoids [9]. Previously-described mobile phases cannot be used satisfactorily at such low UV wavelengths [10]. Secondly, we have found that these compounds resolve optimally when the pH of the mobile phase is maintained at 3.5.

Desiderio et al. [11] have reported the use of a volatile triethylamine—formic acid buffer system for wavelengths below 200 nm. Others have used a solvent system comprising of phosphoric acid and acetonitrile [7]. However, we have found that substantial baseline noise occurs with the triethylamine solvent system while the combination of phosphoric acid and acetonitrile was found to buffer inadequately at pH 3.5. Our solvent system comprised of 2 m*M* potassium dihydrogen phosphate—acetonitrile (70:30) offers excellent UV transparency at 194 nm, gives good baseline stability and buffers well at pH 3.5. However, even with the improvement in sensitivity, normal levels of 6ketoPGF$_{1\alpha}$ and TXB$_2$ in urine are not easily measured by HPLC assay but can be conveniently detected by radioimmunoassay.

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Note

High-performance liquid chromatographic method for determining plasma and urine 3-methoxy-4-hydroxyphenylglycol by amperometric detection

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The levels of urinary, plasma and/or cerebral spinal fluid (CSF) 3-methoxy-4-hydroxyphenylglycol (MHPG) have been used as a biochemical index of noradrenergic function in man and have been extensively studied in their relationship to the major psychiatric disorders. Based on measurements of MHPG there have been attempts to biochemically subtype the depressive disorder [1], differentiate other mental disorders [2] and to select appropriate drug therapy or associate drug response [3]. The analytical methods for determining MHPG content have principally been based on the use of gas chromatography (GC) either with electron-capture detection (ECD) [4] or mass spectrometry (MS) [5]. Recently new procedures have been introduced which use high-performance liquid chromatography (HPLC) with electrochemical detection [6, 7]. These methods in general require extensive pre-purification [i.e., multiple extractions, derivatization, thin-layer chromatography (TLC), etc.] before chromatography which often results in low sample recovery and time-consuming procedures. Furthermore these procedures are often not sensitive enough to detect the low levels of MHPG found in blood. None of the HPLC methods have been used for both plasma and urinary MHPG determinations.

This article presents a HPLC method coupled with electrochemical detection (ED) for determining both free plasma and total urinary MHPG. The procedure is simple, rapid, and highly sensitive. Chromatographic separation involves pre-purification of either hydrolyzed urine or deproteinized plasma by passage through a short mixed-bed anion—cation exchange column. This is followed by extraction of MHPG from the eluate into an organic phase which is then evaporated. Residues are resuspended in the HPLC buffer and run on an

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isocratic HPLC system using a reversed-phase column. The eluted MHPG is then detected electrochemically.

MATERIALS AND METHODS

Chemicals

3-Methoxy-4-hydroxyphenylglycol, piperazine salt, 99% was obtained from Aldrich (Milwaukee, WI, U.S.A.). β-Glucuronidase sulfatase from Sigma (St. Louis, MO, U.S.A.), a crude preparation from *Helix pomatia* containing 100,000 units per ml of β-glucuronidase and 10,000 units sulfatase activity and was used as supplied. All other reagents used were analytical grade, unless otherwise indicated. All water used was double-distilled in glass.

Urine collection

Urine was collected from human subjects over a period of 24 h. To the collection, 0.5 mg/ml sodium metabisulfite was added after total volume was recorded. A 50-ml aliquot was stored in a −20°C freezer prior to analysis. Urine collections were checked for 24-h completeness by determination of creatinine.

Plasma collection

Whole blood was collected from human subjects into evacuated tubes containing heparin. The samples were spun (900 *g* for 15 min) to separate the plasma which was drawn off and stored in a −20°C freezer prior to analysis.

Preparation of anion—cation column

The cation-exchange resin (Bio-Rad AG-50W-X4, 200—400 mesh), in the H^+ form, was converted to the Na^+ form by washing with 2 vol. of 1 *M* sodium hydroxide and rinsing with 4 vol. of double-distilled water. The resin was further washed with 2 vol. of 0.01 *M* sodium phosphate buffer, pH 6.0, and stored at 4°C in this buffer until use. The anion-exchange (Bio-Rad resin AG-1-X4, 200—400 mesh, Cl^-) was conditioned by adding 2 vol. of the same buffer and storing at 4°C. Chromaflex column tubes (Kontes, Martin, IL, U.S.A.), 6 cm × 0.5 cm I.D. were fitted with column tube tips that had a small amount of silanized glass wool placed in the tip to hold the packing. Onto the silanized glass wool was placed 1.0-cm height (volume, 0.19 cm^3) of anion resin. This was followed by the same amount of cation resin. The columns were then washed with 4 vol. of 0.01 *M* sodium phosphate buffer, pH 6.0.

Freeing of urine MHPG conjugates

Duplicate urine samples (2.0 ml) were mixed with 0.1 ml β-glucuronidase sulfatase and 1.0 μl 2% (w/v) ethylenediamine tetraacetate (EDTA) in 1.0 *M* sodium acetate in 15-ml glass centrifuge tubes. Stoppered tubes were incubated 24 h at 37°C with agitation. The crude β-glucuronidase sulfatase preparation gave no peaks on the chromatogram when saline reagent blanks were run through the entire assay.

Deproteinization of plasma

Duplicate plasma samples (1.0 ml) were deprotienized by adding 0.5 ml of formic acid, diluted to 2% (w/v) concentration in water—acetone (1:3), followed by 0.25 ml 10% (w/v) sodium tungstate. After mixing, the samples sat at room temperature for 15 min, then were centrifuged 10 min at 1000 *g*. The supernatants were transferred to 15-ml glass-stoppered centrifuge tubes, the pellets were then resuspended in the formic acid solution and re-centrifuged. The pooled supernatants were extracted in the centrifuge tubes with heptane—chloroform (8:1), using 30 sec vigorous mixing followed by 5 min centrifugation at 300 *g* to separate phases. The organic phase, containing lipophilic material, was discarded. The aqueous phase, much reduced in volume, was adjusted to pH 6.0 by addition of 2 *M* disodium phosphate.

Preparation of standard curves

Standard curves were prepared by replicate additions of MHPG to samples from a single pool of plasma or urine following which the samples containing added MHPG were treated identically with other samples.

For urine, the standard curve consisted of duplicate 2.0-ml samples from a single pool which received 0, 0.2, 1.0, 1.5 or 2.5 mg MHPG per sample. For plasma, the standard curve comprised duplicate 1.0-ml samples from a single pool which received 1, 5, 10, 25 ng MHPG.

Pre-column purification of urine and plasma

Urine and plasma samples were partially purified by eluting 0.5 ml of the hydrolyzed urine or the total aqueous phase of the deproteinized plasma extract, through the anion—cation column with 1.2 ml of 50% methanol. Eluents were collected and reduced to about 0.6 ml volume in a Buchler vortex-evaporator (about 10 min). Each sample was extracted with ethyl acetate (2.5 ml), then samples spun at 300 *g* for 2 min to separate phases. The organic phase was transferred to 5.0-ml conical glass centrifuge tubes, and the ethyl acetate extraction of the remaining aqueous phase was repeated. The pooled ethyl acetate extracts were then evaporated to dryness (under vacuum). Urine samples were resuspended in 1.0 ml and plasma samples in 50 μl of the HPLC buffer.

HPLC

The HPLC apparatus consisted of an Altex pump Model 110-A, a Rheodyne Model 7000 loop injector with 20-μl loop, and a 3-μm Chromantics Spherisorb ODS-2 (100 × 4.6 mm) column (J & S Scientific, Crystal Lake, IL, U.S.A.). Samples were eluted isocratically at 1.5 ml/min using a mobile phase buffered at pH 4.0 with 0.01 *M* sodium acetate—acetic acid, and containing 1 m*M* EDTA.

Detection of MHPG was by an amperometric system (Bioanalytical Systems, West Lafayette, IN, U.S.A.) using a glassy carbon working electrode, a silver—silver chloride reference electrode, and a Model LC4A detector-potentiometer.

The working electrode was maintained at a potential of 0.9 V vs. the silver—silver chloride reference electrode, and the amperometric detector was operated using a 0.1-sec response time constant. Detector response was displayed on a chart recorder (Omniscribe, Houston Instruments).

RESULTS AND DISCUSSION

Resolution

Typical chromatograms of the separation of MHPG in urine and plasma are shown in Figs. 1 and 2, respectively. The left profile of each figure represents endogenous amounts and the right, a duplicate of the same sample containing added MHPG. The MHPG peak is free of obvious interferences.

Precision

Within-assay variation, evaluated by independent replicate determinations of the apparent MHPG content of single samples, was small. For urine, the coefficient of variation (C.V.) for six replicates of a single sample carried through the entire assay (including the hydrolysis step) was only 0.09, while for plasma, eight replicate determinations of free MHPG showed a C.V. of 0.10. This high precision was judged to render the use of recovery standards unnecessary.

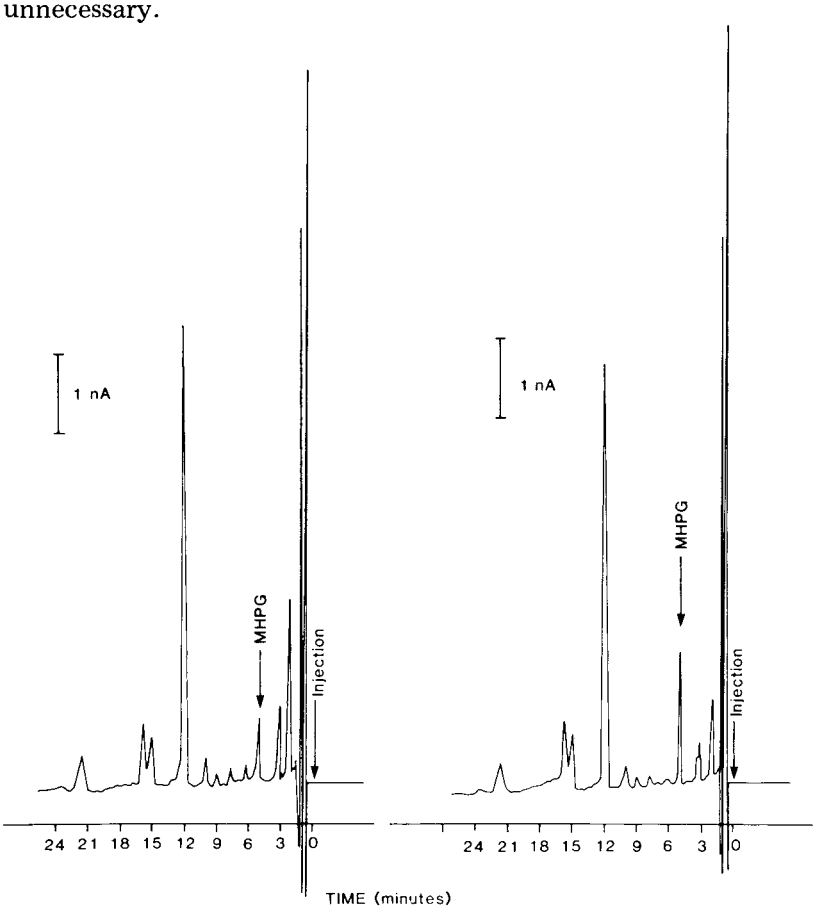

Fig. 1. Shown are typical chromatographic profiles for total urinary MHPG using a 3-μm, C18 reversed-phase column under isocratic conditions and amperometric detection. The left profile is endogenous MHPG and the right an addition of MHPG to the same split sample. From an original 2.0-ml urine extract 20 μl were injected. Sensitivity of the detection system was set at 1 nA with a working electrode potential of 0.90 V.

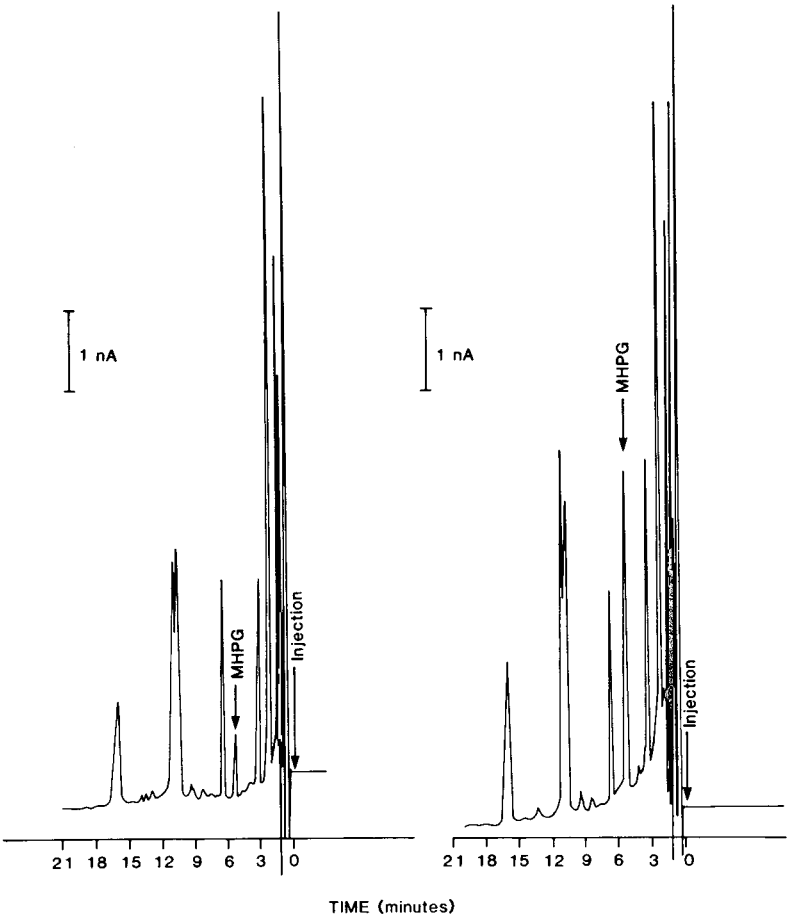

Fig. 2. A typical chromatographic profile for free plasma MHPG using the conditions as in Fig. 1. The left profile is endogenous MHPG and the right an addition of MHPG to the same split sample. From an original 1.0-ml plasma extract 20 μl were injected.

Recovery and accuracy

Recovery, estimated by addition of known amounts of MHPG to samples, was 50% for plasma and 70% for urine. The two steps which gave significant losses were at the anion—cation column step and during the ethyl acetate extraction. Using a greater volume to elute off the column and repeating the ethyl acetate extraction more than twice on the eluents would improve recoveries by 20% or more. Plasma recoveries are lower than urine due to the initial extraction step. However, with the detection limit of the assay at 0.1 ng MHPG per injection, it was felt the extra time involved to improve recoveries was unnecessary. A linear detector response from the lower limit of sensitivity (0.1 ng MHPG per injection) up to 40 mg per injection was verified.

The values for total urinary MHPG in ten subjects and free MHPG in plasma from seven subjects, as determined by this HPLC method, were 1.26 ± 0.58 (S.D.) mg/24 h and 3.34 ± 1.45 ng/ml, respectively. These means compare closely with other procedures using similar subjects populations (Table I).

TABLE I

COMPARISON OF FREE PLASMA AND TOTAL URINARY MHPG AS DETERMINED BY VARIOUS METHODS

All results are taken from the literature and are expressed as the means ± standard deviations with the number of subjects (n) given in each case. All measurements include normal male and female total urinary MHPG determinations.

Method	Reference	Total urinary MHPG (mg/24 h)	n	Free plasma MHPG (ng/ml)	n
HPLC—ED	This method	1.26 ± 0.58	10	3.34 ± 1.45	7
HPLC—ED	[6], [7]	2.65 ± 0.20	12	4.62 ± 1.11	3
GC—ECD	[8], [9]	1.92 ± 0.71	17	5.4 ± 1.5	15
GC—MS	[5], [10]	2.62 ± 0.34	7	4.6 ± 1.0	10

Advantages of this method

The main advantage of the HPLC method described is the ability to assay both urine and plasma samples using the same system. The 3-μm column gives good separation, with MHPG peaks starting from and returning to baseline. Equipment used in this procedure is less expensive than GC equipment and samples do not require derivatization. Other HPLC methods have been used only for urinary MHPG [6, 11] with the exception of one method that requires a more difficult pre-column purification procedure for plasma MHPG [7].

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Note

Measurement of 3-methoxy-4-hydroxyphenylglycol sulfate ester in brain using reversed-phase liquid chromatography and electrochemical detection

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3-Methoxy-4-hydroxyphenylglycol (MHPG) is the major metabolite of norepinephrine (NE) in the mammalian central nervous system. This compound exists either in its free or conjugated forms (mainly as sulfate, MHPG-S) [1]. Its excretion in urine is supposed to reflect the activity of the central noradrenergic system in humans [2]. Changes in concentration of total MHPG or of MHPG-S are the best indicators of variations of NE turnover in the central nervous system. Several methods have been described to quantify the different forms of MHPG in tissue extracts and body fluids. MHPG-S can be hydrolysed to free MHPG either enzymatically or by acid hydrolysis. Free MHPG has been measured by gas chromatography with either flame-ionisation [3], electron-capture [4] or mass spectrometric [5] detection. These techniques require extraction with organic solvents, appropriate derivatization and need sophisticated equipment. A radioimmunological assay of MHPG of extremely high sensitivity has been described recently [6]; this assay has found limited utilization until now. The oldest method based on formation of a fluorophore of MHPG with ethylene diamine [7] lacked sensitivity in our hands. In recent years, methods based on liquid chromatographic separation combined with fluorimetric [8] and electrochemical [9] detection of MHPG have been developed.

We wish to describe here a method of measuring MHPG-S in brain extracts. This method is a new combination of known steps: brain extraction with H_2SO_4 [10], separation of MHPG-S from other constituents of brain extracts [7], hydrolysis to free MHPG by a purified preparation of *Helix pomatia* sulfatase [11], and quantification by high-performance liquid chromatography

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(HPLC) coupled with electrochemical detection [12]. This method is reliable, needs no extraction steps, and no sophisticated equipment in its manual version; 30—40 samples can be analysed by a single person in two working days.

MATERIAL AND METHODS

Reagents

The following chemicals were purchased: MHPG piperazine salt and yohimbine from Sigma, St Louis, MO, U.S.A.; MHPG-S as K^+ salt from Fluka, Buchs Switzerland; lyophilized "Helicase" (sulfatase + glucuronidase from *Helix pomatia*) from IBF, Clichy, France. Monofluoromethyl *p*-tyrosine methyl ester was synthesized in our laboratory by a method to be published elsewhere. All other reagents were of the highest analytical grade available from Merck, Darmstadt, F.R.G.

Liquid chromatography system

The liquid chromatography system contained the following components: a single piston pump (Kontron LC 410) fitted with a pulse dampener (Kontron Model 811) and set to deliver 1 ml/min, and a manual injector (Rheodyne 7125). The column was a LiChrosorb RP-18 (250 × 4.6 mm, 10 μm particle size) from Merck, fitted with a guard column (70 × 2 mm) filled with Co-Pell ODS (30 μm) from Whatman (Clifton, NJ, U.S.A.). The detector was a Model LC-4 electrochemical detector from Bioanalytical Systems (West Lafayette, IN, U.S.A.) with a glassy carbon electrode, the potential of which was set at 0.85 V versus the Ag/AgCl reference electrode. The signal was monitored on a dual-channel recorder (Omniscribe, Houston Instruments, Austin, TX, U.S.A.). The mobile phase was a mixture (89:11, v/v) of 0.15 *M* sodium dihydrogen phosphate and methanol containing 1.3×10^{-4} *M* EDTA; the pH was adjusted to 3.5 with H_3PO_4. Under these conditions, MHPG had a retention time of 6.8 min.

Purification of Helix pomatia sulfatase

It was necessary to purify the extract of *Helix pomatia* as there were a number of parasite peaks on the MHPG chromatogram with the crude extract. This was done by ion-exchange chromatography on DEAE-cellulose with a linear gradient of sodium chloride (0.03 *M* Tris—HCl pH 7.9; sodium chloride from 0 to 0.2 *M*) [11] (Fig. 1). Enzyme activity is determined with nitrocatechol sulfate as substrate. After dialysis of a pool of the most active fractions (hatched area in Fig. 1), the enzyme can be stored frozen at −20°C.

Hydrolysis of a standard solution of MHPG-S and stability of MHPG under hydrolysis conditions

To a solution of MHPG-S (250 ng of K^+ salt) in 3 ml of 0.25 *M* hydrochloric acid, an equal volume of 1 *M* sodium acetate is added so that the final pH is 5.5. Then 300 μl of purified sulfatase are added and the mixture is warmed to 37°C in a shaking water bath. A solution of MHPG piperazine is treated in the same way. Aliquots are withdrawn at given intervals. The enzyme is denatured

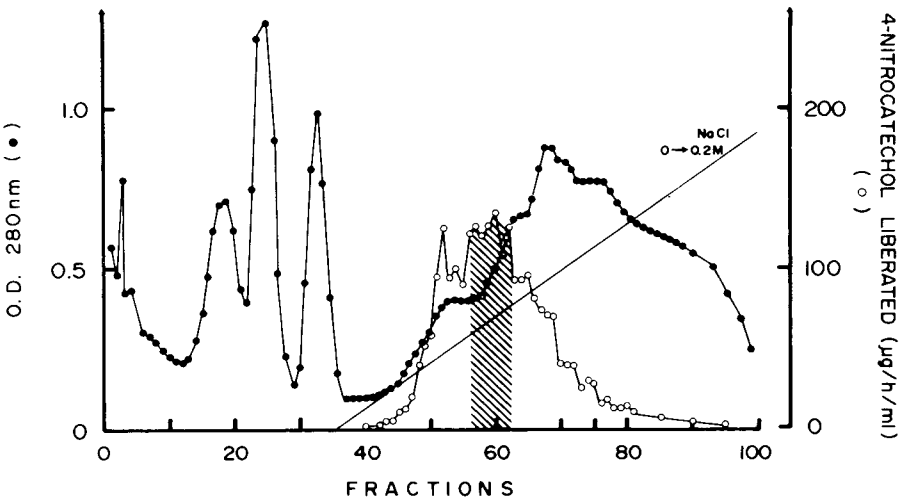

Fig. 1. Purification of the sulfatase from *Helix pomatia*. A lyophilized preparation of digestive juice of *Helix pomatia* (500 mg solubilized in 10 ml of 0.03 *M* Tris—HCl pH 7.9) was applied to a DEAE column equilibrated with the same buffer. The enzyme was eluted by a sodium chloride gradient as described in Materials and methods. The fractions corresponding to the hatched portion of the diagram were collected.

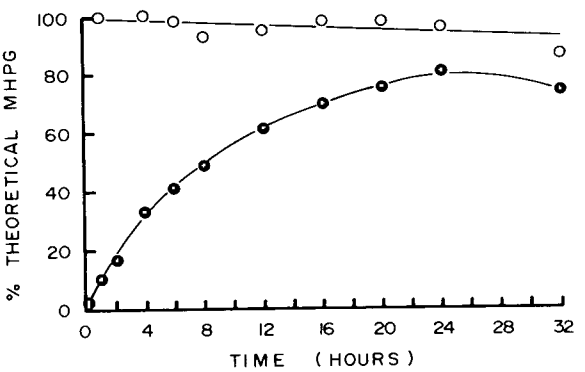

Fig. 2. Rate of hydrolysis of a standard solution of MHPG-S and stability of MHPG under hydrolysis conditions. MHPG-S and MHPG in 0.2 *M* sodium acetate pH 5.5 were allowed to react with a purified preparation of sulfatase (Fig. 1). Aliquots were analysed by HPLC combined with electrochemical detection at given time intervals.

by adding 0.1 volume of 1 *M* perchloric acid containing 1.5% $Na_2S_2O_5$. The amount of protein is so low that no centrifugation is needed before injection on the HPLC column. Fig. 2 shows the rate of MHPG appearance over 32 h. The hydrolysis levels off at 24 h and corresponds to 80% of the theoretical amount. Under the experimental conditions, the recovery of MHPG is superior to 90% after 36 h of incubation.

Tissue extraction and separation of MHPG-S

The procedure of Kohno et al. [10] was followed: homogenization in 0.1 *M* sulfuric acid, neutralization of the supernatant with 0.1 *M* barium

hydroxide up to pH 6.5. After centrifugation of the barium sulfate, MHPG-S was recoveered by ion-exchange chromatography on DEAE-Sephadex A_{25} as described by Meek and Neff [7].

Enzymatic hydrolysis of MHPG-S and HPLC of MHPG

To 3 ml of 0.25 *M* hydrochloric acid containing MHPG-S, 3 ml of 1 *M* sodium acetate are added to bring the pH to 5.5. This solution is incubated with 300 μl of purified sulfatase at 37° C for 24 h. After deproteinization of a 0.9 ml aliquot with 100 μl of 1 *M* perchloric acid containing 1.5% $Na_2S_2O_5$, the samples can be stored in the cold overnight; 25—50 μl are used for analysis in the HPLC system. Fig. 3 shows a typical chromatogram obtained with a standard solution of MHPG and a brain extract. There are no other peaks on the chromatogram so that the analysis time can be limited to 10—15 min. The amount of MHPG formed is calculated by comparing its peak heights to that of the known standard.

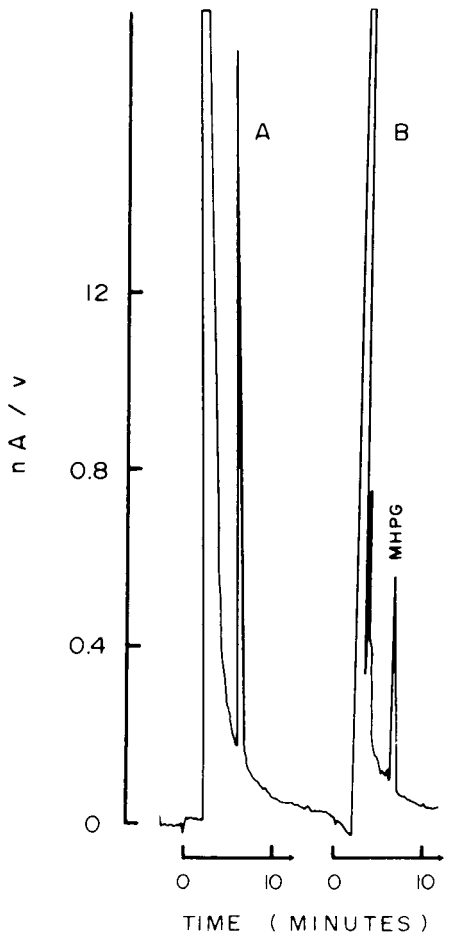

Fig. 3. Chromatogram of a standard solution of MHPG and of a brain extract after hydrolysis. (A) Chromatogram of a standard solution of MHPG. The peak corresponds to an injection of 1 ng. (B) Chromatogram of a brain extract processed as described in Materials and methods. The injection volume was 20 μl; the peak corresponds to 0.28 ng of MHPG.

Calculation of total recovery and reproducibility

A pool of five rat brains was homogenized in 40 ml of 0.1 *M* sulfuric acid. This pool was distributed into 4-ml aliquots, to which different amounts of commercial MHPG-S were added. The samples were processed as described above. There is a linear relationship between the added MHPG-S and the recovered MHPG. The regression line was calculated with a least-squares program on a Hewlett-Packard 9820A calculator: $y = 0.368x + 193$ ($r = 0.995$, $p \leqslant 0.01$).

To test day-to-day reproducibility the recovery was calculated after addition of 992 pmol of MHPG-S to brain extracts on consecutive days. The mean recovery from seven experiments was 369 ± 19 pmol, i.e. 37 ± 2%. No attempt was made to identify the steps responsible for the major loss.

RESULTS

The method of measuring MHPG-S has been applied to quantify brain concentrations of this NE metabolite in control animals, under conditions of increased NE turnover, and under conditions of inhibition of NE synthesis (Table I). Control values calculated by this method are 188 ± 6 ng/g (mean ± S.E.M. $n = 5$). Values in the literature for whole rat brain range from 40 to 60 ng/g ([1], unspecified strain) to 149 ng/g ([10], male Wistar). Our values for Sprague-Dawley are some 20% superior to this last figure. Yohimbine produces an increase of NE outflow and synthesis presumably by blocking presynaptic α_2-receptors. As expected and as reported previously [13], MHPG-S levels are almost doubled in animals treated with 2.5 mg/kg yohimbine. We reported recently that monofluoromethyl *p*-tyrosine methyl ester produces a selective inhibition of catecholamine synthesis in rat brain [14]. We believe that the compound is hydroxylated by the action of tyrosine hydroxylase to monofluoromethyl-DOPA, a potent irreversible inhibitor of aromatic amino acid

TABLE I

BRAIN CONCENTRATION OF MHPG-S IN CONTROL ANIMALS AND UNDER CONDITIONS OF STIMULATION AND INHIBITION OF NE SYNTHESIS

Groups of rats (male Sprague–Dawley, 150 g) were treated as follows. One group received saline intraperitoneally and served as control. A second group was injected intraperitoneally with 2.5 mg/kg yohimbine. A third group was given by gavage 100 mg/kg monofluoromethyl *p*-tyrosine methyl ester (in 1% ascorbic acid). The last group received both yohimbine and the tyrosine analogue simultaneously. The animals were killed 4 h after the different treatments; the brains were split sagitally. One half was processed as described for MHPG-S determinations, the other half was saved for other purposes. Each value represents the mean ± S.E.M. of 5 animals.

	Brain MHPG-S (ng/g)
Control	188 ± 6
Yohimbine	360 ± 20*
Monofluoromethyl *p*-tyrosine	148 ± 10*
Yohimbine + monofluoromethyl *p*-tyrosine	205 ± 3

*$p < 0.01$ (Student's *t*-test compared to control).

decarboxylase (AADC) [15]. Therefore the inhibitory effect, both on AADC and on amine synthesis, should depend on activation of the tyrosine hydroxylase [14]. As reported in Table I, the compound has by itself only a small, albeit significant, effect on brain MHPG-S concentration. However, when given together with yohimbine, monofluoromethyltyrosine blocks almost completely the accumulation of MHPG-S due to α_2 blockade.

DISCUSSION

The method of MHPG-S determination described here combines a series of steps described previously. The extraction with sulfuric acid was found to be more reproducible than that with zinc sulfate. Precipitation by barium hydroxide was best performed on a supernatant rather than on the homogenate as the pH can be adjusted more easily. The isolation of MHPG-S by DEAE-Sephadex had been used by Meek and Neff [7] in their original method. The extract of a 1-g brain sample can be applied in totality to 0.75 ml of resin, so that no correction of volume is needed up to that stage. There is no loss in the washing of the resin with the 0.06 *M* hydrochloric acid and release is quantitative in the subsequent step. In our hands, acid hydrolysis of the eluted MHPG-S by hydrochloric acid or perchloric acid gave low yields and produced an erratic peak interfering with MHPG. For reasons already explained we found it absolutely necessary to purify the enzyme for enzymatic hydrolysis. As can be seen in Fig. 3, the chromatogram shows no other peaks than MHPG. The chromatographic conditions were modified from those used by Wagner et al. [12] to measure catecholamines and their metabolites in cerebrospinal fluid. Addition of $Na_2S_2O_5$ results in a stable baseline which increases the sensitivity of the detection. With adaptation of extraction, elution and hydrolysis volumes, the method should be applicable to measure regional distribution of MHPG-S. Although relatively low, the recovery of MHPG-S is reproducible from sample to sample and from day to day. This method has allowed us to confirm that the α_2-antagonist yohimbine produced an accumulation of MHPG-S in rat brain and that the inhibitory effect of monofluoromethyl *p*-tyrosine on NE synthesis is enhanced under conditions which increase NE turnover.

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Note

Simultaneous determination by high-performance liquid chromatography of tocopherol isomers, α-tocopheryl quinone, and cholesterol in red blood cells and plasma

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In recent years numerous authors [1—9] have reported methods for the analysis of α-tocopherol in both plasma and red blood cells (RBC). Several of these methods have involved high-performance liquid chromatography [1—5], but other techniques such as ultraviolet (UV) spectrophotometry [6, 7] and gas—liquid chromatography [8, 9] have also been employed. Among these methods the reported plasma levels are relatively consistent, but those of the red cells vary significantly, yielding values for the RBC to plasma ratio which range from 0.13 [5] to 0.30 [9]. This discrepancy appears to arise primarily from the oxidation of α-tocopherol during its extraction from red cells when insufficient precautions have been taken to prevent this reaction. This problem does not occur in plasma during normal extraction procedures. To prevent oxidation, pyrogallol or ascorbic acid is usually added either to the red cell suspension or to the alcohol which is added to facilitate the extraction. The amounts added vary widely among the published procedures.

One purpose of this study was to develop a method which would allow us to detect any significant oxidative loss during the extraction of α-tocopherol from red cells. The improved sensitivity afforded by the measurement of α-tocopheryl quinone at 265 nm enables the detection of as little as 0.5% formation of the quinone during red cell extraction. This capability has enabled us to detect conversion of α-tocopherol to the quinone which might occur during previously reported extraction procedures. We report here a method which reduces this oxidation to an undetectable amount, achieving greater accuracy and reproducibility. This method is suitable for rapid analysis of large

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numbers of samples and also for the determination of α-tocopherol in samples of limited size, such as RBC from mice.

EXPERIMENTAL

Materials

α-Tocopherol, γ-tocopherol and α-tocopheryl acetate were the generous gift of Hoffmann-La Roche (Nutley, NJ, U.S.A.). α-Tocopheryl quinone was from ICN Chemicals (Cleveland, OH, U.S.A.). Cholesterol was from Fisher Scientific (Philadelphia, PA, U.S.A.).

Red cells and plasma were obtained from subjects receiving medical care at Mount Sinai Medical Center with informed consent and institutional approval of the research protocol. Blood samples from Swiss-Webster mice were obtained from animals maintained on a standard laboratory diet.

High-performance liquid chromatography

The chromatograph was a Hewlett-Packard Model 1084B, equipped with a programmable, variable-wavelength detector with stopped-flow scan capability, and with an autosampler and integrator. The column was a 25 cm × 4.6 mm I.D. ODS reversible (5-μm particle size) from Regis Chemical (Morton Grove, IL, U.S.A.). A 5 cm × 4.6 mm I.D. guard column packed with Pelliguard LC-18 from Supelco (Bellefonte, PA, U.S.A.) was attached before the analytical column. Elution was performed with methanol—water (96:4) at a flow-rate of 1.05 ml/min, at 30°C. The eluent was monitored at 265 nm for α-tocopheryl quinone, at 292 nm for β- + γ-tocopherol, α-tocopherol, and α-tocopheryl acetate, and at 215 nm for cholesterol.

Sample preparation

Whole blood samples were drawn in heparinized, evacuated tubes (Becton-Dickinson, Rutherford, NJ, U.S.A.).

Red blood cells. The separated red cells were washed with 0.9% saline and brought to a measured hematocrit with 0.9% saline—0.5% pyrogallol. Two milliliters of a methanol solution containing internal standard (α-tocopheryl acetate) and 1.0% pyrogallol were added to 1 ml of the RBC suspension and this mixture was extracted with pesticide grade petroleum ether. The petroleum ether layer was evaporated to dryness with nitrogen; the residue was dissolved in methanol—ethanol (80:20) and an aliquot injected onto the HPLC.

Plasma. This procedure is derived from that of De Leenheer et al. [10]. A methanol solution containing internal standard (α-tocopheryl acetate) was added to an equal volume of plasma and the mixture extracted with petroleum ether. The petroleum ether layer was then treated as described above.

Quantification

Cholesterol, α-tocopherol, and the sum of β- + γ-tocopherol were quantified from a standard curve of their peak height ratios versus α-tocopheryl acetate. A linear relationship was found between the peak height ratios (standard/internal standard) and the concentration ratios (standard/internal standard) for each of the compounds. No detectable α-tocopherol was formed from the internal standard during the extraction.

RESULTS

A typical chromatogram of a red cell extract with no added internal standard or standards is shown in Fig. 1. The same sample with added α-tocopheryl quinone, γ-tocopherol, α-tocopherol, α-tocopheryl acetate, and cholesterol is shown in Fig. 2. Baseline separation is achieved for all peaks of interest.

Ten compounds of interest in the tocopherol system which are well separated under the described analytical conditions are listed in Table I. The characteristics of the method are listed in Table II. The mean values, standard deviations, and ranges for the red cell cholesterol, β- + γ-tocopherol and α-tocopherol concentrations in sixteen normal subjects are shown in Table III. With the exception of the concentraton of α-tocopherol in red cells and its ratio to α-tocopherol in plasma, these levels are in good agreement with those reported in the literature [1—5]. These apparent anomalies are discussed below. The red cell α-tocopherol is plotted against the plasma α-tocopherol for these subjects in Fig. 3. The regression equation for these values is: [α-tocopherol (RBC)] = (1.46 ± 0.31)* μg/ml + (0.11 ± 0.03) [α-tocopherol (plasma)] μg/ml, with significance at the 0.002 level.

The ability of this method to determine tocopherols in small samples was studied by the analysis of red cells and plasma from mice. The concentration of α-tocopherol in 12-week-old Swiss-Webster mice was found to be 1.72 ± 0.28

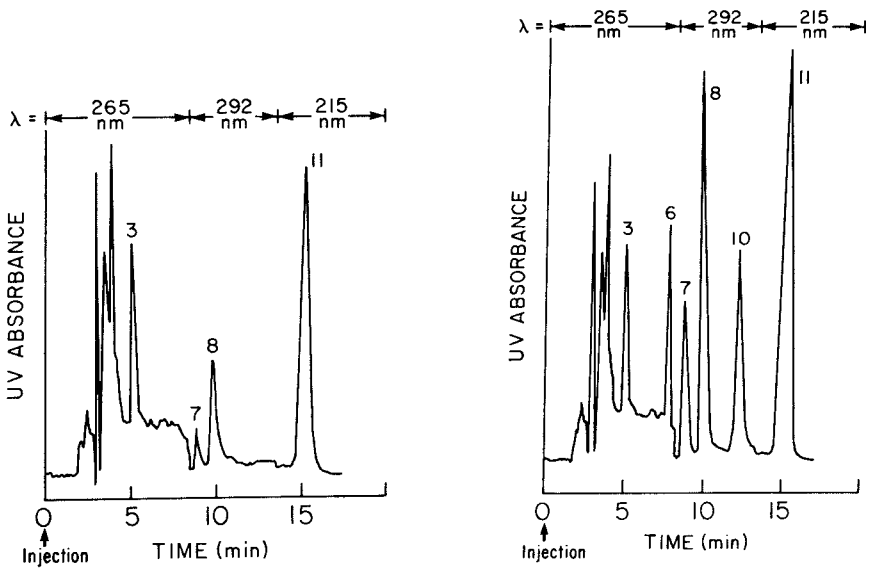

Fig. 1. Chromatogram of a red cell extract with no added standards or internal standard. Peaks: 7 = β- + γ-tocopherol; 8 = α-t opherol; and 11 = cholesterol. The attenuation is changed from 2^4 to 2^9 at 14 min.

Fig. 2. Chromatogram of the same concentration of red cells as in Fig. 1 but with 0.25 μg of α-tocopheryl quinone (peak 6), 0.9 μg of γ-tocopherol (peak 7), 3.1 μg of α-tocopherol (peak 8), 20.0 μg of α-tocopheryl acetate (peak 10), and 280 μg of cholesterol (peak 11) added. The attenuation is changed from 2^4 to 2^5 at 11.7 min, and then to 2^9 at 14 min.

*Mean ± S.D.

TABLE I

SIGNIFICANT CHROMATOGRAPHIC PEAKS

Peak No.	Retention time (min)	Relative retention time	UV absorbance maximum (nm)	Identification
1	3.1	0.24	256 (SH)	Quinone decomposition product
2	4.9	0.39	292	α-Tocopherol hydroquinone
3	5.3	0.42	—	Unknown
4	5.7	0.45	250 (SH)	Quinone decomposition product
5	6.7	0.53	260	γ-Tocopherol oxidation product
6	7.7	0.61	265	α-Tocopheryl quinone
7	9.2	0.72	295	β- + γ-Tocopherol
8	10.2	0.81	292	α-Tocopherol
9	11.5	0.91	301	α-Tocopherol oxidation product
10	12.6	1.00	285	α-Tocopheryl acetate
11	15.9	1.25	<210	Cholesterol

TABLE II

CHARACTERISTICS OF THE HPLC PROCEDURE FOR TOCOPHEROL ISOMERS AND CHOLESTEROL IN RBC AND PLASMA

	β- + γ-Tocopherol	α-Tocopherol	Cholesterol
Coefficient of variation (8 replicate plasma determinations)	4.4%	2.9%	4.4%
Coefficient of variation (8 replicate RBC determinations)	6.4%	4.1%	2.6%
Correlation coefficient (typical plasma standard curve)	0.999	0.998	0.990
Correlation coefficient (typical RBC standard curve)	0.9995	0.9998	0.9995
Rate of recovery from red cells	N.D.*	92.3% ± 0.4%**	95.0% ± 3.4%***
Limit of detection (μg/ml packed RBC)§	0.1	0.1	N.D.*

*Not determined.
**Average of 2 samples.
***Average of 3 samples.
§Limit for α-tocopheryl quinone is 0.02 μg/ml.

μg/ml in plasma (n = 12) and 0.84 ± 0.13 μg/ml in RBC (n = 7). No β- + γ-tocopherol was detected in either the plasma or red cells. The detection limits were 0.02 μg/ml and 0.05 μg/ml, respectively.

Addition of pyrogallol to the red cell suspension but not to the added alcohol has been used by others [2] to prevent the oxidation of α-tocopherol during extraction. Between 2% and 8% of the α-tocopherol is converted to the quinone under these conditions. The quinone is not the only product formed, corresponding to only 15—25% of the total tocopherol lost. Thus, if the α-tocopheryl quinone is measured at a less than optimal wavelength such as 280 nm [2], an undetectable amount of quinone can correspond to a 10% loss of the

TABLE III

CONCENTRATIONS OF TOCOPHEROL ISOMERS AND CHOLESTEROL FOR 16 NORMAL SUBJECTS

	Mean	Standard deviation	Range
RBC β- + γ-tocopherol (μg/ml)	0.52	0.27	0.15 —1.02
RBC α-tocopherol (μg/ml)	2.61	0.41	1.89 —3.42
RBC cholesterol (mg/ml)	1.21	0.13	1.03 —1.51
Plasma β- + γ-tocopherol (μg/ml)	2.0	1.2	0.40 —3.84
Plasma α-tocopherol (μg/ml)	10.2	2.6	4.93 —14.40
α-Tocopherol RBC/plasma ratio	0.267	0.054	0.181—0.381
β- + γ-Tocopherol RBC/plasma ratio	0.299	0.143	0.180—0.717

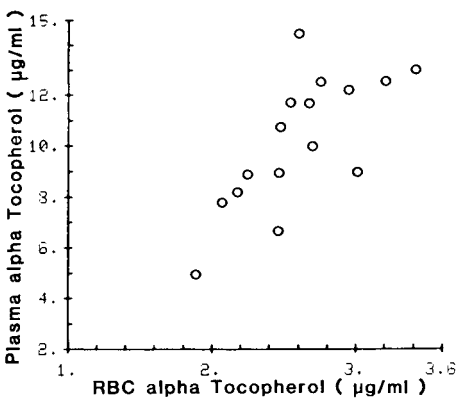

Fig. 3. α-Tocopherol concentrations (μg/ml) in the red cells and plasma of sixteen normal subjects. The R value of the regression fit is +0.71.

tocopherol originally present. Addition of pyrogallol to the alcohol added during extraction allows less than 0.2% (none detected) conversion of the original tocopherol to the quinone. γ-Tocopherol and α-tocopheryl acetate exhibited no detectable oxidation when pyrogallol was added to the red cell suspension and not to the methanol.

DISCUSSION

The prevention of oxidative loss of α-tocopherol during red cell extraction is dependent not only on the anti-oxidant added, but also on the order of the addition of the alcohol and the antioxidant. The oxidative reaction proceeds rapidly upon addition of alcohol to the cell suspension (data not shown). The ten-fold greater sensitivity for α-tocopheryl quinone at 265 nm versus 280 nm allows the detection of minute losses of α-tocopherol. Monitoring the chromatographic effluent at 265 nm also allows sensitive detection of the in vivo quinone which has been postulated to exist [11]. The present method yields values for the α-tocopherol RBC/plasma ratio which are significantly greater than many of the previously described procedures [2, 4—6]. Insufficient

protection against oxidative loss during RBC extraction could lead to 10—50% loss of the membrane α-tocopherol, accounting for these differences.

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Note

Liquid chromatographic separation and quantitation of B_6 vitamers in selected rat tissues

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Vitamin B_6 is the collective term for the metabolically related 3-hydroxy,2-methyl pyridine compounds. Vitamin B_6 exists in three interconvertible forms: pyridoxine (PN, also known as pyridoxol), pyridoxal (PL), and pyridoxamine (PM), each of which has a corresponding 5′-phosphate. The physicochemical properties of the B_6 vitamers, in particular their ionogenic nature, facilitate their assay by high-performance liquid chromatography (HPLC). Vanderslice and co-workers [1—6] have reported separations and quantitations of the non-phosphorylated and phosphorylated B_6 vitamers in human plasma, animal tissues, urine, and selected foods. Their method utilized two anion-exchange columns and a spectrofluorometer which resulted in 1—2 h separations. Gregory and Kirk [7] have reported separation and quantitation of the non-phosphorylated B_6 vitamers in dehydrated food systems by reversed-phase HPLC using an octadecylsilane (ODS) column and an absorbance detector; the vitamers were eluted within 6 min. Gregory [8] used a similar HPLC system to determine PL, PN, and PM as well as pyridoxal 5′-phosphate (PLP) in non-fat dry milk and a fortified breakfast cereal by converting PLP to its semicarbazone derivative; a detection limit of less than 0.5 ng/g was reported. Gregory [9] has also compared the HPLC assay with the conventional microbiological method in analyzing the B_6 vitamer content of fortified breakfast cereal, and found the HPLC method to demonstrate higher accuracy and precision, to facilitate the analysis of larger numbers of samples, and to be a simple and sensitive method. Gregory et al. [10] have also reported quantitation of PL, PM, PLP, and PMP in a variety of animal tissues utilizing derivatization with semicarbazide, a reversed-phase ODS column, and fluorescence detection. The vitamers eluted within 12 min. Lim and co-workers [11, 12] have

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also developed an HPLC method with ultraviolet (UV) detection utilizing an ODS column which separated and quantitated the non-phosphorylated B_6 vitamers in about 11 min, and they applied the method to selected foods. Tryfiates and Sattsangi [13] have reported a reversed-phase ion-pair method for separating the non-phosphorylated and phosphorylated B_6 vitamer standards; the method employed UV detection and required about 40 min for separation of the vitamers.

This communication reports on the use of a reversed-phase ion-pair chromatographic method with fluorometric detection for the analysis of B_6 vitamers in rat liver, kidney, and brain tissues.

EXPERIMENTAL

Analytical instrumentation

A Waters Assoc. HPLC system (Milford, MA, U.S.A.) consisting of the following components was employed in this research: Model 730 data module, Model 720 system controller, two Model 45 solvent delivery systems, Model U6K universal injector, and Model 420-E/AC fluorescence detector equipped with a mercury lamp, 300-nm excitation filter and 375-nm emission filter. The analytical column was a μBondapak ODS column (30 cm × 3.9 mm I.D., 10-μm porous packing, C_{18}) preceded by a guard column (2 cm × 3.9 mm I.D.) packed with Bondapak ODS/Corasil (37—50 μm); both were obtained from Waters Assoc.

Reagents

Water and methanol, HPLC grade, were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Pyridoxal hydrochloride, pyridoxal 5′-phosphate, pyridoxamine dihydrochloride, pyridoxamine 5′-phosphate and pyridoxine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Pyridoxine 5′-phosphate and 4-deoxypyridoxine hydrochloride were purchased from ICN Nutritional Biochemicals (Cleveland, OH, U.S.A.). The buffered ion-pairing reagent was purchased as PIC B-7 from Waters Assoc.

Mobile phase

A binary mobile phase was employed consisting of a mixture of methanol—water (850:150, v/v; solvent A) and PIC B-7 reagent (0.005 *M* heptane sulfonic acid in 1% acetic acid; solvent B). Prior to use, the mobile phase solvents were degassed by vacuum filtration through a 0.3-μm glass fiber filter (Gelman, Ann Arbor, MI, U.S.A.). The mobile phase was delivered at a rate of 1.5 ml/min at ambient temperature.

Separation and quantitation of standards

All handling of B_6 vitamers occurred under red lighting. Deoxypyridoxine (DPN) was selected as the internal standard. Following chromatography of the individual vitamers PL, PN, and PM as well as DPN, 250 μl of an aqueous combined standard solution (containing 625 ng of PL, 500 ng of PN, 250 ng of PM, and 1250 ng of DPN per ml) were injected onto the column. Satisfactory separation of the vitamers was accomplished in about 20 min (Fig.

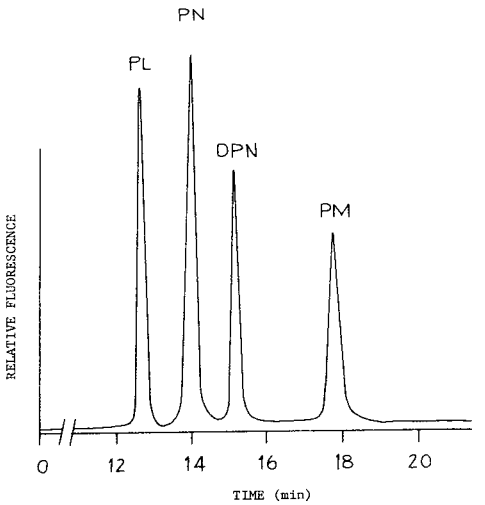

Fig. 1. Separation of B_6 standards by HPLC.

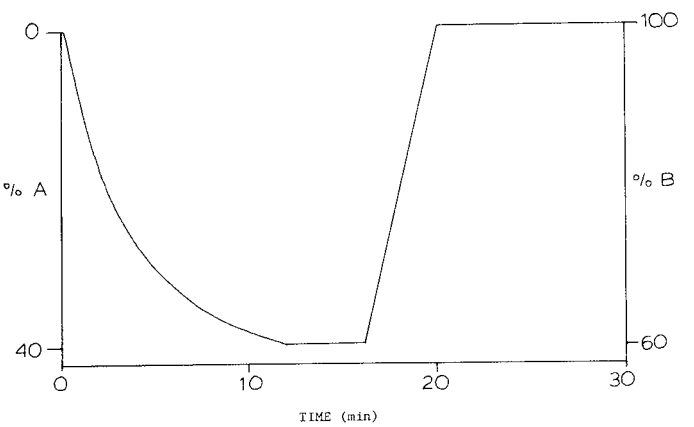

Fig. 2. Illustration of the gradient elution and column reequilibration program.

1) employing a gradient elution program (Fig. 2). Peak identity was confirmed by standard addition (spiking) as well as by extrachromatographic spectrofluorometry on collected HPLC eluates. Detection limits of 5 ng (200 ng/g tissue) for PN and PM and 10 ng (400 ng/g tissue) for PL were found with this method. The coefficient of variation of multiple injections of the same extract was found to be better than 5%. Peak areas, calculated by digital integration, were utilized for vitamer quantitation.

Extraction

B_6 extraction procedures were a modification of techniques reported by Gregory [14], Vanderslice et al. [3], and Thiele and Brin [15]. Samples and reagents were kept at 4° C in an ice—water—sodium chloride mixture until deproteinization was completed, in an effort to regard enzymatic activities. Approximately 1 g of liver, kidney, or brain (frozen, —20° C) was homogenized

with an aqueous solution of DPN (2.5 ml/g tissue) followed by immediate deproteinization with 8% perchloric acid (PCA; 2.5 ml/g tissue) and centrifugation at 12,000 *g* for 10 min at 4° C. An aliquot of the supernatant was adjusted to alkaline pH with 6 *M* potassium hydroxide to precipitate the PCA as insoluble potassium perchlorate; the pH was then adjusted to approximately 5.2 using 1 *M* hydrochloric acid. An equal volume of methylene chloride was added to remove non-polar impurities followed by vigorous shaking and centrifugation at 12,000 *g* for 10 min at 4° C. A 2-ml volume of the supernatant fluid was removed and combined with 2 ml of 0.055 *M* hydrochloric acid; the resulting solution was autoclaved for 5 h at 120° C and 1.04 bar to dephosphorylate the 5′-phosphates. After the solution had cooled to room temperature, the pH was adjusted to 5.2 with 6 *M* potassium hydroxide and dilute acetic acid and put through a 0.2-μm Gelman filter prior to injection into the HPLC system.

Animal treatment

Weanling Sprague-Dawley (Harlan-Sprague-Dawley, Madison, WI, U.S.A.) male albino rats were fed diets containing 15% protein and 7.2 mg of vitamin B_6 per kg of diet in the form of pyridoxine hydrochloride; these levels are considered to be nutritionally adequate [16]. After being on the experiment for almost four months, the animals were sacrificed by electrocution and the livers, kidneys, and brains excised immediately and frozen on dry ice. These organs were kept at —20° C until analyzed.

RESULTS AND DISCUSSION

A typical chromatogram of brain tissue extract is depicted in Fig. 3. Peaks obtained from brain extracts were adequate for quantitation. Satisfactory separation and quantitation of extracts from liver and kidney were also obtained. Peak identity was confirmed by comparison of standard retention times with sample retention times, use of relative retention time, spiking, and extrachromatographic spectrofluorometry of eluates.

Tissue homogenates (three of each tissue) were spiked with known quantities of PL, PN, and PM as well as their phosphorylated forms PLP, PNP, and PMP. B_6 vitamer recoveries were similar for all tissues, and were as follows: PL, 83%;

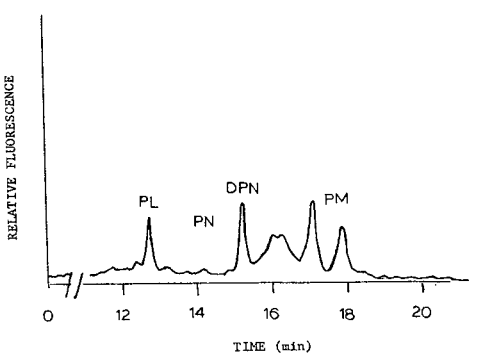

Fig. 3. Separation of B_6 vitamers in a representative brain extract by HPLC.

PN, 89%; PM 93%; PLP, 82%; PNP, 96%; PMP, 96%. The phosphorylated forms were recovered as non-phosphorylated vitamers; losses due to the dephosphorylation step were minimal. The loss of B_6 vitamers from inital homogenization of the tissues to HPLC separation and quantitation was considered acceptable and calculations were based on the recovery of the internal standard which ranged from 85 to 90%.

Vanderslice et al. [3] reported that the thawing, time of deproteinization, and buffer composition utilized with tissues during extraction affected the vitamer recoveries; these researchers observed some interconversions among the vitamers which may have been due to the activities of tissue oxidases. Enzyme-mediated interconversions during the extraction procedure used in the present study should have been minimal due to the low temperatures at which the samples and reagents were maintained throughout the procedure.

The B_6 vitamer concentrations of the three tissues are given in Table I. PM appeared to be the predominant B_6 vitamer in all three tissues with PL being the second most predominant. Vitamer data were in the range of values obtained by microbiological [10, 15, 17] and chromatographic [3, 10, 12, 18] techniques for these same tissues.

TABLE I

B_6 VITAMER CONCENTRATIONS OF RAT TISSUES

Values are given in μg/g and mean ± S.D. (S.D. refers to the range of values among individual rats and not precision). Phosphorylated forms of the vitamers are converted to their respective non-phosphorylated forms during extraction; values represent the sum of free and phosphorylated forms.

Tissue	No. of animals	PL	PN	PM	Total
Liver	10	4.25 ± 0.50	0.59 ± 0.21	6.91 ± 0.91	11.75 ± 1.02
Kidney	10	2.90 ± 1.34	0.62 ± 0.43	7.77 ± 1.74	11.29 ± 1.72
Brain	6	1.97 ± 0.01	nd*	2.45 ± 0.30	4.42 ± 0.30

*nd = not detectable.

The HPLC separation was rapid and the sensitivity adequate for determining physiological levels of the vitamers. Fluorometry provided not only high sensitivity but also selectivity, producing relatively clean chromatograms; spectrofluorometry was also useful in confirming sample vitamer peaks. The HPLC method offers potential for the quantitation of B_6 vitamers in a wide variety of matrices.

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Note

Assay of γ-vinyl-γ-aminobutyric acid (4-amino-hex-5-enoic acid) in plasma and urine by automatic amino acid analysis

Application to human pharmacokinetics

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γ-Vinyl-γ-aminobutyric acid (γ-vinyl GABA, 4-amino-hex-5-enoic acid, GVG; MDL 71.754) is an enzyme-activated inhibitor of GABA-transaminase [1], the enzyme responsible for the degradation of the neurotransmitter, GABA. Oral administration of γ-vinyl GABA to man produces the expected increases in central nervous system GABA concentrations [2]. Further, γ-vinyl GABA treatment has been shown to be of benefit in patients with neuroleptic-induced tardive dyskinesia [3] and in patients with resistant forms of epilepsy [4, 5].

To carry out metabolic, pharmacological and distributional studies, an assay for γ-vinyl GABA in biological specimens was required. Extraction procedures, followed by subsequent concentration of the organic phase, were not suitable due to the hydrophilic character of γ-vinyl GAGA. Conventional amino acid analysis using colorimetry was found to be too time-consuming and insensitive for most samples. A method has therefore been developed where the chromatographic separation has been made with a single buffer so that only part of a normal "physiological" assay is utilized. By using fluorimetric detection of the *o*-phthaladehyde derivative instead of colorimetry, 0.1 nmol of γ-vinyl GABA is 100 μl of injected sample can be easily measured. This method and its application to human pharmacokinetics are herein described.

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METHODS

Analytical

All chemicals were the highest grade commercially available. They were purchased from E. Merck (Darmstadt, F.R.G.) except for the lithium citrate sample dilution buffer which was bought from Pierce (Rotterdam, The Netherlands). γ-Vinyl GABA was synthesized in our centre [1].

The amino acid analyzer was a Liquimat II (Kontron, Paris, France) having two 4-mm I.D. glass columns filled with DC-6 resin (Durrum, Palo Alto, CA, U.S.A.) to a bed height of 30 cm. Automatic valve-switching ensured that the samples were injected on to alternate columns and the effluent of the analysis column was directed to the fluorimeter. A fluoromonitor (American Instrument, Silver Spring, MD, U.S.A.) was used as detector, after changing the light source to a pen-ray lamp (11 LSC - 1 L, Ultra-Violet Products, San Gabriel, CA, U.S.A.). A Corning Glass 7-51 filter was fitted for excitation (340 nm) and a Wratten 2A filter for emission (440 nm). Samples were injected automatically by an APE-100 (Kontron) equipped with a 100-μl loop.

Separation was made with a single buffer of high molarity (lithium citrate, 0.668 *M* lithium, adjusted to pH 4.6 with hydrochloric acid) to elute γ-vinyl GABA rapidly. Buffer flow was 25 ml/h and the column temperature was 45°C. γ-Vinyl GABA eluted after 45 min. Regeneration of the column was carried out after each analysis by pumping lithium hydroxide (0.3 *M*) for 16 min.

Derivative formation with the *o*-phthalaldehyde (OPA) reagent (17 ml/h) was achieved by using a simple "T" to mix the reagent and eluting buffer before passing to the fluorimeter. A reaction coil between the "T" and the fluorimeter was found to have little influence on the fluorescence of the amino acids when using these high buffer flow-rates. The OPA reagent consisted of 200 mg of OPA dissolved in 3 ml of methanol added to 1 l of 0.4 *M* (pH 10.4) potassium borate solution containing 3 ml of Brij and 1 ml of mercaptoethanol. The injection of samples alternately on to two columns in a manner similar to previously reported [6] shortened the analysis time.

Human plasma samples were mixed with 0.5 volume of 20% trichloroacetic acid, allowed to stand for 30 min at 0°C and then centrifuged for 2 min in an eppendorf 3200 centrifuge. The supernatant was then injected directly, or further diluted with lithium citrate sample dilution buffer (0.2 *M*, pH 2.2) as required.

Urine was collected in polyethylene bottles to which 1 ml of 6 *M* hydrochloric acid was added. An aliquot (1 ml) was mixed with 250 μl of trichloroacetic acid (20%), allowed to stand as before to precipitate any proteins, and then centrifuged. The supernatant (200—500 μl) was further diluted to 10 ml with sample dilution buffer, before injection on the column.

Measurements

Sample volumes of 100 μl were injected and areas compared with previously injected reference solutions. Fluorescence was linear over the range 0.2—5 nmol of γ-vinyl GABA injected. When γ-vinyl GABA was added to drug-free plasma and carried through the procedure the recoveries ± S.E.M. for additions

of 0.2, 1 and 3 nmol/ml were 98.7% ± 4.8, 99.% ± 4.8 and 93.8% ± 2.4, respectively. When samples were stored at −20°C for several months no significant changes in the γ-vinyl GABA content were seen.

Subjects

Four healthy male volunteers, age 21—35 years (mean = 26 years), weighing 65—75 kg (mean = 69 kg) consented to be studied. While in the fasting state each subject swallowed four gelatin capsules, each capsule containing 250 mg of γ-vinyl GABA without excipient, with approximately 200 ml of water. To assess intra-individual variation, in two volunteers drug was administered on two separate occasions separated by at least two weeks. At various times up to 24 h after each dose, venous blood (10 ml) was sampled into heparinized tubes and plasma obtained by centrifugation; 24-h urine was collected as described above.

Pharmacokinetic data were evaluated by the strip method [7].

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms from specimens of human plasma carried through the analytical procedure. Similar chromatograms have been obtained with plasma and serum from other species.

Absorption of γ-vinyl GABA following oral administration was rapid, with peak plasma concentrations reached between 0.75 and 2 h, ranging from 172 to 315 nmol/ml (Fig. 2). Mean peak plasma concentration was 192 nmol/ml at 1 h post drug. Thereafter, plasma concentrations declined but were still detectable

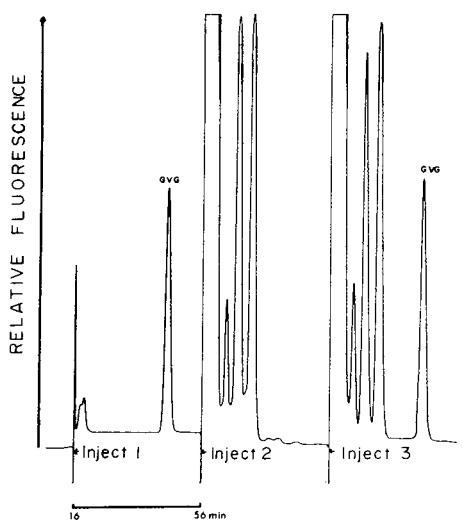

Fig. 1. Chromatograms of samples prepared as described in the text. In practice, the chromatogram is recorded between 16 and 56 min after each injection to avoid recording the early eluting acidic and neutral amino acids. The first injection (1) is of a 6.67% trichloroacetic acid solution containing 1 nmol of γ-vinyl GABA. Injection 2 is a sample of human plasma before administration of γ-vinyl GABA. Injection 3 is obtained from a plasma sample of the same subject 24 h after taking γ-vinyl GABA.

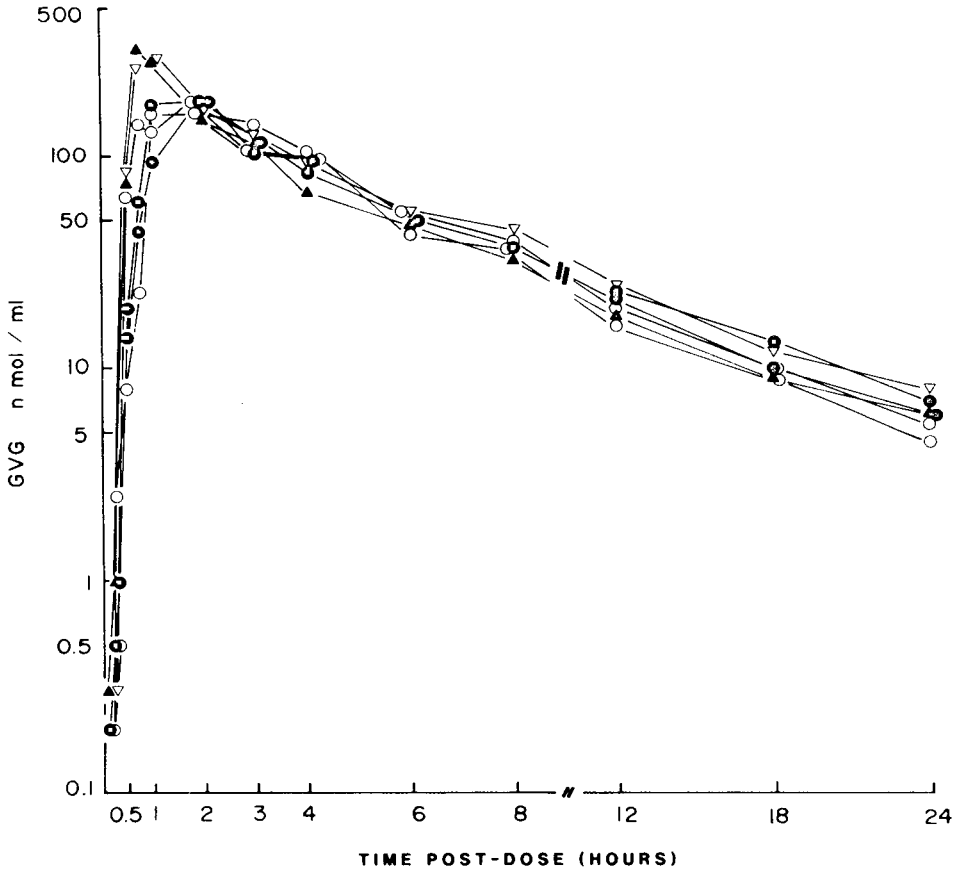

Fig. 2. Plasma concentrations of γ-vinyl GABA (GVG) as a function of time after a 1-g oral dose in four normal volunteers. Each symbol represents a single individual, with two subjects (open and closed circles) being studied twice.

in all cases at 24 h. Both intra- and inter-individual differences were remarkably small. Individual and mean plasma concentration curves could be fitted to an open two-compartment model. Terminal elimination half-lives of individual curves varied from 5.3 to 7.4 h (mean = 6.6 h) with the mean curve having a half-life of 6.8 h. 24-h urinary recovery of γ-vinyl GABA ranged from 746 to 951 mg (i.e. 75–95%; mean = 83%). Thus, γ-vinyl GABA is rapidly and almost completely absorbed following oral administration in man and is mostly eliminated unchanged in the urine within 24 h. No binding to human plasma proteins occurs [8].

The method described allows determination of plasma and urinary drug concentrations following therapeutic doses of γ-vinyl GABA [3–5]. Because of the nature of this drug's action, however, i.e. irreversible enzyme inhibition, it is likely that the kinetics of enzyme turnover will have a greater influence on the time course of the biochemical action than kinetics of the drug itself. Nevertheless, the method should prove useful in the further clinical development of this novel enzyme inhibitor.

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Note

Rapid gas chromatographic determination of pyrimethamine in human plasma and urine

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Pyrimethamine (5-*p*-chlorophenyl-6-ethylpyrimidine-2,4-diamine) possesses cytotoxic activities like many other 2,4-diaminopyrimidines. It passes the blood—brain barrier and inhibits dihydrofolate reductase (EC 1.5.1.4) [1]. It has gained widest use as a malarial suppressant in combination with a sulphonamide (synergistic effect) [2, 3]. It is also used in meningeal leukaemia [4, 5] and as a veterinary additive to rabbit and poultry feeds for the prevention of coccidiosis [6, 7]. The good permeability of pyrimethamine in rat and dog brain tissue [5, 8] has attracted interest in its possible antitumor activity [5, 9—11].

Previous analytical methods were biological [11—13], photometric [9, 14, 15] and fluorimetric [16, 17]. The gas chromatographic method with electron-capture detection described by Cala et al. [18] greatly improved the specificity and sensitivity. The method described was used on chicken tissue and the sensitivity limit was about 100 ng/g. Analysis of pyrimethamine in plasma was first described by DeAngelis et al. [10], who used thin-layer chromatography and an ultraviolet absorption scanning instrument. The methodology was refined by the use of high-performance liquid chromatography [7, 19], and the minimum concentrations measurable [19] were approximately 10 ng/ml. Jones et al. [20] reverted to gas chromatography with the aim of enhancing the sensitivity. They reported the minimum detectable quantity of the pure compound to be 50 pg injected onto the column. However, the analytical procedure involved two extractions from the plasma with the poorly evaporable toluene, programming of the column temperature and a chromatography time of 26 min. Bonini et al. [21] published at the same time gas chromatographic methods to determine some antimalarials including pyrimethamine in biological fluids. The analytes were added and extracted from the cellular whole blood or

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urine and subjected to gas chromatography with a nitrogen—phosphorus detector. No genuine samples were analyzed. Recently, description of a liquid chromatographic method with fluorescence detection to determine pyrimethamine in plasma appeared [22]. The limits of detection were in both cases [21, 22] reported to be about 5 ng calculated per ml of specimen.

This paper describes analytical methods for the rapid and selective determination of pyrimethamine in plasma and urine. The techniques were applied in a pilot study on a female volunteer given a single oral dose of 25 mg.

EXPERIMENTAL

Chemicals and solutions

Pyrimethamine and 2-chlorotriphenylmethanol (internal standard) (Fig. 1) were supplied by the Synthesis Laboratory of Dumex Ltd. All other chemicals were of analytical grade. Methanol was used as solvent for stock and standard solutions of pyrimethamine and 2-chlorotriphenylmethanol.

For the pilot in vivo study a tablet containing 25 mg of pyrimethamine and 500 mg of sulfadoxine was supplied by the Pharmaceutical Research Laboratory of Dumex Ltd.

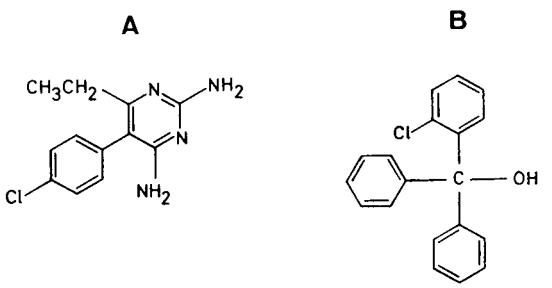

Fig. 1. Chemical structure of (A) pyrimethamine and (B) 2-chlorotriphenylmethanol (internal standard).

Gas chromatographic equipment

A Pye 104 gas chromatograph with an electron-capture detector (^{63}Ni) was used. The glass column was 150 cm long and had an internal diameter of 6.2 mm. The column material was Chromosorb 750 (80—100 mesh) with 3% OV-17.

Analytical procedures

Assay I: plasma concentrations. A 1-ml volume of sample was added to the evaporated residue of 0.6 ng or less of 2-chlorotriphenylmethanol dissolved in methanol. After alkalinization with 150 μl of 0.15 mol/l NaOH the glass tube was rotated at 60 rpm for 5 min. Extraction of the two compounds was performed by adding 6 ml of dichloromethane and rotating the tubes. After centrifugation at about 2000 *g* at ambient temperature and discarding the plasma phase, 5 ml of the remaining organic phase were transferred to a conical glass tube and evaporated under a stream of nitrogen. The residue was redissolved in 100 μl of acetone and 1—2 μl were injected onto the column. The temperature

of the column oven was 235°C, the detector 350°C; the temperature setting of the injection port was position 1. The flow-rate of the carrier gas (argon—methane, 9 : 1) was 60 ml/min.

Assay II: urine concentrations. Urine samples were treated in the same way as plasma, except for the following differences. First, only 100 μl of 0.15 mol/l NaOH were added. Secondly, the organic phase resulting from the extraction was washed with 1 ml of distilled water with a pH of 7.0, after which the internal standard was added. The temperature settings were as given above except that the temperature of the column was 220°C. The carrier gas flow-rate was 50 ml/min.

Quantitation of pyrimethamine. Plasma standard curve samples were prepared by adding drug-free sample to known amounts of pyrimethamine and the internal standard. When urine was analysed only pyrimethamine was added before the extraction (see above). The standard concentrations (ng/ml) ranged from 0 to 400 ng of pyrimethamine whereas the amount of internal standard added was held constant at, for example, 0.6 ng (plasma and 0.3 ng (urine). The peak area ratios of the two analytes in each of the standards were related to the pyrimethamine concentrations and a straight line (linear regression by method of least squares) was obtained. The concentrations of the samples were then computed from this line.

Analytical selectivity. The developed methods were without interferences from commonly prescribed drugs such as acetylsalicylic acid, acetaminophen, acetophenetidin and diazepam.

RESULTS AND DISCUSSION

Analytical procedures

By employing these methods for pyrimethamine quantitation, plasma concentrations were measured in less than 6 min after a simple extraction process. Figs. 2 and 3 demonstrate the ready ability of the methods to separate and identify the two compounds in plasma and urine. The extraction recovery of pyrimethamine from plasma and urine was fully quantitative. The internal standard was extracted from plasma with a yield of about 70% (n = 10, S.E.M. < 5%) and from urine with a yield below 50%. The latter was considered to be unfavorable for an internal standard and therefore this compound was not included in the extraction process of urine.

The concentration limit of quantitation could be lowered to 5 ng/ml when extracting 1 ml of the sample and reducing the volume of redissolution to about 30 μl. The degree of linearity of the plasma standard curve and the results of repetitive analyses of control samples (pools) served as quality characteristics (i.e. accuracy and precision) of the method. From normal plasma standard curves it appeared that the mean coefficient of determination was 0.996, with an interassay coefficient of variation (C.V.) below 1% (n = 10). The mean pool concentration was 95 ng/ml with an interassay C.V. of 7% (n = 10). When an enhancement of the sensitivity was accomplished by reduction of the redissolution volume to 30 μl, an increase in C.V. to about 12% was observed. The quality characteristics of the urine assay differed solely concerning the precision in that the interassay C.V. increased to about 10% (n = 10) at the same

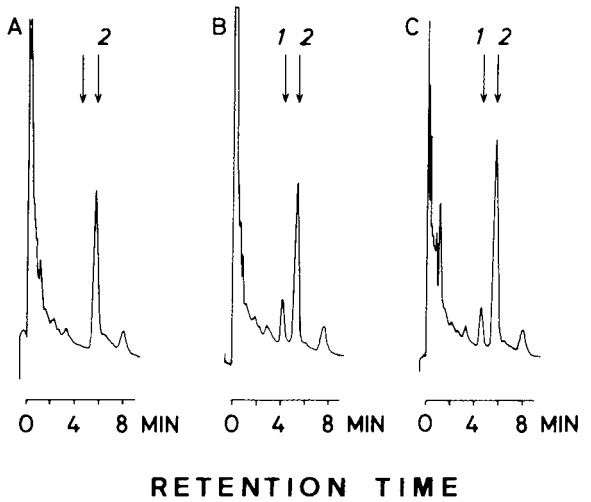

Fig. 2. Chromatograms of (A) blank plasma, (B) plasma pyrimethamine standard of 150 ng/ml, and (C) plasma sample from two days after oral administration of 25 mg of pyrimethamine to an adult volunteer. The peak area ratio was estimated to represent approximately 110 ng/ml. Sample volumes were 0.5 ml and injection volumes 1 μl in all cases. 1 = pyrimethamine, 2 = internal standard.

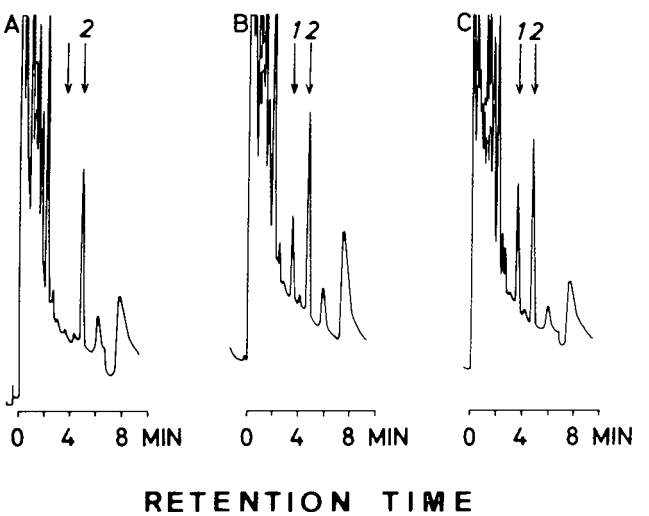

Fig. 3. Chromatograms of (A) blank urine, (B) urine pyrimethamine standard of 150 ng/ml, and (C) urine sample collected 0—3 h after oral administration of 25 mg of pyrimethamine to an adult volunteer. The peak area ratio was estimated to represent approximately 250 ng/ml. Sample volumes were 1 ml and injection volumes 1 μl in all cases. 1 = pyrimethamine, 2 = internal standard.

When developing the methods, different approaches were explored with respect to the extraction and chromatographic procedures. The final extraction procedure resulted from a series of experiments with extraction mixtures of dichloromethane and *n*-hexane at varying pH. Concerning the chromatography there were some difficulties in that extraneous peaks appeared in the chromato-

gram after about twenty injections of plasma samples. However, the retention times of these peaks were 20—25 min, thus far beyond that of pyrimethamine and the internal standard. Raising the temperature of the column oven to about 280°C for 1 h was found to diminish this disturbance.

Application

The methodology was applied in a pilot study in which a female volunteer ingested a tablet of 25 mg of pyrimethamine and 500 mg of sulfadoxine. The analysis of sulfadoxine will be reported later. The plasma concentration of pyrimethamine versus time curve and the corresponding urinary excretion rate curve are shown in Fig. 4.

Assuming that distribution is complete after the third day, the elimination half-life of the drug appears to be about 96 h. This value agrees with other assessments [5, 19], which range from 35 to 175 h. The urinary excretion rate during the first 24 h is estimated as three mean values of about 50 μg/h covering the periods 0—3, 3—8 and 8—24 h. This implies an excretion of about 14% of the dose within 0—24 h and is in agreement with data published on doses of 100 mg of pyrimethamine [23]. To the author's knowledge no data have been published on pyrimethamine urinary excretion after intake of 25 mg of the drug.

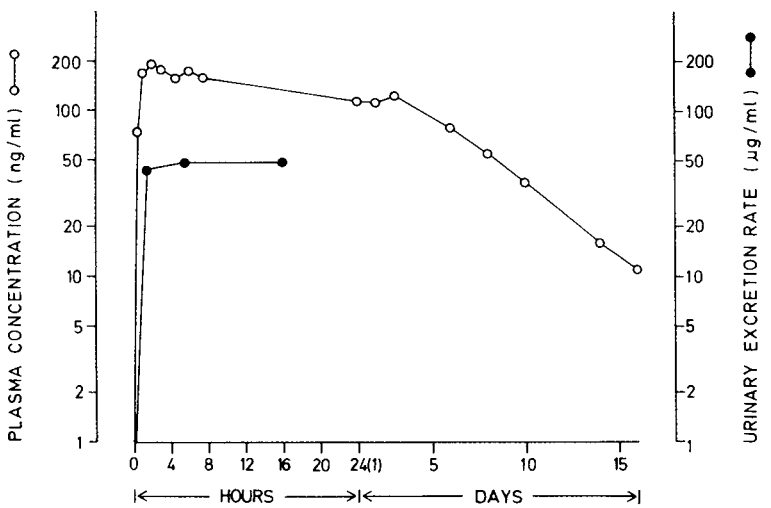

Fig. 4. Plasma concentration and urinary excretion rate versus time curve for a female volunteer who had ingested a 25-mg oral dose of pyrimethamine. The excretion rates are plotted in the midpoints of the collection periods.

CONCLUSION

This paper describes selective and rapid gas chromatographic procedures which permit measurement of pyrimethamine in human plasma and urine for up to at least 16 days after a 25-mg single oral dose of the drug.

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Note

Simple determination of *p*-hydroxyamphetamine by high-performance liquid chromatography with electrochemical detection

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p-Hydroxyamphetamine (pOH-AMPH) is a principal metabolite of amphetamine [1, 2]. This substance has also been suggested to manifest some of the effect of amphetamine [3—5], leading to an interest in the intracerebral distribution of pOH-AMPH. The main procedure for the determination of pOH-AMPH is gas chromatography with electron-capture detection [6]. The substance, however, is not volatile and has to be derivatized prior to injection into the gas chromatographic system. It is generally considered that liquid chromatography is more suitable for the assay of substances that are water-soluble and non-volatile.

The electrochemical reaction has been introduced as a means of detection in liquid chromatography. This reaction is specifically sensitive for a substance having a phenolic hydroxy group in its molecular structure [7]. pOH-AMPH is such a substance and is expected to be detectable with an electrochemical detector. The present report outlines a simple procedure for the determination of pOH-AMPH using high-performance liquid chromatography combined with electrochemical detection.

MATERIALS AND METHODS

Apparatus

The chromatographic system was constructed using commercially available components including a thin-layer voltammetric detector with a glassy carbon electrode (Yanagimoto VMD-101, Kyoto, Japan). The chromatographic

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column consisted of an Ultrasphere-ODS (average particle size 5 μm) prepacked in a 25 cm × 4.6 mm I.D. stainless-steel column (Altex Scientific, Berkeley, CA, U.S.A.). The detector potential was set at +750 mV versus the Ag/AgCl reference electrode.

Reagents

pOH-AMPH was a generous gift from Smith, Kline and French Labs. (Philadelphia, PA, U.S.A.). Isoproterenol, the internal standard for chromatography, was obtained from Sigma (St. Louis, MO, U.S.A.). Reagent grade chemicals, *n*-butanol, *n*-heptane, hydrochloric acid, sodium chloride, ethylenediaminetetraacetate sodium salt (EDTA), methanol and tetrahydrofuran (THF), were all purchased from a single source (Mallinkrodt, Paris, KY, U.S.A.). These chemicals were used as obtained commercially without further purification.

The buffer for chromatography was prepared as follows: 14.7 g of sodium citrate were dissolved in about 400 ml of distilled water. After addition of 50 ml of methanol and 5 ml of THF, the pH was adjusted to 4.5 with citric acid. The volume was finally made up to 500 ml by adding distilled water. The buffer was sonicated under vacuum to eliminate air.

Extraction procedure

Swiss-Webster mice were injected with 10 mg/kg pOH-AMPH. The animals were sacrificed by decapitation 1 h after the injection. The brain was removed as quickly as possible and homogenized in a tube containing 0.2 ml of 0.1 *M* hydrochloric acid, 50 μl of 0.2 *M* EDTA and the internal standard (isoproterenol, 100 ng). The homogenate was mixed with 12 ml of butanol and 4 g of solid sodium chloride and then shaken on a reciprocal shaker for 60 min. Centrifugation was carried out at 1000 *g* for 10 min, and the butanol layer (10 ml) was transferred to another tube. After addition of 0.1 ml of 0.1 *M* hydrochloric acid and 20 ml of heptane to the tube, the mixture was again shaken on the reciprocal shaker for 10 min. The tube was centrifuged at 1000 *g* for 5 min to separate aqueous and organic phases. A portion of the aqueous phase (in general, 50 μl) was injected into the chromatographic column via a six-port injector (Rheodyne, Berkeley, CA, U.S.A.).

RESULTS AND DISCUSSION

In electrochemical detection, the electrode responses vary with the applied voltage [7]. The current—voltage curve for steady-state voltammetry of pOH-AMPH is shown in Fig. 1. pOH-AMPH initiated its electrochemical response at an applied voltage of +600 mV, whereas the parent substance, amphetamine, which has no hydroxy group in its molecule, did not. The applied voltage was higher than that for diphenolic compounds (catechols; for example, noradrenaline and dopamine) and was almost the same as that for the monophenolic amino acid, tyrosine [8]. This means that the response of pOH-AMPH is due to one hydroxy moiety on the benzene ring. The electrochemical response of this substance reached a plateau at about 850 mV, which is also similar to tyrosine. The applied voltage was decided as +750 mV in accordance

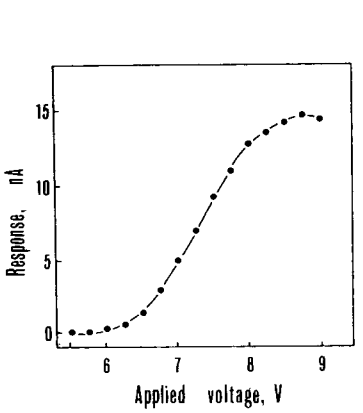

Fig. 1. Current—potential curve for pOH-AMPH.

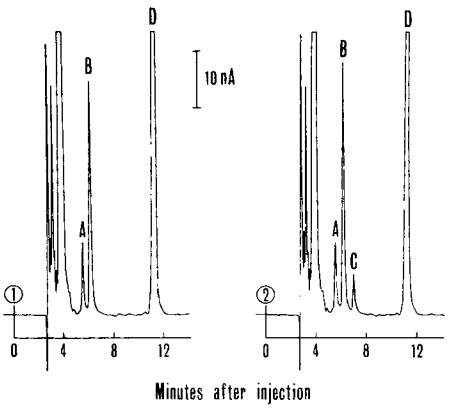

Fig. 2. Typical chromatograms obtained from the brain of animals injected intraperitoneally with 10 mg/kg pOH-AMPH 1 h before sacrifice (2) and with saline (1). Chromatographic conditions: stationary phase, Ultrasphere-ODS (average particle size, 5 μm); mobile phase, 0.1 *M* sodium citrate—citric acid buffer (pH 4.5) containing 1% THF and 10% methanol; detector applied voltage, +750 mV versus Ag/AgCl reference electrode. Peak identifications: A, isoproterenol (internal standard); B, 5-hydroxytryptamine; C, pOH-AMPH; D, tryptophan.

with the following criteria: (1) a voltage which is adequate to obtain a sufficient response and (2) a voltage which does not show significant background noise [9].

pOH-AMPH had a similar retention time to 5-hydroxytryptamine when chromatography was carried out with the buffer utilized for routine assay of monoamines in our department [8]. Thus, the most important aspect of the present study was the selection of a mobile phase which could effectively separate pOH-AMPH from biogenic substances. Some organic solvents are available to separate substances by HPLC with a reversed-phase column. We have employed THF in the assay of monoamine-related substances [8]. However, the organic solvent was not effective in the present separation of pOH-AMPH from 5-hydroxytryptamine. Methanol is also a solvent which can be used for this purpose [8]. While addition of the solvent decreased the retention time, it altered the relative retentions of pOH-AMPH and 5-hydroxytryptamine. Separation and resolution in HPLC were optimized by varying the methanol/buffer ratio of the mobile phase. Addition of 10% methanol was most effective for separating both substances. Fig. 2 shows typical chromatograms obtained for the brain of mice with and without injection of pOH-AMPH, which appeared just after the peak of 5-hydroxytryptamine and was not interfered with by other biogenic substances. Since a combination of butanol extraction and a higher applied voltage meant that the peak of tryptophan was detected [8], one run of the present chromatography required about 12 min to complete. The identification of pOH-AMPH and other biogenic substances was performed on the basis of the chromatographic behaviours and hydrodynamic voltammograms [9].

The recovery and reproducibility were studied by adding known quantities

of pOH-AMPH to blank samples of whole brain and analysing the samples according to the procedure described. The recovery rates by the present butanol extraction from brain samples, after adjusting for solvent loss, were estimated as 85 ± 2% and 75 ± 4% for pOH-AMPH and isoproterenol, the internal standard, respectively. The coefficient of variation was also estimated to be about 4% for both substances. The quantitation was based on peak heights of the resulting chromatogram. Ratios of the peak heights for the pOH-AMPH and the internal standard were compared for samples and standards taken through the entire extraction procedure. By this procedure, ratios of pOH-AMPH and the internal standard varied linearly with the amounts of the drug added in a range between 500 pg and 100 ng. This made it possible to calculate the concentration from simple measurement of the ratio.

The intracerebral concentration of the pOH-AMPH was estimated at 38 ± 9 ng per g wet tissue at 1 h after intraperitoneal injection of 10 mg/kg. This value was lower than that of other central-acting drugs (for example, morphine: 205 ng/g) [10]. This may be because the substance is interfered with in penetrating into the central nervous system at blood—brain barrier. However, even these low concentrations may be sufficient to contribute to the pharmacological effect of the drug [11].

The present study offers a simple and sensitive procedure for measuring the intracerebral concentration of pOH-AMPH. The method involves butanol extraction and quantification by HPLC with electrochemical detection. The sensitivity of the proposed procedure is comparable to that of gas chromatography [6].

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Note

Determination of D-tubocurarine chloride or metocurine iodide in human plasma by high-performance liquid chromatography with ultraviolet detection

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D-Tubocurarine chloride [(+)-7′,12′-dihydroxy-6,6′-dimethoxy-2,2′,2′-trimethyltubocuraranium chloride] is the prototype of the non-depolarizing (competitive) neuromuscular blocking agents (muscle relaxants) [1]. D-Tubocurarine chloride has been widely used since its introduction to the clinical practice of anesthesia in 1942 [2]. Recently the trimethylderivative of D-tubocurarine, metocurine iodide (6,6′,7′,12′-tetramethoxy-2,2,2′,2′-tetramethyltubocuraranium diiodide), has been proposed as an alternative to D-tubocurarine chloride not only because it is twice as potent but also as it causes less autonomic blockade and histamine release [3].

Early techniques for the measurement of plasma concentrations of D-tubocurarine chloride include a micro spectrophotometric method which is sensitive to 1.0 μg/ml [4] and a spectrophotofluorometric method which, though sensitive to 20 ng/ml, is fraught with technical difficulties [5]. A sensitive and specific radioimmunoassay was later developed for the measurement of plasma concentrations of D-tubocurarine chloride [6]; a modification of this technique, using the D-tubocurarine antibody which is not commercially available, is the only method for the measurement of plasma metocurine iodide concentrations [7]. Recently two high-performance liquid chromatographic (HPLC) techniques for the measurement of plasma D-tubocurarine chloride concentrations have been reported; one uses an incompletely resolved internal standard and has a sensitivity of 100 ng/ml [8] while the other has sensitivity to 25 ng/ml but as it uses no internal standard depends on very precise volume measurements [9].

It was the purpose of the present study to develop, for purposes of pharmacokinetic analysis, a simple and sensitive HPLC technique for the determination of plasma concentrations of D-tubocurarine chloride and metocurine iodide. As

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neither is metabolized [10], one could be used as the internal standard for the other. An important feature of this technique is the facile isolation of these quaternary ammonium compounds from an aqueous matrix with inexpensive disposable solid phase extraction columns.

EXPERIMENTAL

Reagents

The D-tubocurarine chloride (Calbiochem, San Diego, CA, U.S.A.) and metocurine iodide (Diosynth, Chicago, IL, U.S.A.) reference standards were used after drying at room temperature in a vacuum desiccator. The dibutylamine was organic reagent grade and the phosphoric acid was reagent grade (Mallinckrodt, Paris, KY, U.S.A.). Acetonitrile, methanol, and tetrahydrofuran were HPLC grade (Waters Assoc., Milford, MA, U.S.A.). All other reagents were analytical reagent grade.

Sample collection and preparation

Blood samples (5 ml) were obtained by syringe through a 16-gauge PTFE-catheter, previously inserted in a radial artery for blood pressure monitoring, and transferred to Vacutainer (Becton-Dickinson, Rutherford, NJ, U.S.A.) collection tubes containing sodium heparin. The plasma samples were removed after centrifugation of the blood for 10 min at 1800 *g*, transferred to polypropylene test tubes, and stored at −30°C until extracted in duplicate.

Sample extraction

Tubocurarine and metocurine were extracted from plasma using Bond-Elut 100 mg C_{18} solid phase extraction columns in conjunction with the Vac-Elut ten-place vacuum manifold (Analytichem International, Harbor City, CA, U.S.A.). The columns were conditioned by sequential flushes (under vacuum) of 2 column vol. of tetrahydrofuran, 2 vol. of methanol, and 2 vol. of water. Then 0.5-ml plasma samples (1.0 ml for the very lowest plasma concentration) were added to the columns along with 50 μl (100 μl with the 1.0 ml plasma samples) of the appropriate internal standard solution, 10 μg metocurine iodide per ml 0.01 mol/l hydrochloric acid for the D-tubocurarine chloride assay and 5 μg D-tubocurarine chloride per ml 0.01 mol/l hydrochloric acid for the metocurine iodide assay, and the vacuum reapplied. The columns were next washed with 2 vol. of water. Subsequent application of 250 μl of the HPLC mobile phase (described below), under vacuum, eluted the D-tubocurarine and metocurine from the columns into 1.5 ml-Eppendorf micro test tubes (Brinkman Instruments, Westbury, NY, U.S.A.) where they were allowed to dry. Prior to injection into the HPLC the samples were reconstituted with 100 μl of the HPLC mobile phase (described below), mixed on a vortex mixer, and centrifuged at 12,800 *g* for 5 min in the Eppendorf Micro Centrifuge Model 5412 (Brinkman Instruments).

Chromatographic apparatus and conditions

The HPLC system consisted of Waters Assoc. Model 6000A solvent delivery system, Model U6K universal liquid chromatograph injector, RCM-100 radial

compression separation system with a Radial-Pak 10-μm CN cartridge and Guard-Pak 10-μm CN precolumn insert, and Model 450 variable-wavelength detector set at 204 nm. D-Tubocurarine and metocurine were eluted isocratically at ambient temperature and at 2.4 ml/min with an acetonitrile—methanol—water—1.0 mol/l dibutylamine phosphate (pH 2.5) (40 : 10 : 10 : 1) mobile phase that had been filtered through a 0.22-μm Durapore Filter (Waters Assoc.). The chromatograms were recorded, the peaks were identified and integrated, and concentrations were reported on the basis of the internal standard area ratio method by the 3390A Reporting Integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Evaluation of the methods

The linearity, accuracy, and precision of the assays were assessed by the measurement of the D-tubocurarine chloride or the metocurine iodide concentra-

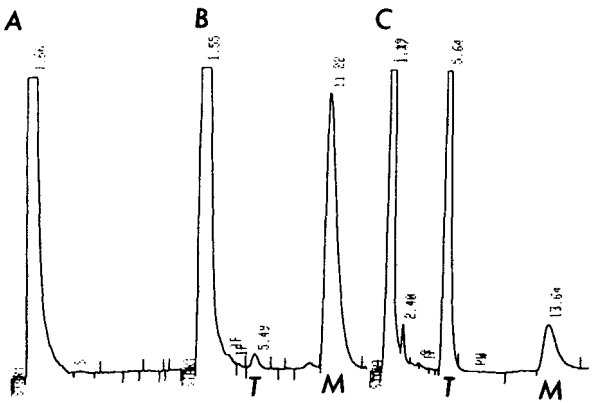

Fig. 1. Chromatograms of D-tubocurarine chloride (T) extracted with the internal standard, metocurine iodide (M), 1000 ng/ml. The ordinate is response and the abscissa is min. The retention times for the peaks are indicated on the chromatograms. A, blank plasma; B, plasma standard with 25.0 ng/ml D-tubocurarine chloride; C, plasma of a patient obtained 5 min after the administration of 0.5 mg/kg D-tubocurarine chloride.

TABLE I

ACCURACY AND PRECISION FOR THE PLASMA D-TUBOCURARINE CHLORIDE ASSAY (n = 8)

D-Tubocurarine chloride added (ng/ml)	D-Tubocurarine chloride measured (ng/ml)*	Mean error (ng/ml)	Relative error (%)	C.V. (%)
25	25.0 ± 2.0	0.0	0.0	7.9
50	50.0 ± 2.4	0.0	0.0	4.8
100	98.0 ± 4.6	2.0	2.0	4.7
250	252.8 ± 9.4	2.8	1.1	3.7
500	488.7 ± 18.7	11.3	2.3	3.8
1000	993.3 ± 41.9	6.7	0.7	4.2
2500	2488.6 ± 86.9	11.4	0.5	3.5
5000	4992.5 ± 171.7	7.5	0.2	3.4

*Mean ± S.D.

tions of replicate plasma standards over a period of several weeks. The plasma standards were made by adding known amounts of D-tubocurarine chloride or metocurine iodide from stock solutions to blank normal human plasma. These plasma standards were in the useful clinical range and contained 25.0, 50.0, 100, 250, 500, 1000, 2500, or 5000 ng/ml of either D-tubocurarine chloride or metocurine iodide. Recovery was evaluated by comparing the peak areas of 25.0, 250, and 2500 ng/ml extracted plasma standards to those of the standard stock solutions.

Clinical study

The usefulness of these methods for clinical pharmacokinetic studies was evaluated, after obtaining institutionally-approved informed consent, in two adult males scheduled to have vascular surgery. Anesthesia was induced in these patients with intravenous thiopental and intravenous succinylcholine was used

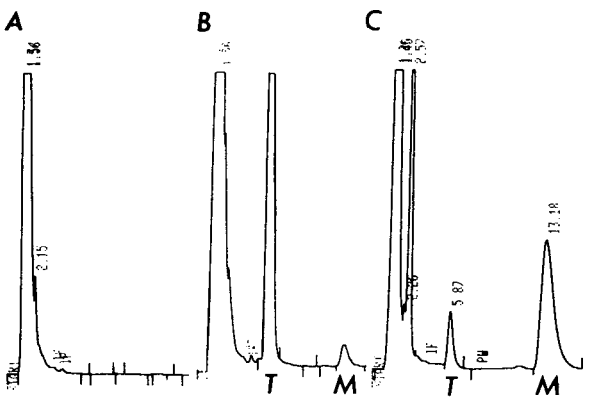

Fig. 2. Chromatograms of metocurine iodide (M) extracted with the internal standard, D-tubocurarine chloride (T), 500 ng/ml. The ordinate is response and the abscissa is min. The retention times for the peaks are indicated on the chromatograms. A, blank plasma; B, plasma standard with 25.0 ng/ml metocurine iodide; C, plasma of a patient obtained 5 min after the administration of 0.3 mg/kg metocurine iodide.

TABLE II

ACCURACY AND PRECISION FOR THE PLASMA METOCURINE IODIDE ASSAY (n = 8)

Metocurine iodide added (ng/ml)	Metocurine iodide measured (ng/ml)*	Mean error (ng/ml)	Relative error (%)	C.V. (%)
25	25.8 ± 1.7	0.8	3.2	6.5
50	48.7 ± 2.3	1.3	2.6	4.8
100	100.7 ± 4.2	0.7	0.7	4.1
250	251.5 ± 8.4	1.5	0.6	3.3
500	493.8 ± 8.5	6.2	1.2	1.7
1000	1004.8 ± 25.9	4.8	0.5	2.6
2500	2495.0 ± 79.5	5.0	0.2	3.2
5000	5000.4 ± 143.2	0.4	0.0	2.9

*Mean ± S.D.

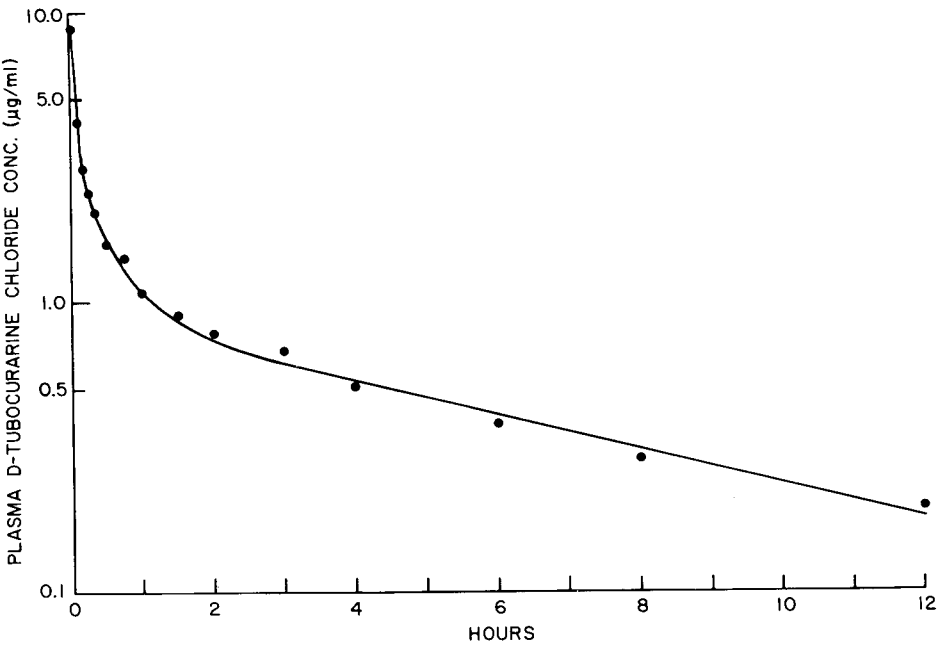

Fig. 3. Plasma D-tubocurarine chloride versus time relationship after the administration of 0.5 mg/kg to a 91-kg 68-year old male for surgical muscle relaxation. The solid line is a computer-derived non-linear least squares regression line through the actual patient points.

to facilitate tracheal intubation. Anesthesia was maintained with 70% nitrous oxide in oxygen and intravenous doses of fentanyl. Muscle relaxation was provided with a rapid (30 sec) intravenous injection of D-tubocurarine chloride, 0.5 mg/kg, or metocurine iodide, 0.3 mg/kg, and was reversed at the end of the anesthetic by intravenous atropine and neostigmine. Blood samples were obtained before and 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 480, and 720 min after the administration of either the D-tubocurarine chloride or metocurine iodide.

RESULTS AND DISCUSSION

High-performance liquid chromatograms of D-tubocurarine chloride and metocurine iodide are shown in Figs. 1 and 2, respectively. None of the drugs administered concomitantly in the clinical study were found to interfere with the assay.

The accuracy and precision of the HPLC techniques for the measurement of D-tubocurarine chloride and metocurine iodide are summarized in Tables I and II, respectively. Linear regression analyses of the standard muscle relaxant concentrations from 25.0—5000 ng/ml versus muscle relaxant : internal standard area ratios verified the linearities of both the D-tubocurarine chloride standard curve ($r = 0.999$; $y = 852x + 4$) and the metocurine iodide standard curve ($r = 0.999$; $y = 571x + 1$). The average recoveries for eight replicate samples at 25.0, 250, and 2500 ng/ml were 79.4, 82.4, and 79.0%, respectively, for

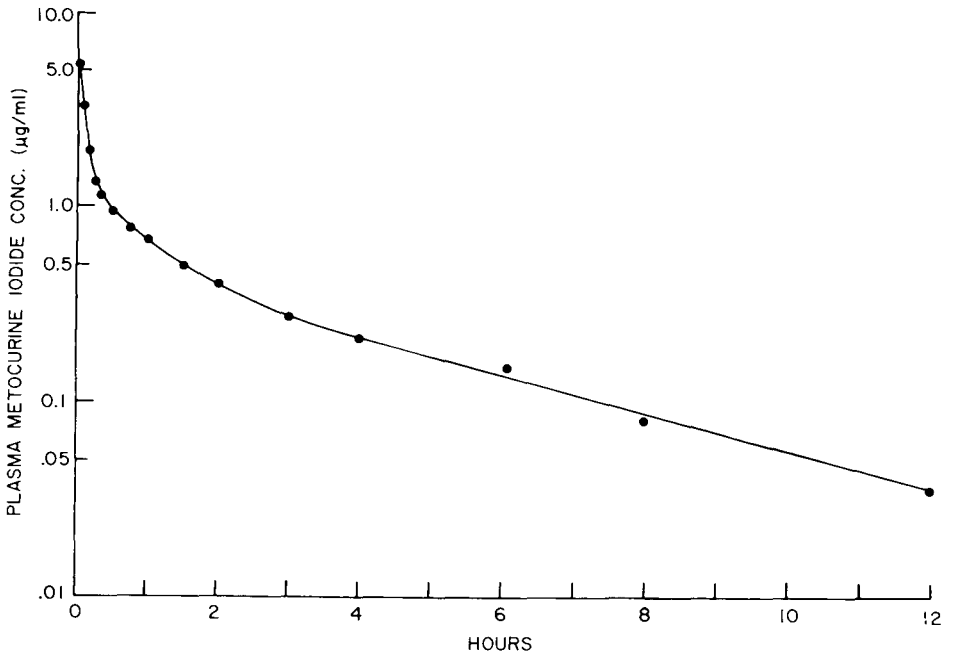

Fig. 4. Plasma metocurine iodide versus time relationship after the administration of 0.3 mg/kg to a 90-kg 50-year old male for surgical muscle relaxation. The solid line is a computer-derived non-linear least squares regression line through the actual patient points.

D-tubocurarine chloride and 78.8, 76.8, and 75.2%, respectively, for metocurine iodide.

Plasma D-tubocurarine chloride concentration and plasma metocurine iodide concentration versus time relationships in the two patients are illustrated in Figs. 3 and 4, respectively. The present HPLC techniques for the measurement of these two drugs are, therefore, able to accurately and easily measure the plasma concentrations of these two drugs for at least 12 h after the administration of the normal clinical doses.

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Note

The direct enantiomeric determination of (—)- and (+)-propranolol in human serum by high-performance liquid chromatography on a chiral stationary phase

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Propranolol [1-isopropylamino-3-(1-naphthoxy)-2-propanol, Ia, Fig. 1] is a widely used nonselective beta-adrenergic blocking agent which is marketed and administered as a racemic mixture (Inderal®, Ayerst). Initial studies of propranolol demonstrated that the *l*-isomer is about 100 times as potent as the *d*-isomer [1] and has a longer plasma half-life [2]. These findings sparked a great deal of interest in the pharmacokinetics of the propranolol enantiomers

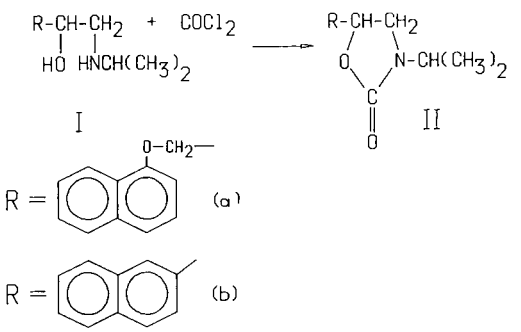

Fig. 1. Synthesis of oxazolidone.

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and resulted in a number of studies that measured the concentrations of the enantiomers in biological fluids [3—6].

Each of these studies faced the analytical question of how to resolve and quantify the propranolol enantiomers. In each, the propranolol enantiomers were first converted into diastereoisomers and then separated either by gas—liquid chromatography (GLC) or by high-performance liquid chromatography (HPLC). Caccia et al. [3] used N-heptafluorobutyryl-L-propyl chloride as the derivatizing agent and separated the resulting diastereoisomers by GLC with a separation factor, α, of 1.40. Silber and Riegelman [4] and Hermansson and Von Bahr [5] used N-trifluoroacetyl-L-prolyl chloride (TPC) and reversed-phase HPLC, achieving separation factors of 1.18 and 1.20, respectively. Hermansson [6] used *tert.*-butoxycarbonyl-L-alanine and *tert.*-butoxy-carbonyl-L-leucine in conjunction with reversed-phase HPLC to resolve the resulting diastereoisomers with α = 1.20 and 1.70, respectively.

The applicability of these diastereoisomeric methods to routine assays is hindered by enantiomeric contamination of the derivatizing agent. For example, Silber and Riegelman [4] found that commercial TPC was contaminated with from 4 to 15% of the (+)-enantiomer and that the reagent rapidly racemized during storage. Efforts to synthesize optically pure TPC were unsuccessful.

These problems illustrate that the determination of enantiomeric purity through the synthesis and separation of diastereoisomeric derivatives inherently contains the danger of inaccurate determinations due to isomeric contamination of the derivatizing agent. An additional complication is that enantiomers may have quite different rates and/or equilibrium constants when they react with another chiral molecule, resulting in the generation of two diastereoisomeric products differing in proportions from the starting enantiomeric composition [7].

Both of these problems can be avoided by resolving the enantiomeric pair as enantiomers. This has been achieved in the case of propranolol by Pettersson and Schill [8] and by Pirkle et al. [9]. Pettersson and Schill [8] resolved propranolol, using ion-pair chromatography with (+)-10-camphorsulfonate in the mobile phase as the counter ion; they reported a separation factor of α = 1.12. Pirkle et al. [9] resolved propranolol as enantiomeric lauryl amides on an HPLC chiral stationary phase (CSP) employing (*R*)-N-(3,5-dinitrobenzoyl)-phenylglycine as the chiral discriminator; they reported α = 1.09. Neither of these methods has yet been applied to the determination of the enantiomeric composition of propranolol in biological fluids.

The present paper describes the development of an HPLC method for the determination of the enantiomeric purity of propranolol using the commercially available CSP developed by Pirkle et al. [9]. This CSP has been shown to have broad applicability in the resolution of molecules of pharmacological interest [9–12]. The enantiomers of propranolol were resolved as their cyclic 2-oxazolidone derivatives (IIa, Fig. 1), which were produced by facile condensation of the amino alcohol with phosgene. The enantiomeric 2-oxazolidones were then resolved by chromatography on the CSP, with α = 1.09. The method is rapid and accurate and was employed in this study to determine concentrations in whole blood as low as 0.5 ng/ml, using both spiked samples and blood from a dosed volunteer.

EXPERIMENTAL

Apparatus

The chromatography was performed with a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 8000 liquid chromatograph equipped with an SP 8000 data system, a Perkin-Elmer (Norwalk, CT, U.S.A.) Model LS-4 fluorescence spectrometer and a temperature-controlled column compartment. The column was a stainless-steel Regis-packed Pirkle Type 1-A (25 cm × 4.6 mm I.D.) with a γ-aminopropyl packing of 5-μm spherical particles modified with (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine (Regis, Morton Grove, IL, U.S.A.). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a 200 MHz Fourier transform NMR spectrometer (Varian XL-200, Varian Assoc., Instrument Group, Palo Alto, CA, U.S.A.). Mass spectra were obtained with a double-focusing, electron-impact mass spectrometer (Varian MAT 311A, Finnigan MAT, San Jose, CA, U.S.A.).

Materials

Racemic propranolol was obtained as the hydrochloride salt from Aldrich (Milwaukee, WI, U.S.A.). The *l*- and *d*-enantiomers were obtained as their hydrochloride salts from Ayerst Laboratories (New York, NY, U.S.A.). Racemic pronethalol was also provided as the hydrochloride salt by Ayerst. Phosgene, 12.5% in toluene, was purchased from MCB Manufacturing Chemists (Gibbstown, NJ, U.S.A.). The HPLC solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). The remaining chemicals and solvents were reagent grade and were used as purchased.

Oxazolidone synthesis

Racemic and enantiomerically pure 3-isopropyl-5-naphthoxy-2-oxazolidones were synthesized from the respective hydrochloride salts according to the procedure described by Hyne [13]. In a typical synthesis employing the racemate, a vigorously stirred mixture of propranolol hydrochloride (1.16 g, 0.004 mol), 12 ml 10% sodium hydroxide solution and 20 ml diethyl ether was cooled to 0°C, and 9.44 ml of 12.5% phosgene in toluene were added dropwise in 30 min. The mixture was stirred for 1 h. The organic layer was then collected, dried over anhydrous sodium sulfate and evaporated under reduced pressure, and the solid residue was recrystallized from absolute ethanol.

Extraction and derivatization from whole blood

Blood samples were collected by using plastic syringes containing 20 U of aqueous sodium heparin per milliliter of blood. Samples were frozen at −80°C before extraction. A 100-μl aliquot of the internal standard (pronethalol, 20 μg/ml in methanol) was added to each blood sample before the addition of 1 ml of carbonate buffer (pH 10). After the addition of 10 ml of diethyl ether, the mixture was shaken for 10 min and centrifuged, and the ether layer was collected and cooled to 0°C. Phosgene (10 μl of a 12.5% solution in toluene) was then added; the mixture was vortexed for 30 sec and centrifuged, and the ether layer was collected. The ether layer was evaporated by using a stream of nitrogen; the resulting solid was redissolved in 50 μl of methylene chloride and analyzed.

Standard curves

Standard curves in blood were constructed by using racemic propranolol at concentrations of each isomer ranging from 0.5 to 100 ng/ml. A standard curve for enantiomeric composition was also constructed by using mixtures of the pure enantiomers.

Chromatographic conditions

The mobile phase was hexane—isopropanol—acetonitrile (97:3:1). A flow-rate of 2 ml/min and a column temperature of 20° C were maintained throughout the analysis. An excitation wavelength of 290 nm was used, and the emission was monitored at 335 nm.

RESULTS

The reaction of phosgene with racemic propranolol proceeds smoothly and can be carried out conveniently with nanogram to milligram quantities. The mass spectrum (molecular ion peak *m/z* 285, base peak *m/z* 56), infrared spectrum (sharp peak at 1750 cm^{-1}) and NMR spectra (both ^{1}H and ^{13}C) of the recrystallized product are consistent with the formation of a single reaction product, 3-isopropyl-5-naphthoxy-2-oxazolidone (IIa, Fig. 1).

Chromatography of the recrystallized product on the CSP produced a chromatogram with two prominent peaks in a 1:1 ratio. The peaks had capacity factors (k') of 57 and 62, respectively, a separation factor (α) of 1.09 and a resolution factor (R_s) of 1.40. The chromatogram of the 2-oxazolidone resulting from the cyclization of *l*-propranolol (*S*-configuration at the asymmetric carbon) contained a single peak with a capacity factor corresponding to that of the first eluted enantiomer. In the same manner, the second eluted enantiomer was identified as the 2-oxazolidone arising from the cyclization of *d*-propranolol (*R*-configuration).

The capability of this method to determine the enantiomeric composition of propranolol was investigated with a series of mixtures of the *l*- and *d*-isomers. Mixtures of the hydrochloride salts ranging in composition from 100% of the *l*-isomer to 100% of the *d*-isomer were prepared, cyclized and analyzed. In this manner it was determined that this method could be used to detect and quantitate as little as 0.5% of one isomer in the presence of the other.

The reaction of phosgene with the hydrochloride salt of racemic pronethalol (α-[(isopropylamino)methyl]-2-naphthalenemethanol, Ib, Fig. 1) also produces a crystalline product. The mass spectrum (molecular ion peak *m/z* 255, base peak *m/z* 196), infrared spectrum (sharp peak at 1739 cm^{-1}) and NMR spectra are consistent with the formation of a single reaction product, 3-isopropyl-5-naphthyl-2-oxazolidone (IIb, Fig. 1). Chromatography of the recrystallized material on the CSP produced a chromatogram with a single prominent peak with $k' = 16$. It is both interesting and fortuitous that the enantiomers from the reaction of pronethalol, unlike those from propranolol, do not resolve under these chromatographic conditions. On the basis of its chemical similarity to propranolol and the chromatographic properties of its 2-oxazolidone, racemic pronethalol was chosen as the internal standard for the whole blood studies.

The chromatogram following the extraction and cyclization of a spiked

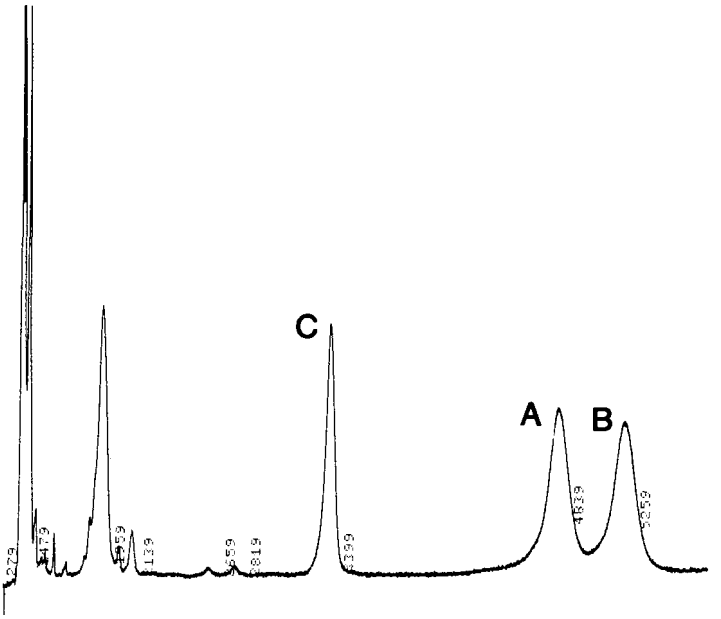

Fig. 2. Chromatogram of whole blood extract containing 50 ng racemic propranolol per ml. Peaks: A = oxazolidone corresponding to *l*-propranolol; B = oxazolidone corresponding to *d*-propranolol; C = oxazolidone corresponding to *dl*-pronethalol.

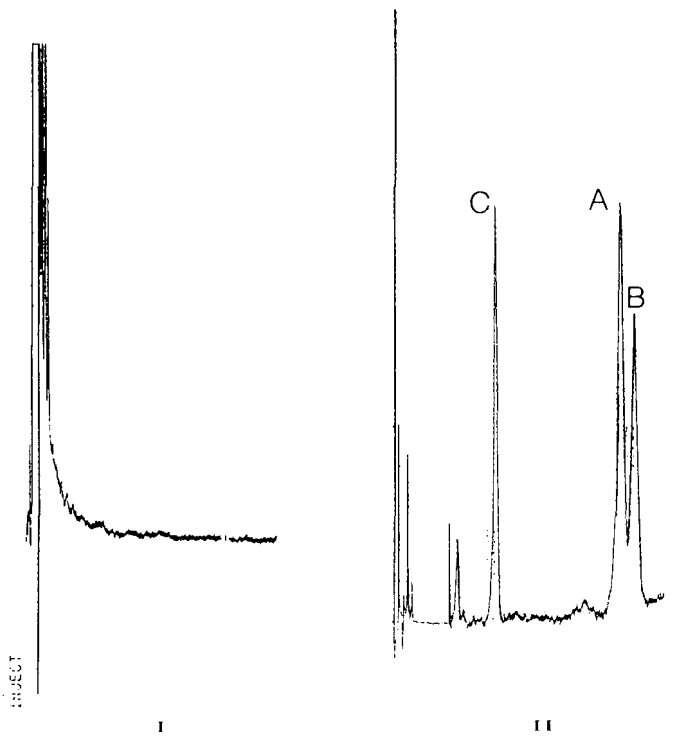

Fig. 3. Chromatograms of whole blood extract from (I) subject before administration of racemic propranolol; and (II) subject 2.5 h after administration of an 80-mg dose of racemic propranolol. Peaks: A = oxazolidone corresponding to *l*-propranolol; B = oxazolidone corresponding to *d*-propranolol; C = oxazolidone corresponding to *dl*-pronethalol.

whole blood sample containing 50 ng racemic propranolol per ml (compounds A and B) and 2 μg racemic pronethalol per ml (compound C) is reproduced in Fig. 2. Standard curves in whole blood were prepared over a concentration range of 0.5 to 100 ng/ml.

The efficiency and reproducibility of the assay were investigated. The extraction efficiency is greater than 99%. This was also reported by Hermansson [6]. The intra-day coefficients of variation (C.V.) (n = 6) at 50 ng/ml are 4.3% for the d-isomer and 5.2% for the l-isomer. The inter-day C.V. (n = 5) is 5.4% for both isomers at 50 ng/ml. The signal:noise ratio at 0.5 ng/ml is 11.3. During a two-week period of storage at room temperature, no degradation was detected in a solution of the pronethalol and propranolol derivatives in the mobile phase.

A human volunteer was dosed with 80 mg of racemic propranolol hydrochloride and blood samples were collected at 0.5, 1.0, 2.0, 2.5, 4, 6, 8 and 12 h after dosage administration. Chromatograms of the whole blood extract (blank) obtained from the subject before and 2.5 h after dosage administration are shown in Fig. 3, I and II, respectively. The blood—concentration curves for the propranolol enantiomers are presented in Fig. 4. These results are similar to those obtained by other investigators [14, 15].

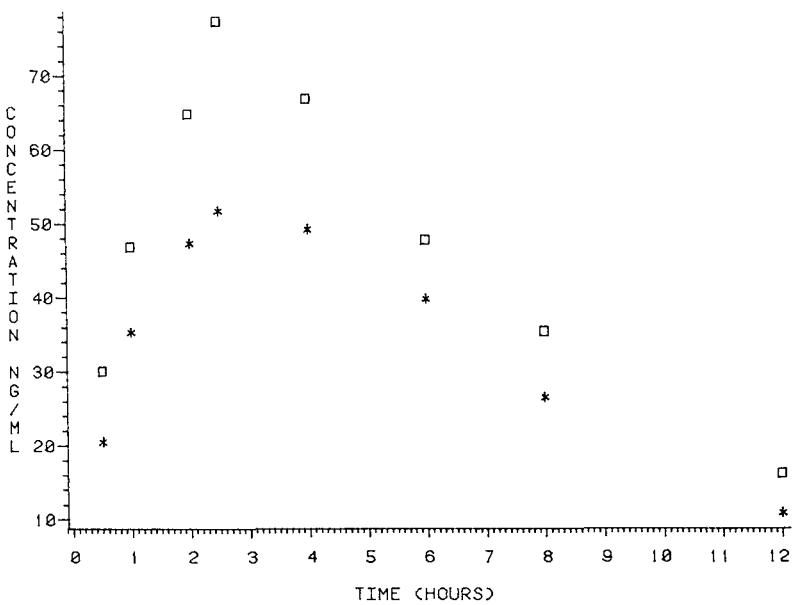

Fig. 4. Blood concentration—time curve for d- (*) and l-propranolol (□) after dosing of one subject.

CONCLUSION

The reaction of propranolol and phosgene produces a rigid oxazolidone ring system. The cyclization proceeds without racemization and the resulting enantiomers can be directly resolved by HPLC using a commercially available

CSP. The analytical approach is a direct and relatively rapid probe of the enantiomeric purity of propranolol that avoids the problems and inherent uncertainties associated with methods using the synthesis of diastereoisomers via chiral derivatizing agents.

This approach can be used with samples ranging in size from nanogram to milligram quantities. It is also applicable to biological samples. Pharmacokinetic and metabolic studies using this analytical method are currently under way.

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Note

Assay for the determination of the tetracyclic antidepressant compound aptazapine in plasma by high-performance liquid chromatography

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Aptazapine {1,3,4,14*b*-tetrahydro-2-methyl-10H-pyrazino[1,2-*a*]pyrrolo-[2,1-*c*][1,4]benzodiazepine maleate} (MW 369.4) is a potential antidepressant drug [1], which is presently being evaluated in clinical studies. This tetracyclic compound is structurally related to mianserin, another antidepressant agent, which is used as the internal standard in the described assay (Fig. 1). Both compounds are basic, contain tertiary nitrogen and are naturally fluorescent. Plasma concentrations of aptazapine were expected to be at the low nanomole per liter level. Fluorometric detection was used to obtain high sensitivity. This paper gives a detailed description of a high-performance liquid chromatographic (HPLC) method for the assay of aptazapine in plasma.

MATERIALS AND METHODS

Solvents

Acetonitrile, methanol and *n*-hexane of HPLC grade were purchased from Fisher Scientific (Springfield, NJ, U.S.A.). *n*-Butylamine (96%) was obtained from Aldrich (Metuchen, NJ, U.S.A.).

Standard solutions

Aptazapine and the internal standard mianserin were obtained from Ciba-Geigy Corporation (Summit, NJ, U.S.A.). A 20 mg per 10 ml stock solution of aptazapine in acetonitrile—methanol (96:4) was prepared and dilutions in this solvent were made as needed. Mianserin (5 mg per 10 ml) was dissolved in the same solvent and then diluted hundred-fold to prepare 5 ng/μl working solutions.

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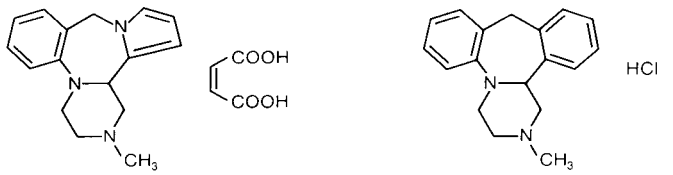

Aptazapine Mianserin

Fig. 1. Aptazapine {1,3,4,14*b*-tetrahydro-2-methyl-10H-pyrazino[1,2-*a*]pyrrolo[2,1-*c*]-[1,4]benzodiazepine maleate} and the internal standard, mianserin.

Mobile phase

The mobile phase was prepared daily and consisted of 960 ml acetonitrile, 40 ml methanol and 100 μl *n*-butylamine. The mixture was degassed by filtration through a Millipore type FH filter.

HPLC instrumentation

The chromatographic system consisted of a Waters Assoc. Intelligent Sample Processor (WISP), a Waters Model 6000A pump delivering 2.0 ml/min (Waters Assoc., Milford, MA, U.S.A.), and a Hewlett-Packard Model 3375A integrator/recorder (Hewlett-Packard, Paramus, NJ, U.S.A.). A Schoeffel fluorescence detector (Kratos, Westwood, NJ, U.S.A.) was used with the excitation wavelength fixed at 238 nm and the emitted radiation passed through a filter with a 379-nm cut-off. A Porasil guard column was fitted in front of a Hibar II silica column, particle size 10 μm, 25 cm × 4.6 mm (Merck, Cincinnati, OH, U.S.A.).

Glassware preparation

All glassware was cleaned with detergent (Micro, International Products, Trenton, NJ, U.S.A.), rinsed with distilled water and heat-treated at 800°C in an oven for 4 h. Just before use, the tubes intended for evaporations were half filled with 1% *n*-butylamine in *n*-hexane, capped and placed on an automatic shaker for 15 min, and then drained and air-dried (deactivated tubes).

Recovery measurement

[^{14}C]Aptazapine with a specific activity of 7.5 μCi/mg (Ciba-Geigy Corporation, Ardsley, NY, U.S.A.) was used to make a 1 mg per 10 ml solution in methanol, and then diluted to 4 ng/μl. Duplicate human control plasma aliquots (1.0 ml) were spiked with 10 μl of radioactive solution. The plasma was diluted with distilled water (8 ml) and 10 ml of *n*-hexane were added. The tubes were rotated for 15 min and centrifuged for 10 min. Aliquots of 9 ml of the organic extracts were transferred to scintillation vials and taken to dryness under a gentle stream of nitrogen in a waterbath at 50°C. The residues were dissolved in 100-μl aliquots of methanol with vortexing for 15 sec, after which 10 ml of Scintisol (Isolab, Akron, OH, U.S.A.) were added for measurement of radioactivity in an Intertechnique liquid scintillation counter Model SL 4000 (Fairfield, NJ, U.S.A.).

Assay procedure

Plasma (0.5—2.0 ml) was pipetted into 40-ml screwcap centrifuge tubes and 5 μl of working standard solution of mianserin (25 ng) was added as internal standard. Calibration standards ranging from 2.5 ng/ml (2.5 ng/ml÷0.369 =6.8 n*M*) to 30 ng/ml were prepared during each analysis day by adding aptazapine to control plasma aliquots to cover the range of expected concentrations for the unknowns. Distilled water (8 ml) and *n*-hexane (10 ml) were added and the tubes closed with PTFE-lined caps. Samples and standards were extracted on a rotator for 15 min and then centrifuged at 1500 *g* for 10 min. Aliquots of 9 ml of the organic extracts were transferred into deactivated tubes and evaporated under a gentle stream of nitrogen in a 50°C waterbath. Just before dryness, the inside of each tube was washed down with 1—2 ml of *n*-hexane which was then taken to dryness. All residues were dissolved in 100 μl of mobile phase with vortexing for 5 sec. The resulting solutions were transferred into Waters WISP vials and 80 μl were injected for chromatography.

Calculations

Concentrations of aptazapine were calculated from peak height ratios of drug to internal standard. Linear regression analysis was carried out with data for the calibration standards, and the resulting slope was used to obtain concentration values for the unknown samples.

RESULTS AND DISCUSSION

Mianserin has been reported to be solvent extractable in the presence of ammonium hydroxide [2]. Extraction of [^{14}C]aptazapine from plasma into *n*-hexane without changing the pH was 80%. In the extraction of aptazapine, addition of base caused endogenous interfering material to be extracted, and it did not increase recovery. Other solvents, such as toluene and chloroform were also tried as extractants, but *n*-hexane with its low polarity gave the cleanest chromatograms from control plasma extracts and was therefore chosen as the extractant for the assay.

Adsorption of drugs and internal standard onto glass, which apparently occurred during evaporation of extracts, caused variation of peak sizes in the nanogram per milliliter concentration range. Reproducibility was not improved by silanizing glassware or prerinsing with diluted hydrochloric acid. Addition of 1% isoamyl alcohol to the extractant resulted in an interfering peak in the chromatogram.

Reported methods for prevention of glass adsorption, such as circumventing the evaporation step by re-extracting into a small amount of acid [3], or wetting the glassware with aqueous alkali [4], could not be applied. In the present assay, chromatography was carried out on silica gel and this packing would not withstand the extreme acid or base conditions. The objective was to find an agent that would deactivate sites on the glassware that caused adsorption of basic amines without interfering with the chromatogram. Treatment of the glass tubes used in the evaporation step with 1% *n*-butylamine in *n*-hexane resulted in excellent reproducibility.

Difficulties with glassware contamination were avoided by using disposable

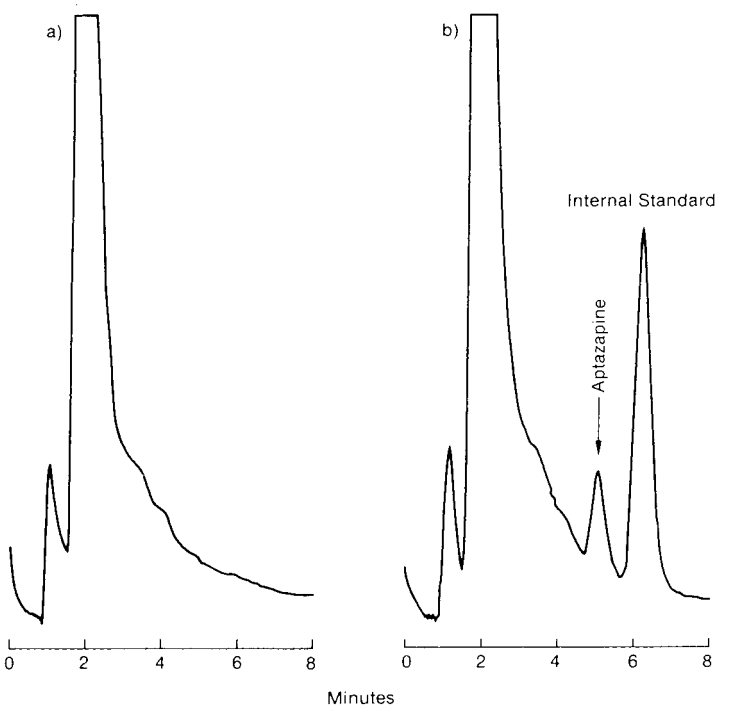

Fig. 2. Chromatograms from 2.0-ml plasma extracts: (a) plasma blank; (b) containing 2.5 ng aptazapine per ml and 25 ng internal standard.

pipets and by routinely heating all other glassware at 800°C for 4 h. This process eliminated the occasional appearance of interfering peaks.

Initial efforts at gas chromatography with a nitrogen detector were abandoned because of poor sensitivity. Reversed-phase HPLC of unextracted drug under a number of conditions produced symmetrical peaks with acceptable retention times. However, plasma extracts contained endogenous materials that interfered with the chromatograms. This interference was not resolved by modifying the chromatographic system. Pre-extraction resulted in improved chromatograms but decreased the recovery of drug. Chromatography on silica HPLC columns with a mildly polar eluent gave rather clean chromatograms but drug and internal standard peaks had long retention times accompanied by tailing. Incorporation of 1 m*M* (100 μl/l) of *n*-butylamine in the mobile phase caused elution of the drug peak, with a much improved shape, immediately after the initial plasma material. The internal standard was well separated (Fig. 2).

Eluents containing aliphatic amines or ammonium hydroxide have been used previously in HPLC separations of tricyclic antidepressants in order to decrease peak tailing and retention time [5—8]. The nature of the mechanism is not clear, although the bases may function as silica surface modifiers as suggested for water [9] and methanol [10]. The modifier presumably masks some of the more active sites on the silica by Van der Waals interaction with neutral molecules, or by acid—base interaction with the amines. The present system represents liquid—solid chromatography in normal phase mode, be-

cause no immiscible or nearly saturated component is present to form a stationary liquid phase.

It has been reported that column efficiency and column life are decreased by exposure to mobile phase of pH 8 or above and that long intervals of baseline stabilization are needed between injections [3]. These problems were not apparent in the present assay, possibly because of the low concentration of *n*-butylamine, and injections could be made every 7—8 min. Retention times decreased slightly over the course of a day's run, but flushing the column with methanol and leaving it in acetonitrile overnight resulted in retention times of the original value at the start of the next day.

The fluorometric response to increasing amounts of aptazapine injected on the column was found to be both linear and precise over the examined concentration range 10—100 ng, as indicated by a correlation coefficient of 0.9998.

The reproducibility of the assay was examined by the analysis of nine replicate aliquots of human control plasma containing 25 ng each of drug and internal standard. The mean peak height ratio of drug to internal standard had a coefficient of variation (C.V.) of 3%, indicating excellent precision.

Standard curves covering the range 2.5—30 ng/ml were obtained on five different occasions as a test of linearity and precision. The mean slope was 0.0076, with a C.V. of 4.7%. Another set of spiked samples was analyzed on each of the five occasions and concentrations were calculated as though unknown. The mean and C.V. for percent recovery were 99.7 and 8.2%, demonstrating excellent assay characteristics. The C.V. for 2.5 ng/ml samples was 7.5%.

The utility of this assay has subsequently been demonstrated by the analysis of aptazapine in plasma samples from toxicological and clinical studies. In one such study, five human subjects received 40 mg aptazapine every 12 h for five days. The mean and standard deviation for plasma concentrations were found to be 16.6 ± 11.6 ng/ml at 3 h after the last dose and 3.25 ± 0.99 ng/ml at 12 h. Concentrations as low as 2.5 ng/ml plasma have been measured and 1.25 ng/ml is measurable if the plasma aliquot is doubled.

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Note

Sensitive high-performance liquid chromatographic assay for platinum in plasma ultrafiltrate

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cis-Diamminedichloroplatinum(II) (CDDP) has proven efficacy in the treatment of germinal neoplasms of the testes, advanced ovarian carcinoma, and head and neck cancer [1]. Several platinum containing fractions have been identified in plasma soon after intravenous administration of CDDP: intact parent drug which is detectable for 3—4 h after administration [2], an ultrafilterable fraction (MW < 50,000) containing 75% CDDP which is detectable for a similar length of time [2] and a bound fraction which persists in plasma for several days [3]. Ultrafilterable platinum appears to represent biologically active platinum in plasma [4] and should therefore be quantitated in preference to total platinum which contains a bound platinum fraction lacking cytotoxic activity [5, 6].

Cisplatin has been measured specifically in plasma ultrafiltrate using a combination of high-performance liquid chromatography (HPLC) and flameless atomic absorption spectrophotometry (FAAS) [2, 7] with limits of detection of 50—100 ng/ml. A more sensitive HPLC method employing electrochemical detection has also been reported [8] and has considerable potential but has not been applied to patient samples. Total ultrafilterable and total plasma platinum have been quantitated by FAAS [2, 3, 7] and again the limits of detection were approximately 50—100 ng/ml. In order to study the pharmacokinetics of ultrafilterable platinum in patients receiving continuous infusion 20 mg/m^2 CDDP

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daily for five days an assay with greater sensitivity was required. Bannister et al. [9] and Borch et al. [10] have quantitated total platinum in urine by HPLC using precolumn derivatization with diethyldithiocarbamate (DDC). This general approach was adapted in the present study to the assay of total platinum in plasma ultrafiltrate and provided an assay with greatly improved sensitivity.

MATERIALS AND METHODS

Reagents and materials

All reagents were analytical grade and aqueous solutions were prepared using glass-distilled water. Chloroform was HPLC grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and was checked for interfering peaks before use. Heptane and isopropanol were HPLC grade (Omnisolv) from MCB Reagents, E.M. Science (Gibbstown, NJ, U.S.A.). Diethyldithiocarbamate (DDC) was obtained from Sigma (St. Louis, MO, U.S.A.), stored at −20°C and a 10% solution (w/v) in 0.1 *M* sodium hydroxide made fresh daily. *cis*-Diamminedichloroplatinum(II) (CDDP) was obtained from Johnson Matthey Research Centre (Reading, U.K.) and palladium chloride and Triton X-100 (octylphenoxypolyethoxyethanol) from Sigma. The DDC adduct of CDDP was synthesized and recrystallized according to the method of Bannister et al. [9].

Standards

Stock solutions of CDDP and the internal standard palladium chloride (1 mg/ml) were prepared in saline with the aid of 1 or 2 drops of concentrated hydrochloric acid and stored at 4°C in glass vials with PTFE-lined caps. The CDDP solution was diluted with sterile saline to provide standard solutions ranging from 50 to 10,000 ng/ml, stored at 4°C in glass and prepared fresh weekly. A working internal standard solution of palladium chloride (10 μg/ml) was prepared in saline and stored indefinitely at 4°C.

Plasma ultrafiltrate

Blood (6—10 ml) was collected into tubes containing EDTA (60 mg), kept in ice and centrifuged as soon as possible. Plasma was removed and stored at −20°C. Plasma ultrafiltrate was obtained from 100—2000 μl of plasma using MW 50,000 cut-off ultrafiltration cones (CF-50A Centriflo from Amicon, Lexington, MA, U.S.A.) by centrifuging at 1000 *g* for 30 min at 21°C. Ultrafiltrate was immediately frozen and stored at −20°C to await analysis.

Flameless atomic absorption spectrophotometric assay

Ultrafiltrate, plasma or red blood cells were diluted with an equal volume of 0.25% Triton X-100 in 500-μl autosampler tubes and 20 μl injected automatically using a Perkin-Elmer AS-40 autosampler into a Perkin-Elmer-Zeenan graphite furnace atomic absorption spectrophotometer, Model 5000. A pyrolytically coated graphite rod was used and the platinum 265.95 line monitored. The program used consisted of drying at 110°C for 20 sec (ramp rate = 10 sec) and at 160°C for 10 sec (ramp rate = 10 sec), ashing at 1400°C for 10 sec (ramp rate = 25 sec) and atomizing at 2700°C for 6 sec (maximum ramp rate). Purge gas flow-rate was normally 300 ml/min, but changed to 20 ml/min at atomiza-

tion. The limits of detection of this assay were approximately 10—25 ng/ml and coefficient of variation 6.5% at 50 ng/ml.

High-performance liquid chromatographic assay

Plasma ultrafiltrate (100—1000 μl) was pipetted into a screw-top disposable 16 × 125 mm borosilicate glass tube (Cat. No. 99447; Corning, Corning, NY, U.S.A.). The total volume was made to 1 ml with sterile saline. Standards were prepared using a volume of blank ultrafiltrate equivalent to that of the unknowns and again the total volume made to 1 ml with sterile saline. A 100-μl aliquot of the palladium chloride internal standard solution (10 μg/ml) was added to all tubes followed by 100 μl of a 10% solution of DDC in 0.1 *M* sodium hydroxide. Then 200 μl of a saturated aqueous solution of sodium nitrate were added and the tubes were capped using PTFE-lined stoppers and left at room temperature for 1 h. Chloroform (3 ml) was added and the tubes shaken at approximately 1500 rpm for 5 min. Centrifugation separated the two phases and the aqueous layer was removed by vacuum aspiration and discarded. Approximately 200 mg of anhydrous sodium sulphate was added to the tubes which were briefly shaken in order to dry the organic phase. The chloroform was decanted into 12 × 75 mm disposable borosilicate glass culture tubes (Cat. No. 14-962-10B, Fisher Scientific, Pittsburgh, PA, U.S.A.) and evaporated under a stream of dry nitrogen at approximately 40°C. The residue was reconstituted in 25 μl of chloroform and to avoid evaporation was immediately transferred to a 0.1-ml micro-vial (Cat. No. 3-3208, Supelco, Bellefonte, PA, U.S.A.) and sealed with a PTFE-lined cap (Microsep®, Type F307 from Canton Biomedical Products, Boulder, CO, U.S.A.). The reconstituted samples were stored at −20°C until ready for injection to avoid slow loss of the platinum adduct from solution. A 10-μl aliquot of each sample was injected automatically onto column.

Chromatography was carried out on a Hewlett-Packard Model 1084B high-performance liquid chromatograph equipped with a variable wavelength UV detector (HP Accessory 79875A) and autosampler (HP Accessory 79842A). A 250 × 46 mm Cyano Spheri-5 column (5 μm) (Model CS-5A, Brownlee Labs., Santa Clara, CA, U.S.A.) was used with a mobile phase consisting of heptane—isopropanol (90 : 10) preheated to 40°C. Flow-rate was 1.5 ml/min and column temperature 40°C. Under these conditions, column back-pressure was in the range of 23—30 bars. The eluent was monitored at a wavelength of 254 nm corresponding to the λ_{max} of the platinum$(DDC)_2$ adduct [9]. To allow both peak height and peak area ratios to be obtained for platinum to palladium, attenuation was automatically changed 6 min after injection.

Reproducibility and recovery

To determine the intra-assay reproducibility of the method, replicate ultrafiltrate samples (4 × 1 ml) containing added CDDP at concentrations of 2.5, 5.0, 25 and 50 ng/ml were assayed in one run. Recovery was determined by comparing the peak area of platinum$(DDC)_2$ in the above samples with that of a known amount of synthesized platinum$(DDC)_2$ injected directly onto the column (n = 5). Reproducibility of the ultrafiltration technique was determined by adding CDDP (1 μg/ml) to plasma, incubating for 1 h at 37°C and filtering

four 1-ml lots of plasma through CF 50A cones. The ultrafiltrate was assayed for platinum after derivatization as described above.

Time course of unbound platinum in blood and plasma at 37°C

Fresh blood (50 ml) was drawn from an arm vein into EDTA and separated into two 25-ml lots. One of these was centrifuged, the plasma (10 ml) removed and both plasma and the remaining blood sample incubated at 37°C. CDDP (1 μg/ml) was added to each and 2.5-ml samples of blood and 1.0-ml samples of plasma removed 10, 20, 30, 60, 90, 120, 180 and 240 min after the addition. Blood samples were centrifuged, the plasma removed and the cells kept. All plasma samples (0.8 ml) were filtered by centrifugal ultrafiltration and the ultrafiltrate (approximately 0.4 ml) collected and assayed for platinum by both the HPLC and FAAS methods. Plasma and red blood cells were assayed for platinum by FAAS only.

Pharmacokinetic study

A patient with diagnosed advanced germ cell cancer was administered an intravenous 20 mg/m^2 infusion of CDDP in saline over 24 h. Blood samples (6 ml) were collected into EDTA (60 mg) immediately before and 10, 20, 30, 40 min, 1, 1.5, 2, 2.5, 3, 6 and 12 h after commencing the infusion. Plasma was assayed for platinum by FAAS and plasma ultrafiltrate assayed for platinum by both HPLC and FAAS.

RESULTS AND DISCUSSION

The platinum and palladium DDC adducts eluted with retention times of 8.1 and 5.3 min, respectively. Chromatograms obtained for a standard mix and for extracts of blank ultrafiltrate, an ultrafiltrate standard and plasma ultrafiltrate from a patient given CDDP (20 mg/m^2) are shown in Fig. 1. Intra-assay coefficients of variation (C.V.) are shown in Table I. Reproducibility of the combined ultrafiltration-assay procedure was 7.3% at 1 μg/ml CDDP for a mean unbound platinum concentration of 0.605 μg/ml. Standard curves were linear over the concentration range 2.5—1000 ng/ml with r^2 always greater than 0.98, and recovery was not significantly different from 100%. The limit of detection of the assay was 2.5 ng/ml for on-column injection of half the extract from 1 ml of ultrafiltrate.

It was noted that considerable loss of the extracted platinum DDC adduct occurred when samples prepared for injection were left standing at room temperature for 24 h. Attempts to inhibit the decomposition by washing the chloroform layer with NaHS before drying were unsuccessful. However, samples were completely stable for at least five days when stored at −20°C and batches of 40 could be injected without significant loss over the period of injection.

A number of metal salts (Table II) were tested to find a suitable internal standard with a retention time greater than that of platinum. The cobalt DDC adduct had the longest retention time but was unsuitable because of variable recovery in the presence of the anticoagulant EDTA.

Time courses of platinum concentrations in plasma and plasma ultrafiltrate

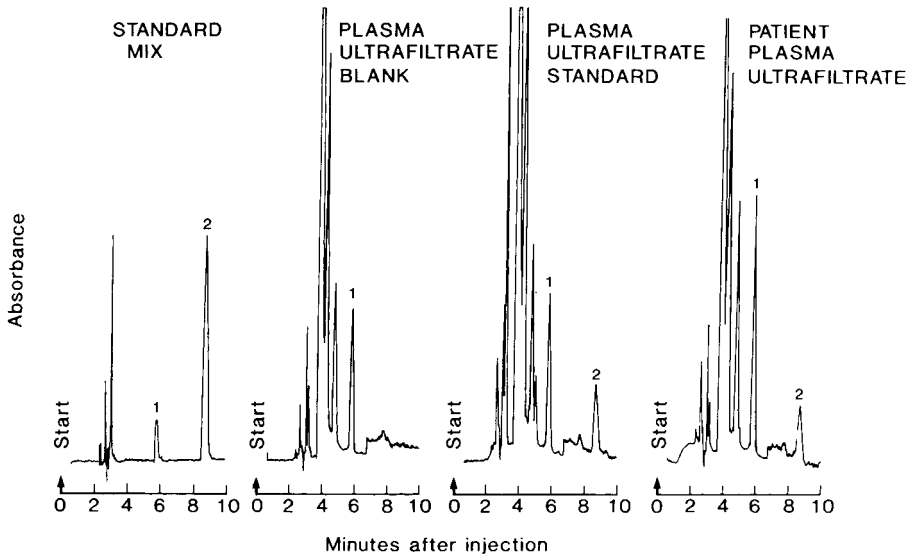

Fig. 1. HPLC chromatograms of a standard mix of the palladium and platinum DDC adducts (50 ng of each), blank ultrafiltrate containing palladium chloride (1 μg/ml), plasma ultrafiltrate containing palladium chloride (1 μg/ml) and platinum (25 ng/ml) and a plasma ultrafiltrate sample from a patient taken during an intravenous infusion of CDDP (20 mg/m²) over 24 h and containing platinum (10.1 ng/ml). Peaks: 1 = palladium; 2 = platinum. Attenuation was automatically changed by a factor of 4, 6 min after the start of the injection to ensure that both palladium and platinum peaks were on scale.

TABLE I

INTRA-ASSAY COEFFICIENTS OF VARIATION FOR HPLC ASSAY OF PLATINUM IN PLASMA ULTRAFILTRATE

Platinum concn. (ng/ml)	50	25	5.0	2.5
C.V. (%)	4.4	4.2	6.1	15.8

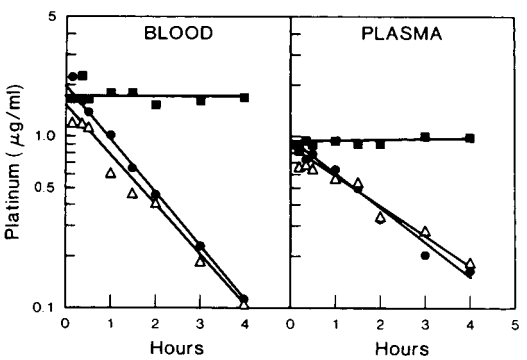

Fig. 2. Time courses of platinum concentrations in plasma and plasma ultrafiltrate following incubation of CDDP (1 μg/ml) in fresh blood and plasma at 37°C. (■——■) Total plasma platinum concentration measured by FAAS; (△——△) ultrafilterable platinum concentration in plasma measured by FAAS; (●——●) ultrafilterable platinum concentration in plasma measured by HPLC.

TABLE II

RETENTION TIMES OF VARIOUS DDC-METAL ADDUCTS

Chromatographic conditions were identical to those described in the text.

Metal	Retention time (min)
Antimony	3.6, 4.0, 6.2
Beryllium	—
Cadmium	3.7
Cobalt(II)	6.4
Germanium	—
Gold(I)	4.2
Iron(II)	3.8
Iron(III)	4.0
Mercury(II)	3.8
Molybdenum	—
Nickel(II)	5.4
Palladium(II)	5.3
Platinum(II)	8.1
Selenium	—
Silver	4.0
Strontium	—
Tungsten	—

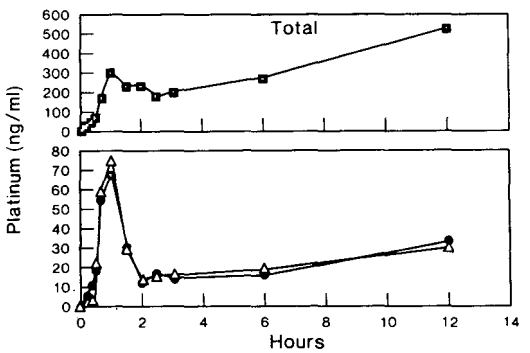

Fig. 3. Time courses of platinum concentrations in plasma and plasma ultrafiltrate in a patient given an intravenous infusion of CDDP (20 mg/m²) over 24 h. (■———■) Total platinum concentration in plasma measured by FAAS; (△———△) ultrafilterable platinum concentration in plasma measured by FAAS; (●———●) ultrafilterable platinum concentration in plasma measured by HPLC.

following incubation of CDDP (1 μg/ml) in blood and plasma at 37°C are shown in Fig. 2. There was essentially no change in total plasma levels over the duration of the experiment in either blood or plasma. Ultrafilterable platinum levels declined with first-order kinetics and the half-life in blood was estimated to be 56 min (r^2 = 0.997) by HPLC and 63 min (r^2 = 0.992) by FAAS. In plasma the half-life was 93 min (r^2 = 0.988) by HPLC and 103 min (r^2 = 0.923) by FAAS. Although there was more variability in the FAAS procedure comparable platinum levels in ultrafiltrate were obtained using HPLC and FAAS.

Time courses of total and ultrafilterable platinum in plasma in a patient infused CDDP (20 mg/m²) over 24 h are shown in Fig. 3. The HPLC and FAAS

methods for ultrafilterable platinum gave essentially identical results. The variation in plasma levels in this patient were a result of changes in drug infusion rate over the course of the study.

ACKNOWLEDGEMENTS

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CHROMBIO. 1977

Note

Analysis of propantheline bromide in serum by high-performance liquid chromatography

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Propantheline is a parasympatholytic drug which has been used for more than twenty years in the treatment of gastrointestinal ulceration and urinary incontinence. Propantheline can be readily measured in vitro [1—3]; however, determination of propantheline in serum has been hampered by the lack of sufficiently sensitive methods, particularly after oral administration of the drug [4]. Until now, the only method available to measure orally administered propantheline involved gas chromatography—mass spectrometry, a highly specialized and expensive technique [5].

The present high-performance liquid chromatographic (HPLC) method involves perchlorate ion-pair extraction of propantheline from serum followed by reversed-phase separation and low-wavelength ultraviolet detection. The assay can be used to measure propantheline in serum following oral or parenteral administration of the drug in normal therapeutic doses.

EXPERIMENTAL

Materials

Propantheline bromide reference material was kindly supplied by G.D. Searle (Chicago, IL, U.S.A.). Potassium dihydrogen orthophosphate was obtained from BDH Chemicals Australia (Port Fairy, Australia). Perchloric acid (70%,

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w/w), hydrochloric acid (32%, w/w) and orthophosphoric acid (85%, w/w) were obtained from Ajax Chemicals (Sydney, Australia). Dichloromethane, acetonitrile and hexane were bought from Waters Assoc. (Sydney, Australia). Diethyl ether (spectral grade) was obtained from E. Merck (Darmstadt, F.R.G.). Unless otherwise stated, all chemicals were analytical grade material.

Standards

For convenience, propantheline standards were routinely prepared in 3% (w/v) bovine serum albumin (BSA) since peak height ratios were similar to those obtained from the assay of propantheline in drug-free serum. Standards containing 100, 50, 20 and 5 ng/ml of propantheline bromide were prepared in bulk, subdivided into 2-ml aliquots and stored at −80°C until assayed by HPLC.

The internal standard (2-dipropylaminoethylxanthene-9-carboxylate methochloride) was synthesized by the following procedure: 2.65 g (16 mmol) of 2-chloroethyldipropylamine [6] and 5.8 g (22 mmol) of potassium xanthene-9-carboxylate were refluxed together in tetrahydrofuran (150 ml) for 13 h. The mixture was filtered and the filtrate evaporated to yield the xanthene ester as a yellow oil (5 g, 88%) which did not distil below 250°C/0.1 mm. (Found: C, 74.5; H, 7.8; N, 4.0. $C_{22}H_{27}NO_3$ requires C, 74.7; H, 7.7; N, 4.0%.) ^{1}H-nmr δ {C^2HCl_3, Me_4Si}, 7.45—6.80, br, 8H; 5.97, s, 1H; 4.66, tr, 2H; 2.52, tr, 2H; 2.30, tr, 4H; 1.26, m, 4H; 0.78, tr, 6H. 2-Dipropylaminoethylxanthene-9-carboxylate (5.1 g, 13 mmol) was refluxed in methyl iodide (25 ml) for 20 h. the pale yellow solid (7.5 g, 87%) was recrystallized from ethanol to give the methiodide, m.p. 130—132°C. (Found: C, 55.4; H, 6.3; I, 24.9; N, 2.6. $C_{23}H_{30}INO_3$ requires C, 55.7; H, 6.1; I, 25.6; N, 2.8%.) ^{1}H-nmr δ {C^2HCl_3, Me_4Si}, 7.5—6.95, br, 8H; 5.08, s, 1H; 4.31, tr, 2H; 3.73, tr, 2H; 3.38—3.02, br, 4H; 2.98, s, 3H; 2.14—1.31, m, 4H; 0.92, tr, 6H. The methiodide (2 g, 5 mmol) was suspended in water (300 ml) and shaken with freshly precipitated and washed silver chloride (from silver nitrate, 8.5 g, 50 mmol) for 1 h. The solid silver chloride—silver iodide was filtered off and the filtrate evaporated in vacuo (0.1 mm, 50°C) to yield a colourless oil (1.4 g, 69%). Trituration of the oil with tetrahydrofuran gave the solid methochloride which was recrystallized as a hygroscopic solid, m.p. 167—169°C, from methanol—ethyl acetate. (Found: C, 68.7; H, 7.3; N, 3.5. $C_{23}H_{30}ClNO_3$ requires C, 68.4; H, 7.5; N, 3.5.) ^{1}H-nmr δ {C^2HCl_3, Me_4Si}, 7.55—7.08, br, 8H; 5.12, s, 1H; 4.52, tr, 2H; 3.92, tr, 2H; 3.28, tr, 4H; 3.00 s, 3H; 2.00—1.30, m, 4H; 0.95, tr, 6H. Melting points were measured in an electrically heated silicone oil bath and are uncorrected. ^{1}H-nmr spectra were recorded on a Varian EM-360 or a Jeol MH-100 spectrometer. Microanalyses were performed by the Department of Chemistry, University of Queensland.

HPLC equipment

Chromatography was performed on a modular system consisting of a Model M45 pump (Waters Assoc., Milford, MA, U.S.A.) connected to a Model 7125 sample injector (Rheodyne, Berkeley, CA, U.S.A.) and a Model 480 Lambda-Max variable-wavelength detector (Waters Assoc.). A 10-μm particle size, 300 × 3.9 mm μBondapak C_{18} column (Waters Assoc.) was used for all

separations. Chromatograms were recorded on a Model R-02 chart recorder (Rikadenki Kogyo, Tokyo, Japan).

Chromatography

The HPLC mobile phase was prepared by slowly adding 400 ml of acetonitrile to 600 ml of rapidly stirred potassium dihydrogen orthophosphate solution (0.1 *M*, previously adjusted to pH 3 with orthophosphoric acid). All HPLC solvents were filtered (0.5 μm, Millipore, Bedford, MA, U.S.A.) and degassed before use. Reagent grade water (Milli-Q, Millipore) was used throughout. The mobile phase was delivered at 1.5 ml/min and the detector set at 210 nm with a 2-sec time constant. Chromatograms were recorded at a chart speed of 10 cm/h using a 10-mV input from the detector.

Extraction procedure

Two millilitres of serum were pipetted into 100 mm × 16 mm Kimax tubes and vortexed for 10 sec with 0.1 ml of aqueous perchloric acid (1 *M*). Dichloromethane (10 ml) containing 300 ng of internal standard was accurately dispensed into the tubes which were capped (PTFE seals) and rotated at 24 rpm on a blood mixer for 10 min. The tubes were then centrifuged (850 *g*, 15 min); the upper (aqueous) phase was aspirated and the lower (organic) phase filtered through 7-cm diameter Whatman 1PS phase separators into 15-ml glass centrifuge tubes.

The organic phase was evaporated to dryness at 35°C under reduced pressure and agitation in a Vortex-Evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.) and reconstituted in 0.1 ml of hydrochloric acid (0.1 *M*). The acidic solution was washed first with 5 ml of diethyl ether (pre-saturated with 0.1 *M* hydrochloric acid) and then with 5 ml of hexane. A 50-μl aliquot of the lower (aqueous) phase was injected onto the HPLC column.

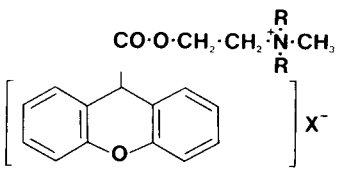
To determine optimum conditions for ion-pair extraction, the above procedure was repeated using 100 ng/ml propantheline bromide in serum with 0.0, 0.25, 0.5, 1.0, 1.5, 2.0 and 4.0 *M* perchloric acid solutions (0.1 ml).

RESULTS AND DISCUSSION

Propantheline and the internal standard (Fig. 1) gave retention times of 6.75 and 9.0 min, respectively. Both compounds displayed symmetrical peaks with baseline resolution and minimal tailing. The chromatograms in Fig. 2 are from the assay of (a) serum from a patient administered 60 mg of propantheline bromide tablets, (b) a propantheline bromide standard (100 ng/ml) in 3% BSA, and (c) drug-free serum. No endogenous serum components interfered with the analysis.

2-Dipropylaminoethylxanthene-9-carboxylate methochloride (Fig. 1) served as a suitable internal standard since it is not a drug and has similar extractive, chromatographic and spectral properties to propantheline. Methantheline bromide (2-diethylaminoethylxanthene-9-carboxylate methobromide, G.D. Searle) can also be used as an internal standard [1]; however, we noted interference with this peak in samples from patients on propantheline who were also taking tricyclic antidepressants.

There was a linear relationship between propantheline bromide concentration and peak height ratio, from 5 ng/ml to at least 100 ng/ml. Calibration plots were constructed daily; the mean data from ten such plots gave the regres-



	R	X
Propantheline Bromide	$CH{\cdot}(CH_3)_2$	Br
Internal Standard	$CH_2{\cdot}CH_2{\cdot}CH_3$	Cl

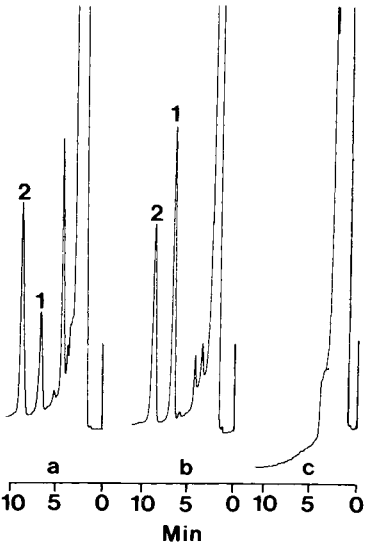


Fig. 1. Structure of propantheline bromide and of the internal standard (2-dipropylamino-ethylxanthene-9-carboxylate methochloride).

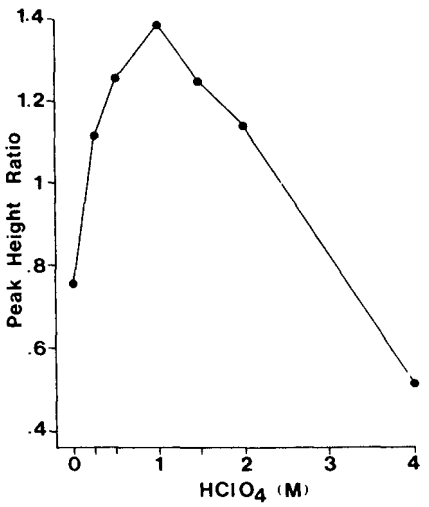
Fig. 2. Chromatograms from the analysis of: (a) serum from a patient administered 60 mg of propantheline bromide 45 min previously, (b) propantheline bromide (100 ng/ml) in 3% BSA, and (c) drug-free serum. Peaks: 1 = propantheline bromide; 2 = internal standard.

sion equation $Y = 0.0138X - 0.009$ ($r^2 > 0.999$), where Y is the peak height ratio of propantheline to internal standard and X is the concentration of propantheline bromide.

The minimum serum propantheline concentration that could be measured was about 2 ng/ml. Further increases in sensitivity might be obtained using larger injection volumes or by using HPLC columns with higher resolution and smaller particle sizes than used here. In our experience, columns which lack silanol "end-capped" packings are unsatisfactory for the assay, due to excessive retention and marked tailing of the propantheline peak. The sensitivity of the method is due in part to the excellent signal-to-noise ratio of the Lambda-Max detector. Further, propantheline has an absorption maximum at 246 nm; however, a marked increase in response is obtained at 210 nm. Since many endogenous components in serum also absorb in this region, it was necessary to develop suitable clean-up procedures. Acid back-extraction followed by ether and hexane washes successfully removed interfering peaks.

As reported by others [4], we found that dichloromethane extraction of propantheline from serum was improved considerably using perchlorate ion-pairing. In our studies, 0.1 ml of 1 *M* perchloric acid added to 2 ml of sample gave optimum extraction of the drug (Fig. 3). Perchloric acid concentrations above 1 *M* gave decreased propantheline extraction, presumably due to occlusion of the drug in protein precipitates [4].

Concentrations of propantheline bromide from 5 ng/ml to 100 ng/ml were stable in 3% BSA for at least three months at −80°C in the presence of 1 *M* perchloric acid (0.1 ml per 2 ml of sample). Assay precision was assessed by repeatedly assaying propantheline bromide standards on the same day. These results appear in Table I.



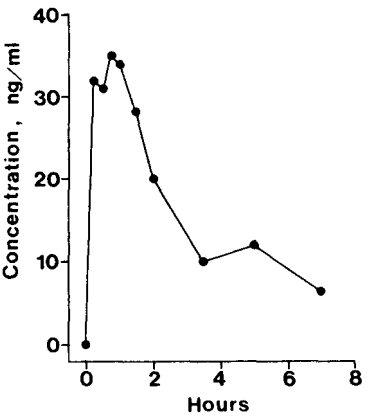


Fig. 3. Effect of perchloric acid concentration on the dichloromethane extraction of propantheline bromide from serum.

Fig. 4. Serum concentrations of propantheline bromide following administration of propantheline bromide tablets (4 × 15 mg) to a fasting, healthy subject.

TABLE I

WITHIN-DAY PRECISION OF THE HPLC ASSAY OF PROPANTHELINE BROMIDE IN SERUM

Propantheline bromide (ng/ml)	*n*	Mean peak height ratio	Standard deviation	C.V. (%)
100	10	1.370	0.1059	7.7
50	10	0.695	0.0502	7.2
20	9	0.253	0.0171	6.8
5	7	0.066	0.0037	5.6

The data in Fig. 4 show propantheline concentrations in serum measured by HPLC following administration of propantheline bromide (60 mg) tablets to a fasting, healthy volunteer. Propantheline could be measured for at least 7 h after drug administration. The assay has been extensively employed in bioavailability studies as well as in propantheline urodynamic investigations in hospital patients.

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Note

High-performance liquid chromatographic determination of amoxicillin in human plasma using a bonded-phase extraction

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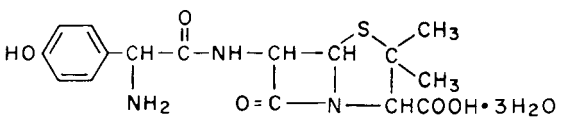
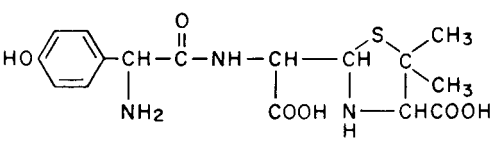
(Received September 29th, 1983)

The analysis of amoxicillin (α-amino-*p*-hydroxybenzyl penicillin, I) (Fig. 1) in biological fluids by high-performance liquid chromatographic (HPLC) assay with UV detection at 225 nm [1, 2], post-column derivatization to the mercuric mercaptide derivative of penicillenic acid and UV detection at 310 nm [3], and post-column derivatization to the fluorescamine derivative with fluorometric detection (excitation/emission 385 nm/490 nm) [4], have been reported. The assay of amoxicillin in plasma [1—3] requires the formation of a protein-free filtrate with perchloric acid and subsequent direct injection of this supernatant which typically results in rapid column deterioration [3]. In addi-



[I]



[II]

Fig. 1. Chemical structures of amoxicillin and its penicilloic acid metabolite.

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tion, the drug is unstable in this supernatant requiring its immediate analysis after protein precipitation [3]. Recent studies have demonstrated that a variety of drugs can be isolated from biological matrices using bonded-phase extraction techniques [5—9]. These methods are extremely rapid and can often avoid the use of solvent extraction and/or protein precipitation steps with strong acids as was reported for amoxicillin [1—3].

The present study describes a sensitive and selective HPLC assay for the determination of amoxicillin in human plasma using a rapid bonded-phase extraction. The methodology avoids the use of the highly acidic protein-free filtrate with resultant drug stability to allow for the assay of amoxicillin using auto-injection.

The bonded-phase extraction method involves the activation of the bonded-phase extraction material, application of the diluted plasma sample onto the bonded-phase resin, clean up and selective elution by the appropriate choice of buffers and water—methanol mixtures. The final eluent is assayed by reversed-phase HPLC using a C_8 column with UV detection at 225 nm. The recovery of amoxicillin in the concentration range of 0.5—7.5 μg/ml of plasma is 90.4 ± 6.6% (S.D.) with a limit of detection equivalent to 0.5 μg/ml plasma using a 1-ml specimen per assay.

The assay was applied to the determination of plasma concentration of amoxicillin following a single 500-mg oral dose of Larotid (amoxicillin) to one human subject.

EXPERIMENTAL

Column

The column used was a 25 cm × 4.6 mm I.D. stainless-steel prepacked column containing approximately 6-μm reversed-phase Zorbax C_8 (DuPont, Wilmington, DE, U.S.A.).

Instrumentation

The HPLC system consisted of a Model 6000A pumping system, a Model 710B automatic sample injector (WISP™) (Waters Assoc., Milford, MA, U.S.A.). An LDC Model spectromonitor III variable-wavelength UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.), was used for quantitation at 225 nm. A back-pressure coil (approximately 3 m × 1.5 mm O.D., 0.3 mm I.D. Altex PTFE tubing) was connected to the sample outlet of the flow cell. The isocratic mobile phase used for reversed-phase chromatography was a mixture of water—methanol—1 *M* phosphate buffer, pH 7 (94 : 6 : 0.5). The chromatographic system was operated at ambient temperature, with a flow-rate of 2.0 ml/min at a head pressure of $1.5 \cdot 10^3$ p.s.i. (10.35 MPa). Under the above conditions the retention time of amoxicillin was approximately 7 min (capacity factor, $k' = 3.3$). The injection of 0.16 μg of amoxicillin per 80 μl yielded a peak of nearly 40% full scale response at a detector sensitivity of 0.01 a.u.f.s. The chart speed on the 10-mV Hewlett-Packard, Model 7132A strip chart recorder was 6.35 mm/min.

Standard solution

Stock solution of 1 mg/ml (Stock A) of amoxicillin, 6-[(*R*)-2-amino 2-(*p*-hydroxphenyl) acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate ($C_{16}H_{19}O_5N_3S \cdot 3H_2O$, mol. wt. 419.45 corrected for 86% potency) was prepared by dissolving 11.63 mg in 10 ml of water. Stock solution A was diluted 1 : 10 with water to prepare solution B containing 100 μg/ml of amoxicillin. Aliquots of 5, 10, 25, 50 and 75 μl of B (equivalent to 0.5, 1.0, 2.5, 5.0 or 7.5 μg of amoxicillin) are added to 1.0 ml of control plasma to establish a calibration curve of the recovered standards for the quantitation of the concentration of amoxicillin in the unknowns and for the determination of percent recovery.

A series of external standards containing 0.5, 1, 2 and 4 μg of amoxicillin per ml of water respectively, are prepared by appropriate dilution of stock solution B. Aliquots (80 μl) of these solutions (equivalent to 0.04, 0.08, 0.16 or 0.32 μg of amoxicillin) are assayed as external standards to verify the performance of the HPLC system and for the calculation of the recovery of the assay.

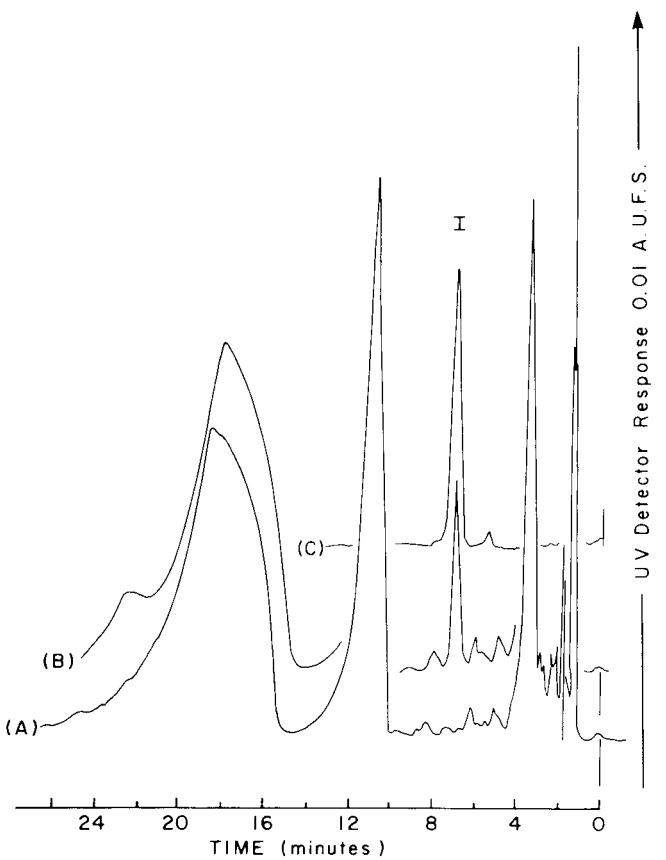

Fig. 2. HPLC analysis of extracts of (A) control plasma (from hospital supply); (B) control plasma containing 2.5 μg amoxicillin per ml added authentic standard (injection aliquot 80/1600 μl, i.e., assuming final volume of collection); (C) authentic standard 0.16 μg (80 μl injection).

Reagents and materials

All reagents are of analytical-grade purity and are prepared in deionized distilled water. Phosphate buffer (1.0 *M*, pH 7.0) is prepared by mixing 390 ml 1 *M* KH_2PO_4 (136.1 g/l) and 610 ml of $K_2HPO_4 \cdot 3H_2O$ (228.2 g/l). (Mix well and adjust to pH 7 with 1 *M* phosphoric acid as needed). Methanol was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

The bonded-phase extraction cartridges containing C_{18} reversed-phase packing (Sep-Pak C_{18} cartridges, Catalogue No. 51910) and the Sep-Pak Cartridge Rack (Catalogue No. 22030) which is capable of holding eight cartridges for simultaneous processing were both obtained from Waters Assoc.

The cartridges were activated with 4 ml of methanol followed by 1 ml of 0.02 *M* KH_2PO_4 at a flow-rate of 1—2 ml/min using a water aspirator as a vacuum source connected to the cartridge rack.

Assay procedure

An aliquot (1 ml) of control plasma, spiked control plasma (0.5—7.5 μg/ml of amoxicillin) or unknown samples was diluted with 1 ml of 0.02 *M* KH_2PO_4 and passed through the respective cartridges. The cartridges were washed with

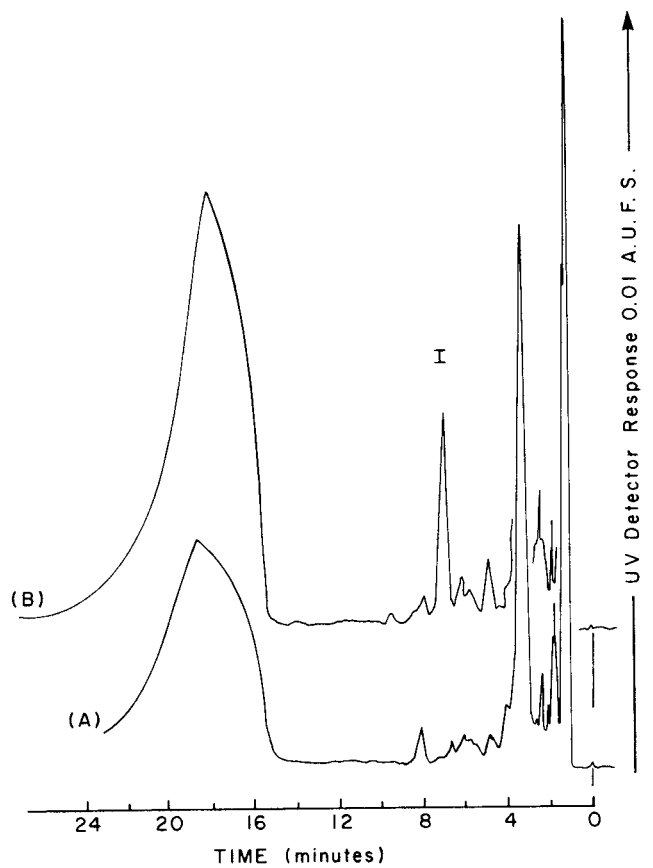

Fig. 3. HPLC analysis of extracts of (A) patient control plasma (0 h) and (B) patient plasma 1 h post 500-mg oral dose of Larotid® (amoxicillin).

1 ml of 0.02 *M* KH_2PO_4 and 0.5 ml of water. The amoxicillin was then eluted from the cartridge with a 2-ml aliquot of water—methanol (85 : 15). The eluent was collected and transferred to a WISP vial, which was placed in the WISP carousel for automatic injection. The WISP was programmed for a 80-μl injection volume and for a 25-min run time per sample. Typical chromatograms are shown in Figs. 2 and 3. The recovered standards were used to establish a calibration curve (linear regression analysis) for the direct quantitation of the concentration of compound I in the unknowns.

Selectivity

Biotransformation studies in urine have indicated that following oral administration of amoxicillin, 50—68% and 22—30% of the dosed drug is excreted in 8 h as the intact drug and penicilloic acid [6-D-(−)-α-amino-*p*-hydroxyphenyl penicilloic acid, II] metabolite, respectively [4], (Fig. 1). The HPLC assay is selective for amoxicillin in the presence of the penicilloic acid metabolite which is far more polar than the parent and is eluted near the void volume using the parameters described. Simultaneous assay for the metabolite is not feasible due to endogenous interferences in the control plasma (Figs. 2 and 3).

The assay is selective for amoxicillin in the presence of ampicillin (retention time, $t_R \geqslant 26$ min). Based upon another study using a C_8 column with a mixture of phosphate buffer and methanol as the mobile phase [10], interferences in the assay of amoxicillin would not be expected from a large number of penicillin antibiotics which all were reported to have retention times much greater than both amoxicillin and ampicillin.

Statistical evaluation of the method

The assay was validated in the concentration range of 0.5—7.5 μg of amoxicillin per ml of plasma. One sample at each concentration (Table I) was taken through the assay procedure using different lot numbers of the C_{18} cartridges on each run in a total of ten analytical runs. The mean inter-assay coefficient of variation for the ten experiments was 5.7% (Table I). The percent recovery of amoxicillin from plasma, calculated from the ratio of peak heights of amoxicillin external and recovered standards (assuming the final volume of collection of the effluent to be equal to 1.6 ml) was 90.4 ± 6.6% (S.D.). The sensitivity limit of the assay was 0.5 μg/ml of plasma.

TABLE I

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR AMOXICILLIN IN PLASMA (n = 10)

Concentration added (μg/ml)	Mean concentration found ± S.D. (μg/ml)	Coefficient of variation (%)
0.50	0.53 ± 0.04	7.6
1.00	0.97 ± 0.07	7.1
2.50	2.47 ± 0.18	7.3
5.00	5.06 ± 0.14	2.8
7.50	7.47 ± 0.27	3.6
	Mean	5.7

Application of the method to biological specimens

Plasma concentrations of amoxillin were determined using the bonded-phase extraction HPLC technique in one human subject following a single oral dose of 500 mg of Larotid. The HPLC data are in fair agreement with the analysis of the same samples by microbiological assay (Table II) and also with previously reported serum concentrations of amoxicillin in normal human subjects by microbiological assay [11]. Additional data will be needed to further statistically validate the correlation between the two assays.

TABLE II

PLASMA CONCENTRATION OF AMOXICILLIN (μg/ml) IN ONE HUMAN SUBJECT FOLLOWING SINGLE ORAL DOSE OF 500 mg OF LAROTID MEASURED BY HPLC AND MICROBIOLOGICAL ASSAY

Sampling time (h)	Concentration of plasma (μg/ml)	
	HPLC	Microbiological assay
0.5	N.M.*	N.M.
1	2.4	1.4
2	8.8	8.1
4.6	2.4	1.6
6.5	0.5	0.4**

*N.M. = Non-measurable, < 0.5 μg/ml of plasma.
**Sensitivity limit for microbiological assay = 0.4 μg/ml plasma.

DISCUSSION

A suitable internal standard with UV and chromatographic properties similar to amoxicillin could not be found. The problem was due to large interfering peaks with retention times of greater than 8 min in hospital supplied control plasma (Fig. 2) and greater than 14 min in patient control plasma (Fig. 3). These peaks also limit the total number of samples per analytical run, since approximately 30 min is required per sample.

Preliminary experiments utilizing an acetonitrile protein precipitation with a diethyl ether wash with or without an evaporation step of the final aqueous phase (similar to that used for amdinocillin [12]) did not yield sufficiently clean chromatograms to allow quantitation below 1.0—2.0 μg/ml.

In addition, experiments with a Bondapak C_{18} column (Waters Assoc.) with an eluting solvent of water—methanol—acetic acid (90 : 10 : 0.5) at a flow-rate of 1.0 ml/min yielded a retention time of 9.0 min for amoxicillin. However, interfering peaks from the control plasma again limited the assay sensitivity to 1—2 μg/ml with an unacceptably long time of analysis of 1.0 h per sample.

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Note

Micro-determination of ketoconazole in plasma or serum by high-performance liquid chromatography

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The relevance of plasma ketoconazole (*cis*-1-acetyl-4-[4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl]-piperazine) levels to clinical response needs further investigation; at present it is probably worth estimating levels only in patients who fail to respond to the drug [1, 2]. To further define this relationship between plasma concentration and therapeutic response in both adults and children, routine monitoring of ketoconazole serum concentration during treatment is necessary, and an accurate, rapid and specific assay method is essential to such studies. Whilst several microbiological procedures have been developed [3—6] they are not specific since they determine the total antifungal activity which might also include a contribution from the active deacyl metabolite [7, 8].

The use of high-performance liquid chromatography (HPLC) for ketoconazole assay has been reported in several recent publications [9—11]. In all of these procedures the sample volume requirement is relatively large (0.5—1 ml), which is a decided disadvantage in cases of severe sample limitation, for example in neonates and small children on whom frequent other tests may also be required. With multiple-step extraction [9] or column elution [10] contributing to relatively poor recoveries but necessary to decrease the amounts of interfering endogenous compounds in plasma, these assays are also time-consuming; in addition, without employment of an internal standard [9, 10] or examination of possible interference from metabolites [10, 11], they may lack accuracy.

In response to requests for drug levels in neonates treated with ketoconazole suspension for systemic mycoses, we have developed a rapid, selective and sensitive assay which involves liquid chromatography and is now in routine use

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for both therapeutic monitoring and pharmacokinetic studies. In contrast with other HPLC methods, for the method we describe as little as 20 μl of plasma or serum are required, sample preparation is simple with the one-step extraction of ketoconazole conducted quickly and conveniently in small vials, and analytical recovery is complete.

EXPERIMENTAL

Reagents and glassware

Acetonitrile (ultraviolet cut-off 190 nm) and methanol are HPLC grade while diethylamine is analytical reagent grade (Ajax Chemicals, Sydney, Australia). Ketoconazole, the three postulated metabolites of ketoconazole [9], *cis*-1-acetyl-4-(4-hydroxyphenyl)piperazine (I), *cis*-1-acetyl-4-[4-(1,2-dihydroxyethyl)methoxyphenyl] piperazine (II), *cis*-1-[4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl] piperazine (III), and triaconazole, *cis*-1-[4-{[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy}phenyl]-4-(1-methylethyl)piperazine, were supplied by Janssen Pharmaceutica (Beerse, Belgium). The stock ketoconazole solution, 100 μmol/l (53.2 mg/l) in methanol, is stable for at least six months at 4° C. The working ketoconazole solution, 1 μmol/l in methanol, is prepared freshly on the day of analysis.

To prepare the quality control (QC) serum (drug-free pooled human serum containing added ketoconazole), rapidly stir 100 ml of serum and slowly add about 2.7 mg of ketoconazole; continue stirring the mixture for 1 h. Dilute 1 ml to 10 ml with serum and store in 50-μl aliquots at −15° C; this is stable for twenty weeks.

The stock triaconazole internal standard solution, 90 μmol/l (47.9 mg/l) in acetonitrile, is stable for at least six months at 4° C. The working triaconazole internal standard solution, 9 μmol/l in acetonitrile, is prepared freshly on each day of analysis.

Glass vials, 0.3 ml (Pierce Reactivials, Pierce, Rockford, IL, U.S.A.) were obtained complete with screw caps and PTFE-faced discs.

Sample preparation

To a 0.3-ml Reactivial add 20 μl of plasma or serum. At the same time prepare reagent blank, control (QC) and standard vials. In the standard vials place 10, 20, 40, 80 and 150 μl of ketoconazole working solution and evaporate to dryness at 40° C in a stream of dry nitrogen. To the blank and standard add 20 μl of drug-free pooled human serum; to the control add 20 μl of QC serum.

To each vial add 30 μl of working internal standard solution, cap securely, and vortex-mix for 60 sec; centrifuge at 2000 *g* for 1 min.

High-performance liquid chromatography

Liquid chromatographic analysis is performed using a Model 320 isocratic liquid chromatograph equipped with a Model 165 variable-wavelength detector (Beckman Instruments). The analytical column is a pre-packed 250 × 4.6 mm I.D. Ultrasphere ODS, average particle size 5 μm, and the guard column is

50 × 4.6 mm I.D. dry-packed with Ultrasphere ODS 20 μm (Beckman Instruments).

The samples are eluted isocratically with a water—methanol—diethylamine (25:75:0.1, v/v) mixture at a constant flow-rate of 1 ml/min. The solution is prepared daily using double-distilled water, filtered (0.45 μm) and degassed before use. With detector sensitivity 0.01—0.005 A.f.s., peak heights at 240 nm are recorded with a 10-mV recorder at a chart speed of 0.25 cm/min.

Inject 20 μl of the supernate into the chromatograph and elute with the mobile phase. Under the above conditions, the retention time for ketoconazole is 9.2 min and for the internal standard 12.9 min. The ratio of peak heights of ketoconazole standard to triaconazole is calculated and the value of QC and unknown specimens calculated by direct proportion.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of blank plasma and a chromatogram of plasma from a patient being treated with ketoconazole. Concentrations as low

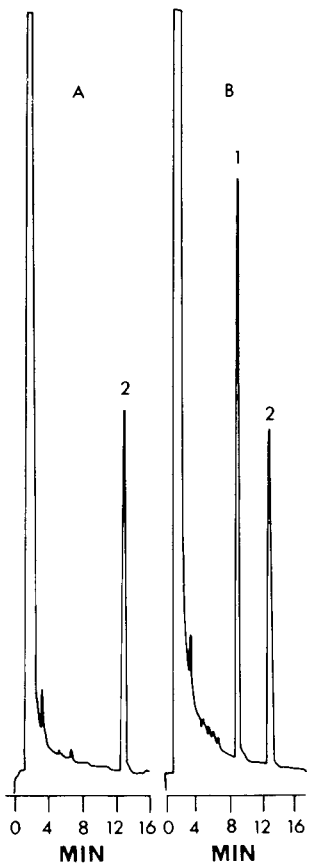

Fig. 1. Chromatograms of (A) extract of drug-free plasma, (B) extract of plasma sample containing 5 μmol/l ketoconazole. Peaks: 1 = ketoconazole; 2 = triaconazole (internal standard).

as 0.1 μmol/l can be measured accurately. Concentration and peak height were linearly related throughout the concentration range investigated, 0.1—20 μmol/l. This adequately covers the range of clinically significant concentrations of the drug in human adult plasma [3, 12—14]; however, in practice in our laboratory, most patient samples were found to contain ketoconazole in the concentration range 0.1—9 μmol/l. Absolute recoveries of ketoconazole at the level 5 μmol/l ranged from 98 to 102%; the correlation coefficient of 0.990 (n = 16) for ketoconazole determinations on paired plasma and serum samples shows that the results are interchangeable. The intra-batch coefficients of variation for replicate 20-μl aliquots of serum (n = 10 in each case) containing 1.0 and 5.0 μmol/l ketoconazole were 4.8% and 3.1%, respectively. Over a period of five months, the between-run coefficient of variation of the assay for samples (n = 21) having a concentration of 5 μmol/l was 4.5%.

The three potential metabolites of ketoconazole (retention times 3.7, 4.8 and 40.1 min, for I, II and III, respectively) were all well resolved from the parent drug and triaconazole. Extracts of plasma samples from patients on griseofulvin, miconazole, nystatin and amphotericin B showed no interfering peaks. Lignocaine (sometimes found in samples as an artefact following its use as a local anaesthetic during blood collection) elutes with ketoconazole; this interference can be eliminated by monitoring the column effluent at 291 nm rather than 240 nm but with a reduction of about eight-fold in sensitivity. The internal standard, triaconazole, is employed as it is a homologue of ketoconazole and, although possessing antifungal activity [15], is not used as a drug. Sample preparation by acetonitrile deproteinization simplifies and speeds the assay; at a 3:2 (v/v) ratio of acetonitrile to serum, the supernate obtained is clear with no micro-precipitate [16]. Techniques previously considered mandatory and designed to either overcome obstruction of the ketoconazole peak by contaminants coming off in the void or quantitatively elute ketoconazole from the column, viz. lengthy extraction procedures [9], the use of Sep-Pak C_{18} cartridges for deproteinization [10], monitoring with a fluorescence detector [11] or the use of a buffer in the mobile phase [9—11] are avoided by using a less polar solvent system and a column with higher theoretical plates. With the procedure described, the later emerging contaminants which Andrews et al. [10] observed when using protein denaturants and which made it necessary for them to extend the individual assay times to 20 min were not evident. The distinct separation of ketoconazole from contaminating peaks allows monitoring of the drug at its maximum absorption at 226 nm when increased sensitivity (0.05—0.1 μmol/l) is required (although with a concomitant increase in baseline drift and noise); even at 240 nm, sensitivities are equal to [9] or in excess of [10, 11] other methods.

The method described is currently being used for pharmacokinetic studies of ketoconazole suspension in neonates and for the therapeutic monitoring of children on oral ketoconazole for severe chronic mucocutaneous candidiasis. Requiring only 20 μl of plasma or serum it offers advantage over other methods in monitoring these paediatric patients.

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Note

Detection of marijuana metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in human urine by bonded-phase adsorption and thin-layer chromatography

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Thin-layer chromatography (TLC) is a widely used, cost-effective method for the qualitative assay of abused substances in urine. In recent years a new technique, bonded-phase adsorption chromatography, has been developed for selective extraction of drugs, pesticides, and vitamins from biologic and other materials [1]. We now report a qualitative method for the identification of the primary urinary metabolite of marijuana, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCA) [2]. The technique involves extraction of THCA onto a selective solid-phase material (Bond Elute-THC®) coupled with identification by TLC. The advantage of solid-phase extraction is that extracts obtained from hydrolyzed urine are concentrated in a small volume of solvent and are free of interfering substances.

EXPERIMENTAL

Materials

Fast Blue RR was obtained from Calbiochem-Behring (San Diego, CA, U.S.A.) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid from Applied Science Labs. (State College, PA, U.S.A.). Bond Elute-THC columns (500 mg) were purchased from Analytichem International (Harbor City, CA, U.S.A.) and E. Merck silica gel 60 thin-layer plates (25 × 75 mm) from Applied Analytical (Wilmington, NC, U.S.A.). Thin-layer developing tank (No. 13265) is available from Fisher Scientific (Springfield, NJ, U.S.A.). A Baker 10 extraction system was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). All solvents and reagents were either HPLC or reagent grade and used without further purification.

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Method

Urine samples are hydrolyzed and extracted according to the following procedures:

A 10-ml aliquot of urine is transferred to a siliconized 15-ml screw-cap type centrifuge tube and 0.9 ml of 10 *M* sodium hydroxide is added. The tube is loosely capped and allowed to stand for 15 min in a boiling-water bath. After cooling the tube, the pH of the mixture is adjusted to a range of 1—3 by the addition of approximately 0.7 ml of concentrated hydrochloric acid. THCA is extracted from the urine using a Baker 10 extraction apparatus fitted with a Bond Elute-THC column (500 mg). The extraction column is activated by successive column full rinses of: methanol, water, methanol, water. Then 10—20 ml of hydrolyzed urine are drawn through the column followed by rinses with 10 ml of 0.1 *M* hydrochloric acid and 25 ml of 50 m*M* phosphoric acid (10% acetonitrile). THCA is eluted from the column into a siliconized 10 × 75 mm culture tube with exactly 1 ml of acetone. Methylene chloride (0.5 ml) is added to the eluate, the mixture is vortexed, then centrifuged briefly, and the upper phase removed by aspiration. To the remaining phase 0.5 ml of hexane is added, the mixture vortexed, and then centrifuged briefly. The upper organic phase is pipetted into a clean siliconized 10 × 75 mm tube leaving behind a small water droplet at the bottom of the tube. The solvent then is removed by evaporation at 60° C using a stream of nitrogen gas. The dry residue is dissolved in about 10 μl of acetone and spotted with a capillary tube onto a 25 × 75 mm thin-layer plate. The chromatogram is developed in 10 ml of ethyl acetate—methanol—water—concentrated ammonium hydroxide (12:5:0.5:1). THCA is visualized as a red spot at R_F 0.43—0.50 following spraying with freshly prepared 0.5% Fast Blue RR (50 mg/10 ml of 1:1 methanol—water).

A 10-ml pooled urine aliquot, which shows a positive EMIT® cannabinoid [3] reading at or above the medium calibrator level (equivalent to 75 ng/ml THCA), is run as a control in parallel with each assay.

RESULTS AND DISCUSSION

The method described here represents a significant modification of a THCA Bond Elute-THC extraction—HPLC assay [4]. The new method yields an eluate which is made free of water and thus suitable for TLC. Most of the water in the organic phase is separated by the addition of methylene chloride. The remaining water is then completely partitioned from the organic phase by the addition of hexane, a highly water-insoluble solvent. The final organic solvent volume is less than 2 ml and requires under 5 min for evaporation. Most liquid—liquid extraction procedures require the evaporation of a final solvent volume of 30—50 ml as well as repeated addition of other organics to remove remaining traces of water [5, 6].

The solid-phase adsorption residue left after evaporation of the solvent is dry, concentrated, and quite clean. The use of a 25 × 75 mm thin-layer plate reduces the development time to 7 min. The customary 20 × 20 cm plate requires 40 min development time. The TLC mini-chamber system is cost-effective since it uses much less solvent (10 ml) and a less expensive plate.

The solvent developing system places the THCA hydrolysis product midway in the chromatogram at R_F 0.43—0.50. The chromatogenic agent, Fast Blue RR, has been shown to be a sensitive and specific visualization reagent for marijuana [6, 7]. In addition, it is observed that the red color of a developed THCA spot sprayed with Fast Blue RR remains for many weeks when covered with a glass plate. In contrast to the marijuana chromatogenic spray reagent Fast Blue B, which has been linked with carcinogenicity [8], the question of safety has not been raised with the use of Fast Blue RR.

The Bond Elute-THC—TLC assay may serve as a confirmation method for the EMIT cannabinoid [3] drug screen procedure. It can detect 20 ng/ml of THCA in urine when the volume assayed is 10 ml. This sensitivity is sufficient to confirm the presence of THCA in a 10-ml clinical urine specimen which shows a positive EMIT cannabinoid response at the medium calibrator level [9]. However, a urine volume of 20 ml often is required to detect THCA when the positive EMIT cannabinoid response is at the low calibrator level [9].

We feel the combination of Bond Elute-THC extraction coupled with TLC assay represents a new and reliable technique for the qualitative detection of THCA in urine. It should prove particularly useful clinically when EMIT screening for cannabinoids is positive and a relatively simple confirmatory test is required.

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Letter to the Editor

Liquid chromatographic determination of mitomycin C in human plasma and urine

Sir,

In the paper of Eksborg et al. [1] on the liquid chromatographic analysis of the antitumor agent mitomycin C in plasma and urine, it was claimed that the aim of the study was to simplify a previously described sample treatment [2] and to find conditions under which the degradation of mitomycin C is negligible for a proper handling of biological samples as well as for the use of a suitable liquid chromatographic isolation procedure. However, we wish to comment on both parts of this study.

As far as the sample pre-treatment procedure is concerned, evaporation of 1—10 ml of chloroform—2-propanol (1:1, w/w) takes some hours [2]; but, because of the varying sample size and accordingly varying amount of extraction solvent, a skilled analyst can arrange the treatment of the samples in such a way that the first extracts are ready for chromatographic analysis at the time when all the extracts are prepared. The alternative proposed by Eksborg et al. [1], Sep-Pak C_{18} column separation of plasma substituents and drug, needs column pre-treatment and includes evaporation of 4 ml of methanol. We think it is very doubtful whether this procedure, which is moreover much more expensive, is simplified and less time-consuming. The main draw-back of this sample treatment is, however, the varying recovery at different concentration levels. If one prefers column isolation above a liquid—liquid extraction, we suggest the use of Amberlite XAD-2 columns, which do not introduce non-linearity in the recovery, although it needs experience to produce these non-commercial columns reproducibly [3].

A thorough investigation into the pharmacokinetics of mitomycin C in 36 patients showed that the batch-wise extraction procedure is reliable and fast enough for routine analysis [4].

With regard to the second aim of Eksborg et al. [1], the authors only studied the instability of pure mitomycin C in acidic media, a subject which is already well-documented in the literature [5]. The authors did not solve the main problems in the handling of biological samples containing mitomycin C such as the proper conditions for the separation of plasma and red blood cells, stability of mitomycin C during storage, stability in daylight, etc. [6].

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Without having investigated these subjects, a study into the conditions for a proper handling of samples seems at the least incomplete.

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Letter to the Editor

Liquid chromatographic determination of mitomycin C in plasma and urine

Sir,

Den Hartigh et al. state [1] that the main drawback of our method [2] for the determination of mitomycin C in plasma and urine samples compared to other published methods [3, 4] is the varying recovery obtained at different concentration levels using a Sep-Pak C_{18} extraction procedure. However, in neither ref. 3 nor ref. 4 are the recovery and precision at different concentration levels presented. The conclusion of a concentration-independent recovery in refs. 3 and 4 is based on the fact that linear regression analysis of the calibration curves gives correlation coefficients in the order 0.999. The difficulties in interpreting the results from a linear regression analysis with a wide range of the variables (ref. 3: 1—1500 ng/ml; ref. 4: 5—1000 ng/ml) have been discussed in refs. 5 and 6. For example, linear regression analysis of the data in ref. 2 gives r values of 1.0000 and 0.9997 for plasma and urine samples, respectively. In summary, no conclusion regarding the recovery and precision of mitomycin C at different concentration levels can be drawn from the data presented in refs. 3 and 4.

The stability of mitomycin C was studied at pH < 7. The aim of the study was to find suitable conditions for the chromatographic procedure (the chromatographic support materials available are only stable at pH values below 7).

We are well aware of the problems associated with the handling of biological samples containing low concentrations of cytostatic drugs (see ref. 7). This has not been discussed in ref. 2, or in the paper by Den Hartigh et al. [3].

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Author Index

Subject Index

Erratum

J. Chromatogr., 305 (1984) 325—334
Page 330, text line 1, "1.5 h" should read "15 h".

PUBLICATION SCHEDULE FOR 1984

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

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Biomedical Applications		305/1	305/2	306	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 264, No. 3, pp. 491–494. A free reprint can be obtained by application to the publisher.)

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