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VOL. 272, NO. 2 JOURNAL OF CHROMATOGRAPHY FEBRUARY 11, 1983

(Biomedical Applications, Vol. 23, No. 2)

CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index)

Ischemic change of organic acids in kidney
by T. Niwa, N. Yamamoto, H. Asada, A. Kawanishi, M. Yokoyama, K. Maeda and T. Ohki (Nagoya, Japan) (Received September 28th, 1982) 227

Rapid simultaneous assay of serum estrone, estradiol, and estriol in pregnant women using methyl ether acetate derivatives by capillary gas chromatography and electron-impact mass spectrometry
by K.-Y. Tserng, R.K. Danish and J.S. Bendt (Cleveland, OH, U.S.A.) (Received August 30th, 1982) 233

Determination of 20α-hydroxy-9β,10α-pregna-4,6-dien-3-one in plasma by selected ion monitoring
by A. Takasu, K. Ohya and M. Sano (Tokyo, Japan) (Received September 22nd, 1982) 243

High-performance liquid chromatographic determination of free fatty acids with 1-naphthylamine
by M. Ikeda, K. Shimada and T. Sakaguchi (Niigata, Japan) (Received September 23rd, 1982) 251

Immobilized 3α-hydroxysteroid dehydrogenase and dansyl hydrazine as a pre-labeling reagent for high-performance liquid chromatography with fluorescence detection of bile acids
by T. Kawasaki, M. Maeda and A. Tsuji (Tokyo, Japan) (Received September 22nd, 1982) 261

Separation and estimation of retinyl fatty acyl esters in tissues of normal rat by high-performance liquid chromatography
by P.V. Bhat and A. Lacroix (Montreal, Canada) (Received September 17th, 1982) 269

Determination of polyamines in hydrolysates of uremic plasma by high-performance cation-exchange column chromatography
by T. Takagi, T.G. Chung and A. Saito (Nagoya, Japan) (Received September 28th, 1982) 279

Determination of urinary 5-S-cysteinyldopa by high-performance liquid chromatography
by B. Kågedal and A. Pettersson (Linköping, Sweden) (Received August 3rd, 1982) 287

Dosage plasmatique et urinaire du 3,3,5-triméthylcyclohexanol-*cis* libre et conjugué par G. Andermann et M. Dietz (Kaysersberg, France) (Reçu le 9 septembre 1982) 299

(Continued overleaf)

Gas chromatographic analysis of chloroquine after a unique reaction with chloroformates
by J.O. Kuye, M.J. Wilson and T. Walle (Charleston, SC, U.S.A.) (Received September 22nd, 1982) 307

Determination of the calcium antagonist flunarizine in biological fluids by gas—liquid chromatography
by S. Campos Flor (Raritan, NJ, U.S.A.) (Received September 28th, 1982) 315

Determination of acetylsalicylic acid and metabolites in biological fluids by high-performance liquid chromatography
by U. Reidl (Copenhagen, Denmark) (Received September 9th, 1982). 325

Quantitative determination of O-(β-hydroxyethyl)-rutosides in serum by high-performance liquid chromatography
by W. Kuhnz, K. Zech, R. Lupp, G. Jung and W. Voelter (Tübingen, G.F.R.) and F. Matzkies (Bad Neustadt/Saale, G.F.R.) (Received September 22nd, 1982) 333

High-performance liquid chromatographic method for the quantitation of flecainide, a new antiarrhythmic, in human plasma and urine
by S.F. Chang, T.M. Welscher, A.M. Miller and R.E. Ober (St. Paul, MN, U.S.A.) (Received September 22nd, 1982) 341

Determination of tamoxifen and metabolites in human serum by high-performance liquid chromatography with post-column fluorescence activation
by R.R. Brown, R. Bain and V.C. Jordan (Madison, WI, U.S.A.) (Received September 22nd, 1982) 351

High-performance liquid chromatography of pefloxacin and its main active metabolites in biological fluids
by G. Montay, Y. Blain, F. Roquet and A. Le Hir (Alfortville, France) (Received September 20th, 1982). 359

Notes

Reversed-phase high-performance liquid chromatography method for separation of collagen tryptic peptides
by A.J. Banes and M. Yamauchi (Chapel Hill, NC, U.S.A.) and R.T. Moody (Wilmington, DE, U.S.A.) (Received September 9th, 1982). 366

Isocratic reversed-phase liquid chromatographic separation of 3′,5′-cyclic ribonucleotides
by S.P. Assenza and P.R. Brown (Kingston, RI, U.S.A.) and A.P. Goldberg (Wilmington, DE, U.S.A.) (Received September 8th, 1982). 373

Simple and sensitive method for the determination of propylthiouracil in blood by high-performance liquid chromatography
by C. Kim (Toronto, Canada) (Received September 23rd, 1982) 376

Determination of trimethadione and its metabolite by gas chromatography with flame-thermionic detection and its application to pharmacokinetic studies of the drug in carbon tetrachloride-treated rats
by E. Tanaka, H. Kinoshita, T. Yoshida and Y. Kuroiwa (Tokyo, Japan) (Received September 22nd, 1982). 380

Dosage du diltiazem plasmatique par chromatographie en phase gazeuze
par R. Calaf (Marseille, France), P. Marie (Toulon, France) et Cl. Ghiglione, M. Bory et J. Reynaud (Marseille, France) (Reçu le 22 septembre 1982) 385

Detection of picogram concentrations of fentanyl in plasma by gas—liquid chromatography
by J.A. Phipps, M.A. Sabourin, W. Buckingham and L. Strunin (Calgary, Canada) (Received September 14th, 1982). 392

Quantitative determination of cardiotonic agent MDL 17,043 in plasma by reversed-phase high-performance liquid chromatography
by K.Y. Chan, J.F. Lang and R.A. Okerholm (Cincinnati, OH, U.S.A.) (Received September 9th, 1982) 396

Determination of dihydroergocristine and dihydroergotamine in plasma by high-performance liquid chromatography with fluorescence detection
by L. Zecca, L. Bonini and S.R. Bareggi (Milan, Italy) (Received September 23rd, 1982) 401

High-performance liquid chromatographic determination of ethyl biscoumacetate in human plasma
by M. Arman and F. Jamali (Tehran, Iran) (Received August 5th, 1982) 406

High-performance liquid chromatographic assay with fluorometric detection of ketanserin, a new antihypertensive agent and serotonin S_2 antagonist in human plasma, blood and urine
by P.O. Okonkwo and I.W. Reimann (Stuttgart, G.F.R.), R. Woestenborghs (Beerse, Belgium) and U. Klotz (Stuttgart, G.F.R.) (Received August 31st, 1982) 411

Determination of ketanserin in human plasma by high-performance liquid chromatography
by A.T. Kacprowicz, P.G. Shaw, R.F.W. Moulds and R.W. Bury (Parkville, Australia) (Received August 2nd, 1982). 417

Analysis of adriamycin and adriamycinol in micro volumes of rat plasma
by A.M.B. Bots, W.J. van Oort, J. Noordhoek, A. van Dijk, S.W. Klein and Q.G.C.M. van Hoesel (Utrecht, The Netherlands) (Received September 28th, 1982) 421

High-performance liquid chromatographic procedure for the quantitation of propafenone in serum and tissues
by R. Kannan, D. Tidwell and B.N. Singh (Los Angeles, CA, U.S.A.) (Received August 3rd, 1982) 428

Book Review

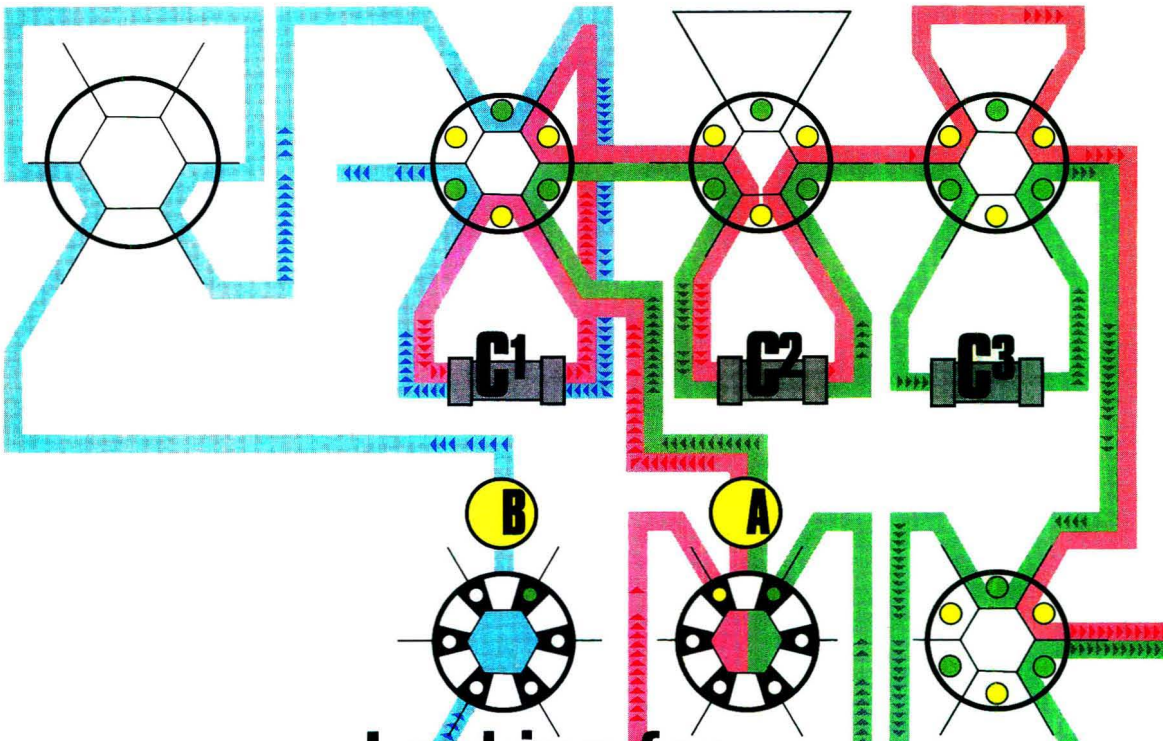
Plasma and cellular modulatory proteins (edited by D.H. Bing and R.A. Rosenbaum), reviewed by Z. Deyl. 432

Related articles published in Journal of Chromatography, Vols. 250—255 433

Author Index 437

Subject Index 442

HPLC 600 TRACER MCS 670 K



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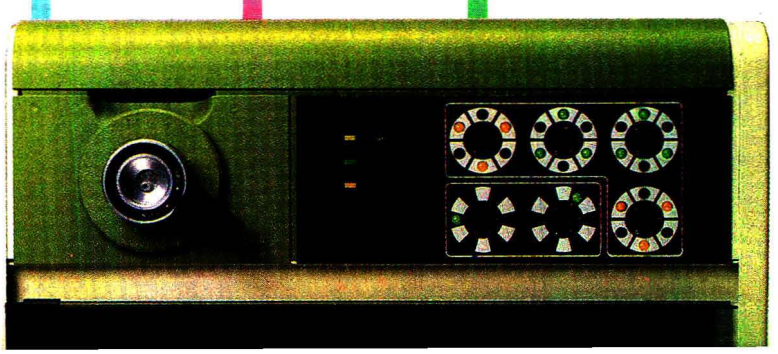

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ISCHEMIC CHANGE OF ORGANIC ACIDS IN KIDNEY

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(First received August 2nd, 1982; revised manuscript received September 28th, 1982)

SUMMARY

Organic acids in rabbit renal tissue biopsy were analyzed by capillary column gas chromatography—mass spectrometry. The change of these organic acids under ischemic conditions was determined over 60 min after clamping the renal artery and vein. The results showed that lactic acid, glycolic acid, 2-hydroxybutyric acid, 3-hydroxypropionic acid, 2-methylglyceric acid, glyceric acid and malic acid increased at 4 and 6 min after clamping, but then decreased at 15 min. Glycerol increased 2 min after clamping and then decreased. However, 3-deoxyaldonic acids of 3-deoxytetronic acid, 3-deoxy-2-C-hydroxymethyltetronic acid and 3-deoxypentonic acid decreased in the renal tissue biopsy from 2 min after clamping.

INTRODUCTION

In a previous study, the authors analyzed organic acids in renal tissue biopsy from patients with renal disease, and identified 4-hydroxybutyric acid and 4-hydroxy-2-butenoic acid in renal tissue for the first time [1]. Recently, the organic acids in various tissue specimens have been analyzed by gas chromatography—mass spectrometry (GC—MS) [2]. Haraguchi et al. [3] analyzed the organic acids in rat heart muscle under ischemic conditions using GC—MS and showed that lactic acid, glycolic acid and 3-deoxyaldonic acids increased until 4 min after decapitation, but then decreased at 6 min after decapitation; 2-deoxytetronic acid and dideoxypentonic acid were markedly increased 6 min after decapitation.

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We have attempted to determine the metabolic change of organic acids in renal tissue under ischemic conditions using GC—MS. Renal ischemia was caused by clamping the renal artery and vein, a method often used as an experimental ischemic acute renal failure model [4].

MATERIALS AND METHODS

Sample preparation

Biopsied samples of the right kidney were obtained from five male rabbits (2–2.5 kg) before and after clamping the renal artery and vein. Renal biopsy samples were obtained at 2, 4, 6, 10, 15, 30, and 60 min after clamping to examine the time-related change of organic acids. The biopsy samples obtained were immediately frozen in dry ice—acetone. Renal tissue (5 mg wet weight) was removed from each specimen, and homogenized in 0.2 ml of cold saline. After the addition of 0.3 ml of cold saline and 50 μg of *p*-(*n*-amyl)benzoic acid (Tokyo Kasei Co., Tokyo, Japan) as an internal standard, the homogenized solution was deproteinized with 3 ml of cold ethanol, and centrifuged at 25,000 *g* for 10 min. The precipitate was washed with cold ethanol and centrifuged again. The collected supernatant was concentrated to 0.5 ml under a stream of nitrogen, and 0.5 ml of distilled water was added to the concentrate. The solution was acidified to pH 1 with 1 *N* hydrochloric acid and extracted thrice with 3 ml of ethyl acetate. The organic phase was dehydrated over anhydrous sodium sulfate and dried under a nitrogen stream. The keto groups were methoximed with 1 mg of methoxylamine hydrochloride at 60°C for 30 min. After evaporation under a nitrogen stream, the organic acids were trimethylsilylated with 40 μl of bis-(trimethylsilyl)-trifluoroacetamide (Pierce, Rockford, IL, U.S.A.) and 10 μl of trimethylchlorosilane (Pierce) at 60°C for 1 h. Aliquots of the samples were subjected to GC and GC—MS analysis.

Gas chromatography and gas chromatography—mass spectrometry

For quantitation of the compounds, a Shimadzu GC-6A gas chromatograph equipped with an OV-101 open tubular glass capillary column (30 m × 0.25 mm I.D.) was used. The column temperature was programmed from 70°C to 260°C at 3°C/min. Peak areas and retention times were determined with an on-line Shimadzu Chromatopac C-RIA computer.

For identification of the compounds, a JEOL JMS D-300 mass spectrometer with a Hewlett-Packard 5710 A gas chromatograph and a JMA 2000 data processing system were used. Mass spectra were recorded at an ionizing voltage of 70 eV, an ionization current of 300 μA and an accelerating voltage of 3 kV.

RESULTS

The profile of organic acids in 5 mg of non-ischemic rabbit renal tissue is shown in Fig. 1 (upper chromatogram). Some thirty compounds were detected in the gas chromatogram. Identification of these peaks was performed by comparing their mass spectra and retention times with those of authentic compounds or from references in the literature.

The change of organic acids in the renal tissue was determined by the time

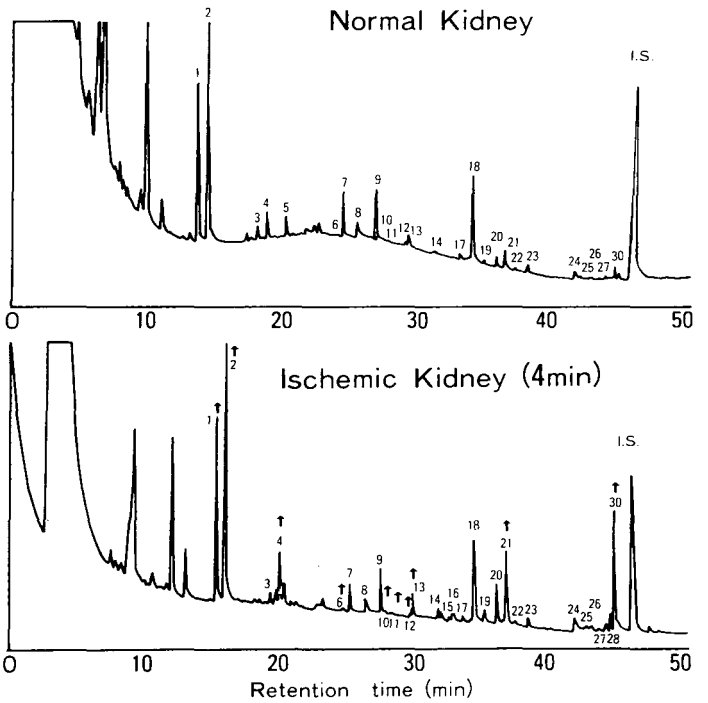

Fig. 1. Gas chromatograms of trimethylsilyl derivatives of organic acids from 5 mg of renal tissue biopsied before clamping the renal artery and vein (upper chromatogram) and 4 min after clamping (lower chromatogram). The biopsied samples were immediately frozen. The peaks identified were as follows: 1 = lactic acid, 2 = glycolic acid, 3 = 2-hydroxybutyric acid, 4 = 3-hydroxypropionic acid, 5 = 3-hydroxybutyric acid, 6 = 4-hydroxybutyric acid, 7 = diethyleneglycol, 9 = glycerol, 10 = 4-hydroxy-2-butenoic acid, 11 = succinic acid, 12 = 2-methylglyceric acid, 13 = glyceric acid; 14 = 3-deoxy-2-C-hydroxymethyltetronol-1,4-lactone, 17 = 3-deoxytetronic acid, 18 = 2-deoxytetronic acid, 21 = malic acid, 23 = 2,3-dideoxypentonic acid, 27 = 3-deoxy-2-C-hydroxymethyltetronic acid, 28 = 3-deoxypentonic acid, 29 = 3-deoxy-2-C-hydroxymethylpentono α-1,4-lactone, 30 = 3-deoxy-2-C-hydroxymethylpentono β-1,4-lactone. I.S. = internal standard. The peaks indicated by arrows in the lower chromatogram were increased in intensity compared with the upper control gas chromatogram.

elapsed after clamping the renal artery and vein to examine the ischemic metabolic change. The gas chromatogram of the organic acids in the ischemic renal tissue obtained 4 min after clamping is shown in Fig. 1 (lower chromatogram). Many peaks were increased over the control level, especially those of lactic acid, glycolic acid, 3-hydroxypropionic acid, glyceric acid, and malic acid. The levels of these organic acids, however, decreased in the ischemic renal tissue 15 min after clamping.

Fig. 2 shows the change of lactic acid, glycolic acid, 2-hydroxybutyric acid, 3-hydroxypropionic acid, 4-hydroxybutyric acid, and glycerol after clamping renal vessels. It can be seen that lactic acid, glycolic acid, and 2-hydroxybutyric acid increased in the renal tissue at 4 and 6 min after clamping, but then decreased from 15 min after clamping. 4-Hydroxybutyric acid increased in the renal tissue from 10 to 30 min after clamping. Glycerol increased in the renal tissue at 2 min after clamping but decreased from 6 min after clamping.

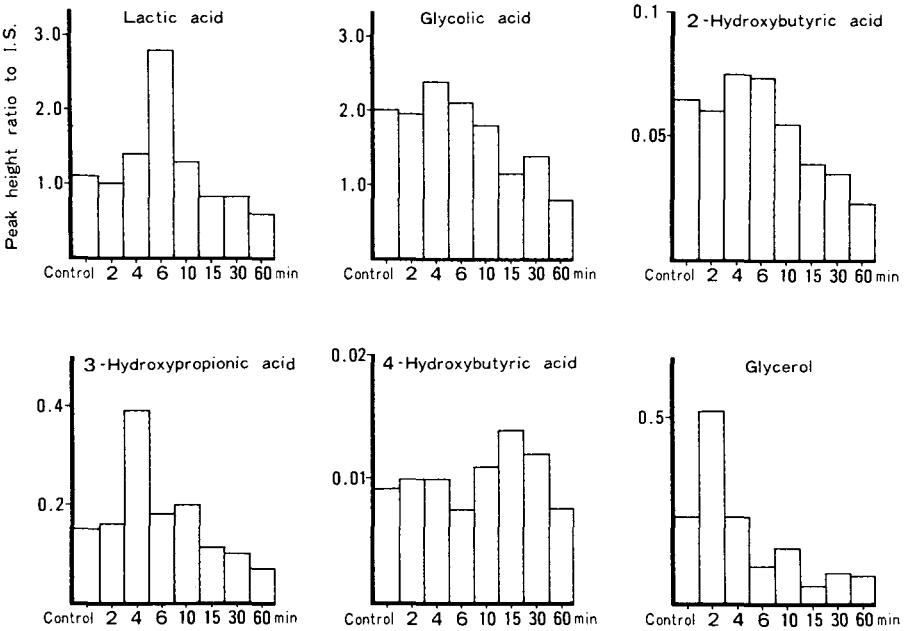

Fig. 2. The change in lactic acid, glycolic acid, 2-hydroxybutyric acid, 3-hydroxypropionic acid, 4-hydroxybutyric acid, and glycerol in renal tissue after clamping the renal artery and vein. Each bar represents the value averaged from five specimens. The abscissa represents 2 min, 4 min, 6 min, 10 min, 15 min, 30 min, and 60 min time elapsed, and the ordinate represents peak height ratio with respect to an internal standard.

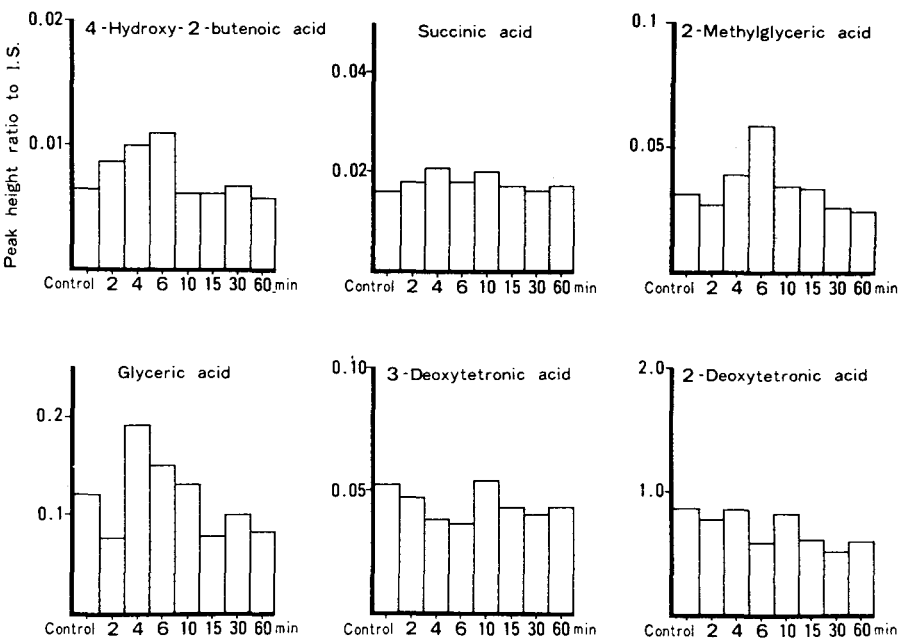

Fig. 3. The change in 4-hydroxy-2-butenoic acid, succinic acid, 2-methylglyceric acid, glyceric acid, 3-deoxytetronic acid, and 2-deoxytetronic acid in renal tissue after clamping the renal vessels.

Fig. 3 shows the change of 4-hydroxy-2-butenoic acid, succinic acid, 2-methylglyceric acid, glyceric acid, 3-deoxytetronic acid, and 2-deoxytetronic acid in the renal tissue after clamping renal vessels. 4-Hydroxy-2-butenoic acid increased in the renal tissue from 2 to 6 min after clamping, but then remained at around the control level. Succinic acid increased slightly in the renal tissue after clamping. 2-Methylglyceric acid and glyceric acid increased at 4 and 6 min after clamping and then decreased. 3-Deoxytetronic acid decreased in the renal tissue after clamping except at 10 min. 2-Deoxytetronic acid slightly decreased in the renal tissue after clamping.

Fig. 4 shows the change of malic acid, 2,3-dideoxypentonic acid, 3-deoxy-2-C-hydroxymethyltetronic acid, and 3-deoxypentonic acid in the renal tissue after clamping renal vessels. Malic acid increased at 4 and 6 min after clamping, but then decreased slightly. 2,3-Dideoxypentonic acid increased at 2 min after clamping, but then remained at around the control level. 3-Deoxy-2-C-hydroxymethyltetronic acid and 3-deoxypentonic acid decreased in the renal tissue after clamping.

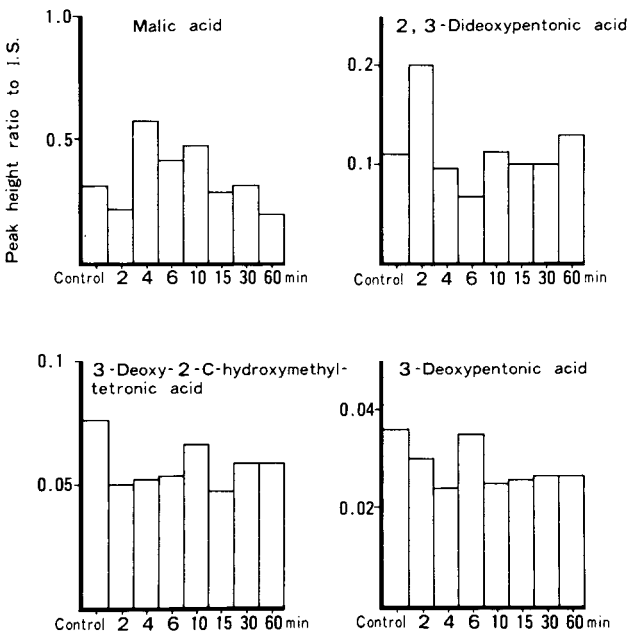

Fig. 4. The change in malic acid, 2,3-dideoxypentonic acid, 3-deoxy-2-C-hydroxymethyltetronic acid, and 3-deoxypentonic acid in renal tissue after clamping the renal vessels.

DISCUSSION

Profiling analysis of the organic acids in the renal tissue specimen was used effectively to identify the compounds and to examine the change in the compounds under ischemic conditions.

Lactic acid, glycolic acid, 3-hydroxypropionic acid, 2-methylglyceric acid, glyceric acid, and malic acid increased at 4 and 6 min after clamping renal vessels, and then decreased. Lactic acid is well known to increase in the hypoxic

condition, due to the increased NADH/NAD^+ ratio. Glycolic acid is formed from reduction of glyoxylic acid or glycol aldehyde by the action of glyoxylic reductase or glycol aldehyde dehydrogenase, respectively. The increase of glycolic acid in the renal tissue at 4 and 6 min after clamping would appear to reflect the increase of the NADH/NAD^+ ratio in the hypoxic state. Our observation that lactic acid and glycolic acid increased in an early period of ischemia is consistent with findings reporting [3] that both acids increase in the rat heart muscle in early ischemia. 2-Hydroxybutyric acid is also known to change in parallel to lactic acid. In lactic acidosis the urinary excretion of 2-hydroxybutyric acid is increased [5, 6]. 2-Hydroxybutyric acid is derived from 2-ketobutyric acid by the action of a subfraction of lactate dehydrogenase [7]. The increase of 2-hydroxybutyric acid may be related to the increased NADH/NAD^+ ratio. 3-Hydroxypropionic acid is derived from malonic acid semialdehyde by the action of 3-hydroxypropionic dehydrogenase. The increase of 3-hydroxypropionic acid in an early period of ischemia may also be due to the increased NADH/NAD^+ ratio in hypoxia. Glyceric acid is formed from hydroxypyruvic acid by the action of gyceric dehydrogenase. The increase of glyceric acid in early ischemia may again reflect an increased NADH/NAD^+ ratio. Although 2-methylglyceric acid changed in parallel to lactic acid, the metabolic pathway for the formation of 2-methylglyceric acid is not yet known.

Glycerol rapidly increased in the renal tissue after clamping and then decreased at 6 min. Succinic acid increased slightly in ischemic renal tissue. This finding is in contrast to the marked increase of succinic acid in hypoxic heart muscle [8].

3-Deoxyaldonic acids, such as 3-deoxytetronic acid, 3-deoxy-2-C-hydroxymethyltetronic acid, and 3-deoxypentonic acid, decreased in the renal tissue after clamping. This result is also in contrast to the report that these acids increased in heart muscle in the early period of ischemia, and then decreased [3]. The metabolism of these deoxyaldonic acids in the kidney may be different from that in the heart.

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RAPID SIMULTANEOUS ASSAY OF SERUM ESTRONE, ESTRADIOL, AND ESTRIOL IN PREGNANT WOMEN USING METHYL ETHER ACETATE DERIVATIVES BY CAPILLARY GAS CHROMATOGRAPHY AND ELECTRON-IMPACT MASS SPECTROMETRY*

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(First received June 25th, 1982; revised manuscript received August 30th, 1982)

SUMMARY

A simple gas chromatographic—mass spectrometric method capable of measuring estrone, estradiol, and estriol simultaneously with a sensitivity close to that of radioimmunoassay has been developed. The estrogens in serum were extracted with diethyl ether, and internal standards (3-O-C^2H_3-estrone, 3-O-C^2H_3-estradiol, and 3-O-C^2H_3-estriol) were added, followed by converting to methyl ether compounds with an extractive alkylation procedure. The methyl ethers were then acetylated. Analyses were performed using a SP-2250 capillary column gas chromatograph coupled with an electron-impact mass spectrometer.

The estrogen methyl ether acetate derivatives were more stable chemically and gave less fragmentation upon electron impact than the conventional trimethylsilyl derivatives. The use of selected ion monitoring of molecular ions and that of the corresponding internal standards (M + 3) provides a sensitivity down to 10 pg for estrone and estradiol and to 200 pg for estriol. The time required for the preparation of multiple samples is within 4 hours.

INTRODUCTION

The measurement of estrone (E_1), estradiol (E_2), and estriol (E_3) serum levels is usually done by radioimmunoassay (RIA), but this requires three separate procedures [1]. Alternatively, measurement by gas chromatography—mass spectrometry (GC—MS) with selected ion monitoring permits simultaneous measurement of these three classical estrogens with sensitivity comparable to that of RIA [2]. Over the past several years, a number of GC—MS methods for the analysis of estrogens have been developed [3—7]. However, these methods require elaborate and lengthy procedures, which make them

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impractical for multiple sample studies in clinical investigations. In evaluating dehydroepiandrosterone sulfate (DHAS) loading test for predicting fetus outcome [8] in our laboratory, the serum levels of E_1, E_2, and E_3 are to be monitored. We have developed a new GC—MS method using a methyl ether acetate derivative which simplified and shortened the preparation procedure and, in conjunction with the use of a capillary column, provided increased sensitivity. This method is simple enough to permit measurement of multiple samples. It also offers the opportunity to study estrogen metabolism safely with the use of stable isotopically labelled estrogens.

MATERIALS AND METHODS

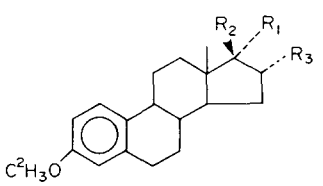
Reagents

Estrone, estradiol, and estriol were obtained from Steraloids (Wilton, NH, U.S.A.). Pyridine and acetic anhydride (commercial reagent grade) were distilled before use. Methylene chloride, diethyl ether, and methanol were of glass-distilled grade (Burdick and Jackson Labs., Muskegon, MI, U.S.A.).

0.1 M Tetrabutylammonium hydrogen sulfate solution. Tetrabutylammonium hydrogen sulfate (TBAHS) was obtained from Aldrich (Milwaukee, WI, U.S.A.). An amount of 1.963 g was dissolved in 4 ml of water, and after washing with methylene chloride (4 × 4 ml), the aqueous solution was diluted to a final volume of 50 ml with water.

Synthesis of 3-O-C^2H_3-estrone, 3-O-C^2H_3-estradiol and 3-O-C^2H_3-estriol, internal standards

Estrone, estradiol, and estriol (1 mg of each) were dissolved in 1 ml of 0.5 *N* sodium hydroxide solution, followed by the addition of 0.5 ml of 0.1 *M* TBAHS, 2 ml of methylene chloride, and 0.2 ml of [$^2H_3$]iodomethane (99+ atom % deuterium, Aldrich). The mixture was shaken for 20 min. An additional 0.2 ml of [$^2H_3$]iodomethane was then added with repeat shaking for 20 min and then centrifuged to separate the layers. The methylene chloride layer was washed with 0.5 *N* sodium hydroxide (2 × 2 ml), water (3 × 2 ml), and evaporated to dryness. The residue was dissolved in 8 ml of methanol and stored at —20°C. The recovery of estrogens was quantitative.



Ia. $R_1 + R_2 = =O$, $R_3 = H$

b. $R_1 = H$, $R_2 = OH$, $R_3 = H$

c. $R_1 = H$, $R_2 = R_3 = OH$

Fig. 1. Structural formulae of internal standards, 3-O-C^2H_3-estrone (Ia), 3-O-C^2H_3-estradiol (Ib), and 3-O-C^2H_3-estriol (Ic).

Internal standard solution (E-d_3)

The above internal standard stock solution was diluted 50-fold with methanol to a final concentration of 2.5 μg/ml of each estrogen. It was stored at −20°C, and was stable for at least one year under these conditions.

E_1, E_2, and E_3 standards

Unlabelled E_1, E_2, and E_3 were dissolved in methanol to a final concentration of 10 ng per 20 μl, 2.5 ng per 20 μl, 1 ng per 20 μl, 500 pg per 20 μl, 50 pg per 20 μl, and 10 pg per 20 μl for each estrogen.

Standard curve

A 20-μl aliquot of internal standard solution (E-d_3), containing 50 ng of each estrogen, and 20 μl of a mixture of E_1, E_2, and E_3 standard solutions were combined and mixed with 0.25 ml of 0.5 *N* sodium hydroxide, 0.125 ml of 0.1 *M* TBAHS, 2 drops of iodomethane (freshly distilled), and 0.5 ml of methylene chloride. The mixture was mixed by a vortex mixer for 1 min, then centrifuged to separate the layers. The upper layer was discarded and the lower organic layer was washed with water (2 × 0.5 ml), and evaporated to dryness in a stream of air. To the dry residue, 0.15 ml of pyridine and 0.1 ml of acetic anhydride were added. The solution was kept at room temperature for 0.5 h. Water (0.5 ml) was then added. After mixing, the aqueous solution was extracted with methylene chloride (0.4 ml). The lower organic layer was transferred to a 1-ml serum vial (Wheaton Scientific, Millville, NJ, U.S.A.) with the aid of a syringe equipped with a flat-tipped needle and was evaporated to dryness with an air stream. The vial was then capped (PTFE-lined serum cap, Fisher Scientific, Fair Lawn, NJ, U.S.A.) and stored at −20°C. Prior to analysis by GC—MS, 7 μl of isooctane were introduced into the vial by a Hamilton microsyringe (Hamilton, Reno, NV, U.S.A.). After mixing well, 1 μl of this solution was used for GC—MS analysis.

Analysis of serum samples

Serum samples (200 μl) were diluted with equal volumes of water, then extracted with diethyl ether (freshly opened, 3 × 3 ml). The combined extract was dried and the residue was reconstituted with 200 μl of methanol. Aliquots (25—100 μl) of this solution were used for the GC—MS analysis of E_1, E_2, and E_3. The rest of the solution was used for RIA of E_2. The methanol solution (25—100 μl) was evaporated to dryness and 0.25 ml of 0.5 *N* sodium hydroxide was added. This solution was washed with cyclohexane (2 × 0.3 ml), followed by the addition of 20 μl of internal standard solution (E-d_3), 0.125 ml of 0.1 *M* TBAHS, 0.5 ml of methylene chloride, and 2 drops of iodomethane (freshly distilled). The procedure for the preparation of the standard curve was then followed.

Gas chromatography—mass spectrometry

A Hewlett-Packard (Palo Alto, CA, U.S.A.) 5985A GC—MS system was used, equipped with a Hewlett-Packard 18835A capillary inlet system. The

original interface between gas chromatograph and mass spectrometer ion source was removed (the isolation valve section was kept), and replaced with a glass-lined stainless-steel tube (GLT) from Scientific Glass Engineering (Austin, TX, U.S.A.). The ends of the glass capillary (WCOT SP-2250, 10 m × 0.25 mm, J & W grade AA, supplied by Supelco, Bellefonte, PA, U.S.A.) were straightened with a glass capillary end straightener (Supelco), then coated with Carbowax 20M (Supelco) according to the recommended procedure. The outlet of this glass capillary column was connected to the GLT interface with a low-dead-volume valve (Scientific Glass Engineering). A splitless mode of analysis was used. Initially the column temperature was held at 90°C, programmed to rise at 30°C/min for 5 min, then changed to 2°C/min for the rest of the 13-min run. The helium flow-rate was 1 ml/min and the solvent purging valve was activated at 0.7 min after injection. The various zone temperatures of this system were as follows: injection port, 250°C; GC—MS interface, 250°C; ion source, 200°C; and analyzer manifold, 200°C.

The sample was ionized with an electron beam at 70 eV, then analyzed with selected ion monitoring (software provided by the manufacturer). From 5 to 8 min it was focused on *m/z* 284 vs. *m/z* 287 with dwelling times 50 msec and 200 msec, respectively. It was then switched to *m/z* 328 vs. *m/z* 331 from 8 to 10 min; then to *m/z* 386 and *m/z* 389 from 10 to 13 min. In a typical analysis, E_1 has a retention time of 7.5 min; E_2, 8.3 min; and E_3, 12.3 min. The total analysis time was 13 min.

Radioimmunoassay

Estradiol in serum was analyzed by the following procedure. An aliquot (25 μl) of methanol solution of E_2 extracted from plasma was diluted to a final volume of 500 μl. Three different sample volumes (10 μl, 30 μl, 50 μl) of the methanol solution were pipetted into separate tubes and evaporated to dryness. The residue was mixed with 200 μl of first antibody tracer solution ([2,4,6,7-^{3}H]estradiol, phosphate buffer (pH 7.8), rabbit gamma-globulins, and anti-E_2 antibody), and kept at 38°C for 2 h, then chilled at 0°C for 0.5 h. The bound E_2 was precipitated by the addition of a second antibody (goat anti-gamma-globulins precipitating antibody). After incubating at 0°C for 1 h, the supernatant (100 μl) was pipetted into a scintillation vial and scintillation mixture was added. The radioactivity was determined with a Packard (Downers Grove, IL, U.S.A.) 3320 liquid scintillation counter. The data were treated by the standard logit—log procedure.

RESULTS AND DISCUSSION

Internal standards

In a GC—MS assay with stable isotope labelled internal standard, the isotopic purity of this labelled compound should be high in order to increase the sensitivity. Besides, this internal standard should be added in the early stage of the procedure. A number of stable isotope labelled estrogens were used for GC--MS procedures by various investigators [4—7]. However, they were either being added late in the procedure [4, 5] or were of insufficient isotopic

purity for a sensitive assay [6, 7]. In addition, the procedures for synthesizing some of these standards are lengthy and time consuming.

The internal standards used in the present procedure, 3-O-C^2H_3-E_1, 3-O-C^2H_3-E_2, and 3-O-C^2H_3-E_3, are easy to prepare. They can be synthesized conveniently from [$^2H_3$]iodomethane. The high isotopic purity of [$^2H_3$]iodomethane (99+ %) made the labelled end-products devoid of residual unlabelled species, therefore the sensitivity of this method was increased. The disadvantage of these internal standards is that losses in the initial extraction procedure were not corrected. However, when recovery was checked by the addition of trace amounts of radioactive labelled E_2, the yields were consistently greater than 90%. The extractive methylation was quantitative and the washing with cyclohexane in the analysis of serum samples did not affect the recovery (see below).

Derivative

Conventionally, the trimethylsilyl (TMS) derivative of estrogen is used for GC—MS procedures [4—7]. The methyl ether acetate derivative used in the present method offers several advantages over the TMS derivative. First, it is more stable and results in less fragmentation. Secondly, extractive alkylation, used to prepare the methyl ether derivative, also serves a purification purpose. The mass spectra of the methyl ether acetate derivative of E_2 and 3-O-C^2H_3-E_2 are shown in Fig. 2.

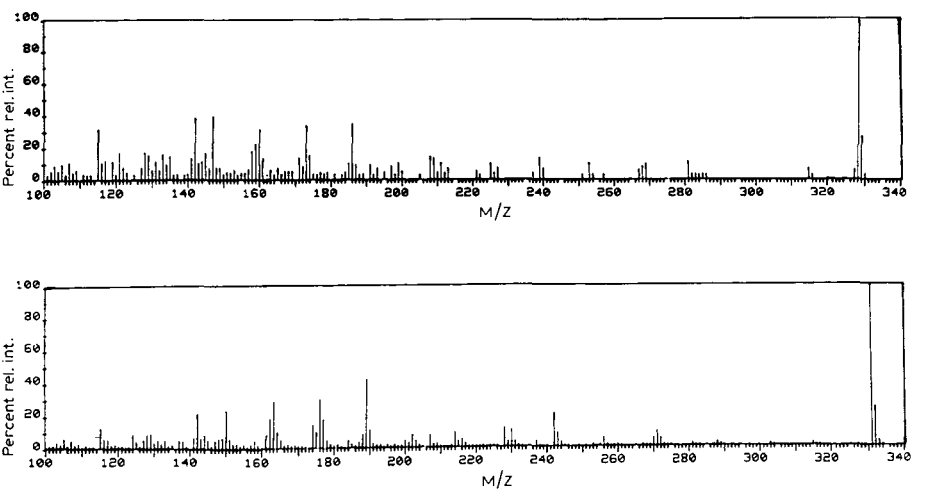


Fig. 2. Electron-impact (70 eV) mass spectra of estradiol methyl ether acetate (top), and 3-O-C^2H_3-estradiol acetate (bottom).

Analytical procedure

A chromatographic (column or thin-layer) purification procedure usually precedes the GC—MS analysis as a preliminary clean-up in estrogen analysis [3—7]. However, column or thin-layer chromatographic pre-cleaning is usually the most time-consuming step, and results in both sample loss and contamination. It prolongs the total time required for a complete analysis. The present

procedure eliminated all the chromatographic pre-cleaning procedure. The cleaning of the sample resulted from solvent washing and differential extractive methylation. The final removal of interfering peaks was achieved with a high-resolution capillary column.

The estrogens were dissolved in 0.5 *N* sodium hydroxide solution. For plasma samples, the alkaline solution was then washed twice with cyclohexane. This step removed cholesterol, triglycerides, neutral steroids, and other non-polar neutral compounds. The solvent washing was necessary when E_3 was analyzed. It removed cholesterol, which was eluted immediately before E_3 in GC and elevated the baseline to mask the E_3 peak. However, cyclohexane washing was not necessary when only E_1 and E_2 were to be analyzed. None of the neutral, non-polar components in the plasma interfered with the assay of E_1 and E_2. The difference between the standard curve and plasma sample is the cyclohexane step. Even though the possible sample loss during this step was not compensated by internal standards, the data in Table I show that

TABLE I

EFFECT OF CYCLOHEXANE WASHING ON THE ASSAY RESULTS OF ESTRONE AND ESTRADIOL

Method 1 is without cyclohexane washing and method 2 is with cyclohexane washing.

Plasma sample No.	E_1 (ng/ml)		E_2 (ng/ml)	
	Method 1	Method 2	Method 1	Method 2
1	4.8	4.3	24.0	26.5
2	4.5	4.2	23.0	24.8
3	5.0	5.0	23.0	21.0
4	5.1	5.5	23.5	25.5
5	9.7	9.0	83.0	85.0
6	10.2	9.9	63.0	67.5
7	11.3	10.7	60.0	62.4

essentially no estrogen loss occurred when a series of plasma samples were analyzed with and without cyclohexane washing for E_1 and E_2.

Daley et al. [9] used tetrahexylammonium as counter ion to synthesize estrogen methyl ethers in high yield. Rosenfeld and Taguchi [10] applied the same principle for the analysis of 2-hydroxyestradiol. It is known that by varying the type and concentration of the counter ions, anions of different ionization constants can be differentially extracted into an organic solvent, thereby achieving a degree of purification [11]. Experimentally, it was found that tetrabutylammonium ion in a concentration of 0.024 *M* served this purpose. Higher concentration or counter ions with higher hydrophobicity increased the background of GC—MS analysis. Lower concentration or counter ions with higher hydrophilicity ran the risk of incomplete recovery.

Gas chromatography—mass spectrometry

Estrogen methyl ether acetates have good GC properties. They showed

symmetric peaks when the glass capillary column was used under the conditions described above. Their 70-eV mass spectra (Fig. 2) show prominent molecular ions and were used for selected ion monitoring (Fig. 3). The area ratio of the molecular ion of estrogen versus the corresponding deuterated standards was plotted against the amount of unlabelled estrogen in the sample. The standard curves (Fig. 4) were linear over a range of at least 1000-fold, from 10 pg to 10 ng. Estrone and estradiol had similar standard curves, and the detection limits were 10 pg. The standard curve for E_3 shows a different slope from E_1 and E_2 and had a lower sensitivity (200 pg). The deviation of the E_3 standard curve is unclear at this moment. Since it was reproducible and was linear over the range studied, the possibility that it was due to incomplete recovery or decomposition of E_3 was highly unlikely. The reproducibility of this procedure was checked by analyzing a set of plasma samples in triplicate. The average coefficient of variation was 3.5% for sample concentrations ranging from 5—70 ng/ml of estrogen (n = 18). Theoretically,

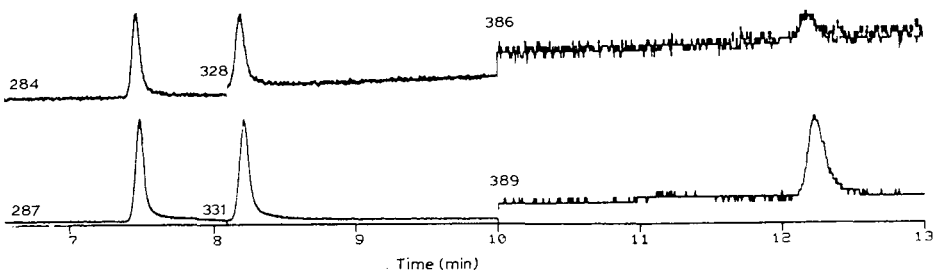


Fig. 3. Selected ion monitoring chromatogram of a serum sample for estrone (m/z 284 vs. 287), estradiol (m/z 328 vs. 331), and estriol (m/z 386 vs. 389). The displays are normalized to the maximum of each individual peak.

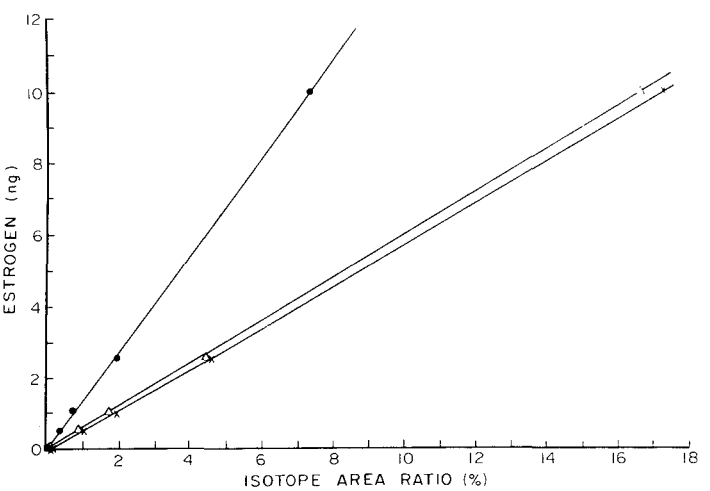


Fig. 4. Standard curves of estrogens: (△——△) estrone; (×——×) estradiol; (•——•) estriol. Isotope area ratio (%) was obtained by dividing the peak area of unlabelled estrogen by that of labelled estrogen.

chemical ionization results in less fragmentation which would tend to increase sensitivity. The methane chemical ionization of these estrogens did show the protonated molecular ion (MH^+) as the only fragment. Unfortunately, the sensitivity was not increased.

Clinical study

A typical result of a dehydroepiandrosterone sulfate loading test is shown in Fig. 5. The E_1 serum level rose slowly and peaked at the end of study (2 h), whereas the E_2 level rose rapidly after the loading dose, reaching a peak level at about 0.5 h after dose. The E_3 level was unchanged throughout the study. The average of four basal serum levels of unconjugated E_1, E_2, and E_3 in this subject were 8.2 ng/ml, 29.7 ng/ml, and 12.6 ng/ml, respectively. These are consistent with RIA assayed values reported in the literature for women in this stage of pregnancy [12]. The pattern of estrogen after DHAS loading is also consistent with the literature results measured by RIA [12—14].

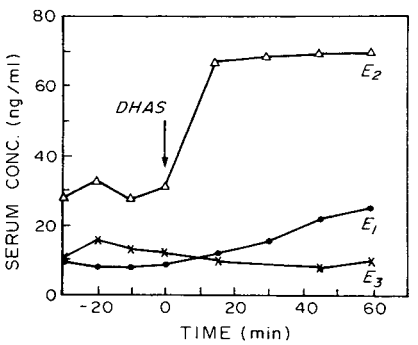


Fig. 5. Estrone, estradiol, and estriol levels before and after intravenous administration of a 50-mg bolus of dehydroepiandrosterone sulfate (DHAS). The patient was a woman in her 39th week of pregnancy, which resulted in delivery of a healthy, normal baby a week later.

TABLE II

CORRELATION OF E_2 PLASMA LEVELS AS MEASURED BY THE GC—MS PROCEDURE AND BY RIA

Sample No.	E_2 (ng/ml)	
	GC—MS	RIA
1	30.0	28.3
2	35.0	39.5
3	29.0	28.5
4	32.6	37.5
5	73.2	64.0
6	74.5	79.0
7	75.8	82.3
8	75.8	83.7

Correlation of E_2 plasma levels as determined by GC—MS and by RIA

The correlation of E_2 serum levels as determined by the GC—MS procedure and by RIA is shown in Table II. The samples were obtained from a DHAS loading study of a pregnant woman. Samples 1—4 were basal values before the administration of DHAS. Samples 5—8 were E_2 values after DHAS loading. Regression analysis of data obtained from RIA (Y) and GC—MS (X) gave the equation $Y = 1.0281\ X + 0.5187$; the correlation coefficient (r^2) was 0.9479.

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DETERMINATION OF 20α-HYDROXY-9β,10α-PREGNA-4,6-DIEN-3-ONE IN PLASMA BY SELECTED ION MONITORING

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SUMMARY

A selected ion monitoring (SIM) method has been devised for the determination of metabolites of dydrogesterone, 20α-hydroxy-9β,10α-pregna-4,6-dien-3-one (DHD) and DHD glucuronide, in plasma. Using testosterone as an internal standard (IS), DHD and IS were extracted with *n*-hexane and were purified by means of magnesium oxide column chromatography. The purified DHD and IS were converted to their diheptafluorobutyryl derivatives (DHD diHFB and testosterone diHFB) with heptafluorobutyric anhydride in acetone for analysis by SIM.

SIM was carried out with a 2% OV-17 column (1 m) at 230°C by monitoring the molecular ions of the derivatives (*m/z* 706 for DHD diHFB, *m/z* 680 for testosterone diHFB). DHD was determined from a calibration curve using a peak area method. The determination limit of the devised method was about 5 ng DHD per ml of plasma and the reproducibility was within ± 6% of the coefficient of variation for 30 ng of DHD per ml of plasma or above.

INTRODUCTION

Dydrogesterone (6-dehydro-9β,10α-progesterone) is a progestationally active steroid hormone synthesized in 1960 [1]. It is a retrosteroid characterized by the β-position of the C-9 hydrogen atom and the α-position of the C-10 methyl group.

The absorption, metabolism and excretion of dydrogesterone have been studied in detail by use of the ^{3}H-labelled drug. It has been revealed that dydrogesterone is mainly metabolized to 20α-hydroxy-9β,10α-pregna-4,6-dien-3-one (DHD) and further to DHD glucuronide, as shown in Fig. 1 [2]. In addition, it has been reported that the plasma level of dydrogesterone in female subjects who received orally 10 mg of [^{3}H]dydrogesterone was 1 ng/ml of plasma or below, and that of DHD was about 10 ng/ml of plasma [3].

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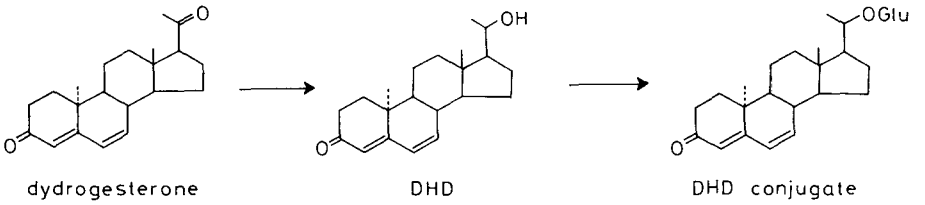


Fig. 1. Metabolic pathway of dydrogesterone.

On the other hand, no chemical methods for the determination of dydrogesterone and the metabolites have been presented. This would be due to the difficulty of measuring dydrogesterone and/or DHD because of their very low plasma levels. In order to examine the pharmacokinetics of dydrogesterone in man after oral administration, we devised a convenient and sensitive selected ion monitoring (SIM) method for the determination of DHD and DHD glucuronide in plasma. This paper describes the method in detail and its application to the pharmacokinetic study of DHD and DHD glucuronide in man.

EXPERIMENTAL

Chemicals and reagents

DHD was provided by Philips Duphar (Amsterdam, The Netherlands). Testosterone of reagent grade was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Heptafluorobutyric anhydride (HFBA) of reagent grade was purchased from Tokyo-Kasei Kogyo (Tokyo, Japan). β-Glucuronidase/arylsulfatase (from *Helix pomatia*, 5.2 U/ml for β-glucuronidase and 2.6 U/ml for arylsulfatase) was purchased from Boehringer Mannheim (Mannheim, G.F.R.). All other chemicals of reagent grade were used without further purification.

A stock DHD solution was prepared by dissolving DHD in ethyl acetate at a concentration of 1.0 μg/ml. An internal standard (IS) solution was prepared by dissolving testosterone in ethyl acetate at a concentration of 2.0 μg/ml. These solutions were stored at 5°C protected from light. The magnesium oxide (MgO) column was a 2.7 cm × 0.5 cm I.D. silanized glass tube. MgO of reagent grade was used after drying in an oven at 300°C overnight and then grinding in a mortar.

Sample preparation

To 1.0 ml of plasma samples in 10-ml glass tubes was added 1 ml of pH 7.0 Michaelis phosphate buffer solution. Subsequently, to these solutions were added exactly 0.1 ml of IS solution (corresponding to 200 ng of testosterone) and 5 ml of *n*-hexane. The tubes were shaken vigorously for 15 min and centrifuged for 5 min at ca. 1000 *g*. The aqueous layer was frozen in a dry-ice–acetone bath, and then the organic layer was transferred to a 10-ml glass tube and evaporated in vacuo to dryness. The residue was dissolved in 0.1 ml of isooctane and passed through the MgO column [4]. The column was washed with 2 ml of isooctane and 2 ml of isooctane–ethyl acetate (20:1) mixture to remove contaminating plasma components; then DHD and IS were eluted with

8 ml of isooctane—ethyl acetate (3:1) mixture. The eluate was evaporated in vacuo to dryness. The residue was reacted with 10 μl of HFBA in 100 μl of acetone at room temperature for 1 h. Then the reaction mixture was evaporated in vacuo to dryness. The residue was dissolved in 50 μl of acetone to give the sample solution. The sample solutions were analyzed by SIM.

In the case of the determination of total DHD including the glucuronide, enzymatic hydrolysis was carried out before the extraction procedure as follows. To 1.0 ml of plasma sample were added 1 ml of pH 4.5 Michaelis acetate buffer solution, 50 μl of β-glucuronidase and 10 μl of toluene, and these solutions were incubated at 37°C for 48 h. Then the solutions were purified in the same manner as described above to prepare the sample solutions for SIM.

Instrumentation

A JEOL JMS-D300 mass spectrometer equipped with a JMA 2000 data processing system was used. A 1 m × 2 mm I.D. silanized glass column packed with 2% OV-17 coated on 80—100 mesh Gas-Chrom Q was used. The column and the injection port were maintained at 230°C and 250°C, respectively. Helium was used as a carrier gas at a flow-rate of 90 ml/min. SIM chromatograms were obtained by monitoring the molecular ions at *m/z* 706 for DHD diHFB and *m/z* 680 for testosterone diHFB at an ionizing energy of 70 eV.

Calculation

The concentration of DHD in plasma samples was determined from a calibration curve obtained using the peak area ratio of the peak at *m/z* 706 against that at *m/z* 680. The standard samples were prepared by adding DHD stock solution in a concentration of 0—140 ng/ml and IS solution (corresponding to 200 ng of testosterone) to 1 ml of blank plasma, and then the calibration curve was obtained from the standard samples treated in the same manner as the plasma samples. The concentration of DHD glucuronide in plasma samples was determined from the difference between the concentration of DHD plus DHD glucuronide and that of DHD alone.

RESULTS AND DISCUSSION

Derivatization, mass spectra and gas chromatographic behaviour

The mass spectrum of underivatized DHD gave intensive peaks at *m/z* 161 and *m/z* 296 as shown in Table I. But the attempt to use these ions for SIM failed in sensitive detection because DHD gave the broad gas chromatographic (GC) peak caused by adsorption on the GC column, and many fragment peaks due to thermal decomposition.

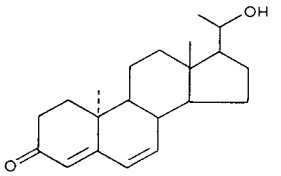
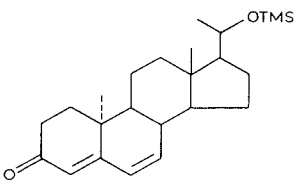
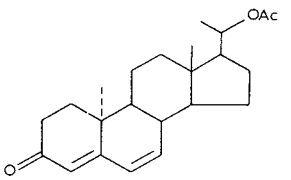
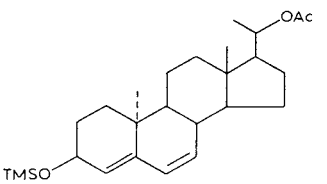
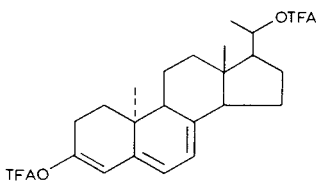
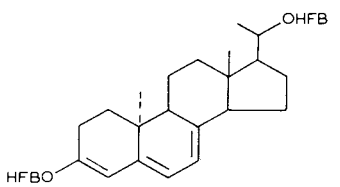
We examined the various derivatives of DHD favourable for SIM measurement, such as 20-trimethylsilyl (TMS), 20-acetyl (Ac), 3-TMS-20-Ac, ditrifluoroacetyl (diTFA) and diheptafluorobutyryl (diHFB) derivatives. The major intense peaks characteristic of the mass spectra of these derivatives are shown in Table I.

It seemed to be convenient for the SIM analysis to choose the DHD derivatives giving intense ions at a higher mass range where little interference from

TABLE I

CHARACTERISTIC PEAKS FOR MASS SPECTRA OF DHD DERIVATIVES

The values in parentheses represent the relative intensity (%) and the underlines indicate the base peaks of respective spectra.

Derivatives	Peaks observed at 70 eV
DHD 	314 (2, M^{+}), 296 (88) 281 (44), 161 (100)
DHD 20-TMS 	386 (1, M^{+}), 371 (2) 296 (3), 271 (3), 117 (100)
DHD 20-Ac 	356 (1, M^{+}), 296 (45), 281 (14), 161 (100)
DHD 3-TMS-20-Ac 	430 (63, M^{+}), 415 (6), 370 (8), 340 (11), 75 (100)
DHD diTFA 	506 (12, M^{+}), 491 (2) 392 (20), 377 (10), 79 (100)
DHD diHFB 	706 (63, M^{+}), 691 (12) 370 (100), 357 (93), 355 (56)

plasma components was observed. Among the derivatives examined, DHD 3-TMS-20-Ac and DHD diHFB derivatives have intense peaks at a higher mass range such as the peak at *m/z* 430 for the former and that at *m/z* 706 for the

latter derivative. In addition to these mass spectral features, both the derivatives exhibited sharp and symmetric GC peaks. Of the two derivatives, we chose the DHD diHFB derivative in view of the simplicity of derivatization. The mass spectrum of DHD diHFB is shown in Fig. 2a.

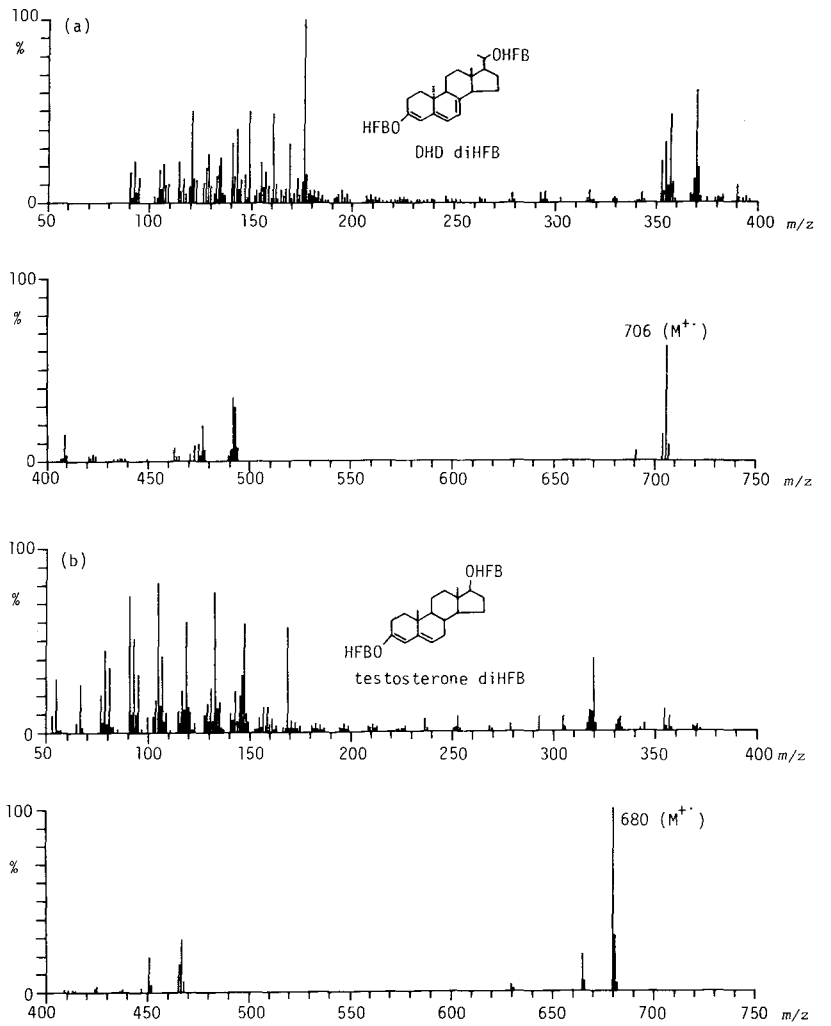


Fig. 2. Mass spectra of (a) DHD diHFB and (b) testosterone diHFB.

Purification of plasma samples

In order to purify DHD from the plasma components, organic solvent extraction using *n*-hexane, diethyl ether and *n*-heptane was examined. Among these solvents, *n*-hexane was found to be appropriate in view of the removal of the plasma components and higher recovery of DHD (about 90%). But the purification could not be achieved by *n*-hexane extraction alone. It was necessary to make a further purification for the more sensitive measurement of DHD in

plasma by the SIM method. Therefore the column chromatographic purification of *n*-hexane extracts was examined.

Consequently MgO column chromatography, which was reported to be effective for the purification of steroid hormones [4], was found to be very effective for the purification of DHD in plasma. This purification method was simple and rapid and was readily applicable to routine analysis of the plasma samples. On the other hand, silica gel column chromatography was inadequate to separate DHD from the plasma components. Use of Ameberlite XAD-2 and Sephadex LH-20 column chromatography was tedious for the treatment of many samples.

In the MgO column chromatographic method, nonpolar components in plasma were eluted in the isooctane and isooctane—ethyl acetate (20:1) fractions and DHD was eluted in the isooctane—ethyl acetate (3:1) fraction.

Choice of internal standard

As recognized in general, DHD labelled with a stable isotope seemed to be the most suitable IS for SIM analysis. Therefore we tried the synthesis of deuterium-labelled DHD by several methods, but these attempts failed because of the long synthetic route. The isotopic purity of deuterated DHD synthesized was too poor to use for an IS. So we examined the use of testosterone, methyltestosterone and progesterone because of their structural resemblance to DHD. Among these compounds, testosterone was suitable for the IS from both its chromatographic and mass spectral behaviour. Testosterone showed a similar behaviour to DHD during the clean-up of plasma samples. The retention time of testosterone diHFB in GC was close to that of DHD diHFB and the mass spectrum of testosterone diHFB gave the base peak at m/z 680 (M^+) as shown in Fig. 2b. It has been reported that testosterone is an endogenous steroid presenting at the maximum plasma level of a few nanograms per ml in male subjects [5]. But the effect of trace amounts of endogenous testosterone was considered to be not important for this SIM method, because testosterone was used as IS in higher amounts such as 200 ng/ml of plasma.

SIM chromatograms, calibration curve and precision

The SIM chromatograms of DHD in plasma are shown in Fig. 3. DHD diHFB and testosterone diHFB exhibit retention times of about 2 min and 1 min, respectively, and there is no interference from plasma components.

Known amounts of DHD (0—140 ng) were added to plasma containing a fixed amount (200 ng) of IS and these samples were analyzed by the devised SIM method. The calibration curve obtained by the peak area method showed good linearity ($r \geqslant 0.993$), and the determination limit seemed to be 5 ng of DHD per ml of plasma. The reproducibility of this method is shown in Table II. DHD in plasma could be determined within ± 6% of the coefficient of variation (C.V.) at the concentration of 30 ng DHD per ml plasma or above, although the C.V. at the concentration of 5 ng DHD per ml plasma was about ± 17%. It was considered that such sensitivity and precision would be acceptable for the pharmacokinetic studies of DHD and DHD glucuronide in plasma.

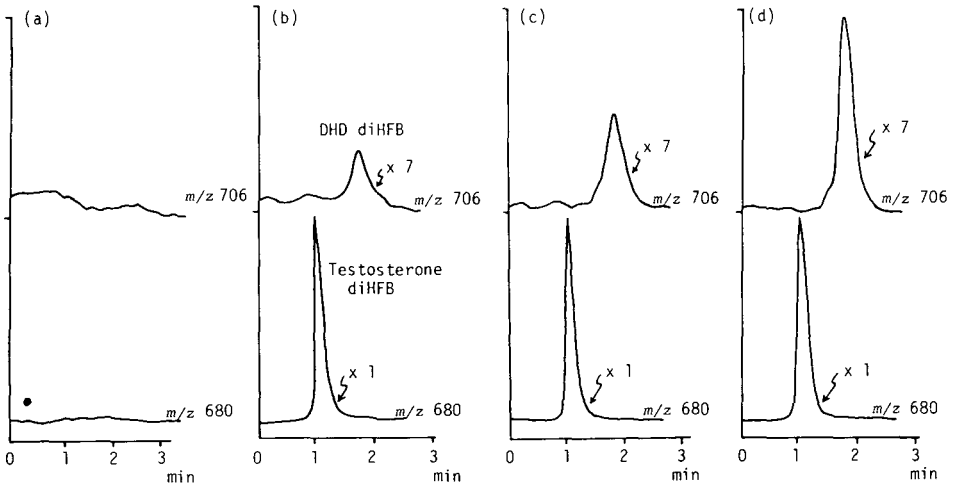


Fig. 3. Selected ion monitoring chromatograms of (a) blank plasma and standard plasma samples, (b) 20 ng DHD per ml of plasma, (c) 40 ng DHD per ml of plasma, and (d) 75 ng DHD per ml of plasma.

TABLE II

REPRODUCIBILITY OF THE METHOD

DHD concentration (ng/ml plasma)	DHD found (ng/ml, mean ± σ_n, $n = 7$)	C.V. (%)
5.0	4.1 ± 0.7	17.1
35.0	35.6 ± 2.1	6.35
110.0	112.4 ± 4.9	4.74

Application of the SIM method

The SIM method was applied to the determination of plasma levels of DHD and DHD glucuronide in five healthy male subjects who each received 10 mg of dydrogesterone in two tablets. Fig. 4 shows the time course of the concentrations of DHD and DHD glucuronide. It is apparent that dydrogesterone is rapidly absorbed into the blood and is readily metabolized to DHD and DHD glucuronide. There were some individual differences; however, the plasma concentrations of DHD and DHD glucuronide reached the maximum levels at 1 h after the single oral administration of 10 mg of dydrogesterone. In addition, the elimination process of DHD and DHD glucuronide could be interpreted in terms of a one-compartment model with elimination half-lives of 1.9 h for the former and 1.5 h for the latter compound. The elimination rate constants k_{el} were calculated by computer fitting as 0.39 h^{-1} and 0.48 h^{-1}, respectively.

This SIM method has been extensively and conveniently utilized for the routine analysis of DHD and DHD glucuronide in plasma.

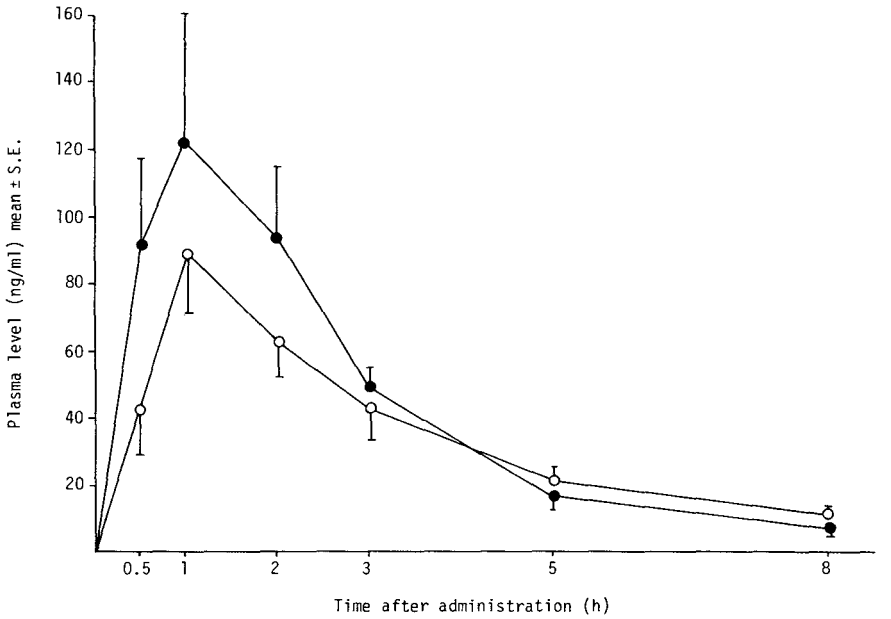


Fig. 4. Time course of DHD (○) and DHD glucuronide (●) in five healthy male subjects after the oral administration of 10 mg of dydrogesterone in the form of commercial tablets.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE FATTY ACIDS WITH 1-NAPHTHYLAMINE

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SUMMARY

A 1% benzene solution of oxalyl chloride was added to saturated and unsaturated fatty acids and the mixture was allowed to react at 70°C for 30 min; by this procedure, each fatty acid was converted into its acid chloride in a considerably quantitative manner. By reacting this acid chloride with 1-naphthylamine at 30°C for 15 min, naphtylamine derivatives were produced, which showed strong ultraviolet absorption around 280—290 nm. Experiments were made on the recovery of the fatty acids added to 0.5 ml of human serum, and the recovery was found to fall in the range of 94—106% (coefficient of variation = 0.5—4.1%) when the following amounts of six fatty acids were added: $C_{14:0}$, 2 μg; $C_{16:0}$, 20 μg; $C_{16:1}$, 5 μg; $C_{18:0}$, 4 μg; $C_{18:1}$, 20 μg; $C_{18:2}$, 10 μg.

INTRODUCTION

In the quantitative analysis of fatty acids by high-performance liquid chromatography (HPLC), UV- and fluorescence-labeling are widely utilized to increase the detection sensitivity of fatty acids. The main derivatizing reagents reported so far are phenacyl bromide [1], 2-naphthacyl bromide [2], *p*-bromophenacyl bromide [3], 1-benzyl-2-tolyltriazene [4], and O-*p*-nitrobenzyl-N,N'-diisopropyl-isourea [5]. Many of the labeling reagents for carboxylic acids reported in the past have been developed mainly for the purpose of direct reaction with carboxylic acids. Therefore, there was a considerable limitation to the types applicable as labeling reagent.

Generally, when synthesizing the amides or esters of carboxylic acids, they are reacted with amines or alcohols after they have been derivatized to acid chorides. We attempted to apply this method to the labeling of free fatty acids (FFA). Thus, instead of producing derivatives from FFA in the free form, by changing the FFA into their acid chlorides it is easy to introduce the amines

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with UV or fluorescence properties into the FFA, thus facilitating the micro-detection of FFA. In our previous report [6], we examined the reaction conditions to make the acid chlorides by reacting thionyl chloride ($SOCl_2$) with saturated fatty acids, and binding 1-naphthylamine (NA) containing a primary amine structure to the acid chlorides. As a result, it was found that saturated fatty acids were converted into their acid chlorides by $SOCl_2$, and that the acid chlorides reacted easily with NA, forming NA derivatives exhibiting strong UV absorption around 280 nm. On the basis of these results, we performed the present study in an attempt to establish a more accurate method for the quantitative analysis of FFA, which also involves a labeling method applicable to unsaturated FFA.

EXPERIMENTAL

Reagents

Myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$, PT), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), linoleic acid ($C_{18:2}$, Ll), were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Oleic acid ($C_{18:1}$) and palmitoyl chloride (PT · Cl) were purchased from Sigma (St. Louis, MO, U.S.A.). Thionyl chloride ($SOCl_2$) and margaric acid ($C_{17:0}$) were from Nakarai Chemicals (Kyoto, Japan). Linoleoyl chloride (Ll · Cl), chrysene, 1-naphthylamine (NA) and triethylamine (TEA) were from Tokyo Kasei Kogyo (Tokyo, Japan). Oxalyl chloride, $(COCl)_2$, was purchased from Wako Pure Chemical (Osaka, Japan).

Apparatus

A Hitachi high-performance liquid chromatograph Model 635A equipped with a Hitachi multiwavelength UV monitor was used. Melting point was determined with the Yanagimoto micro melting point apparatus. For measuring the infrared (IR) spectra, a Hitachi grating IR spectrometer 215 was used. ^{1}H-NMR spectra were determined on a JEOL Fx-200 NMR spectrometer with using tetramethylsilane (TMS) as an internal standard. UV spectra and mass spectra were measured with a Shimadzu UV-210A and a Hitachi RMU-7MG, respectively.

HPLC conditions

Column: μBondapak C_{18} (30 × 0.4 cm I.D., particle size 8—10 μm). Detection wavelength: 280 nm. Mobile phase: methanol—water (81:19). Flow-rate: 2.0 ml/min. Column temperature: 40° C.

Preparation of N-linoleoyl-1-naphthylamine (Ll · NA)

Ll · Cl (1 mmole), NA (1 mmole) and TEA (1 mmole) were dissolved in benzene (10 ml) in a reaction vial (15 ml), and were allowed to react with stirring for 30 min in an oil bath at 50° C. The solvent was removed at reduced pressure, and the residue was recrystallized from methanol–water to give Ll · NA: m.p. 41—42° C. IR $\nu_{max}^{CHCl_3}$ cm^{-1} : 1680 (—NHCO). NMR (C^2HCl_3) δ : 0.89 (3H, t, J = 7 Hz, —CH_2CH_3), 1.0—1.56 (16H, m, —$(CH_2)_5$— and —$(CH_2)_3$—), 1.9—2.14 (4H, m, 2 × (—CH_2—C=)), 2.51 (2H, t, J = 7 Hz, —CH_2—CO—), 2.77 (2H, t, J = 5 Hz, =C—CH_2—C=), 5.19—5.48 (4H, m, 2 ×

(—CH=CH—)), 7.4—8.13 (7H, m, aromatic protons). Mass spectrum *m/z*: 405 (M^+).

Preparation of NA solution

NA (57.3 mg, 400 μmoles) was dissolved in benzene to give a total of 10 ml. The prepared solution was kept shielded from the light.

Preparation of TEA solution

TEA (404 mg, 4 mmoles) was dissolved in benzene to give a total of 10 ml.

Method for the quantitative analysis of PT · Cl and Ll · Cl

PT · Cl (4—200 nmoles) or Ll · Cl (14—200 nmoles) dissolved in 0.1 ml of benzene was put into reaction vials to which 0.1 ml of NA solution and 0.01 ml of TEA solution were added; the reaction was carried out at 30°C for 15 min. Then 0.1 ml of benzene solution containing chrysene (50 μg) as an internal standard was added, and the mixture was shaken sufficiently: 2 μl were injected into the chromatograph. On the chromatogram obtained, the ratio of the peak height for the internal standard to that for each acid chloride was measured, and the amount of PT · Cl or Ll · Cl was calculated using the regression equation made in advance.

Method for making the NA derivatives of FFA

FFA (2—1000 nmoles) dissolved in 0.6 ml of benzene was put into a reaction vial and shaken sufficiently after adding 0.6 ml of 2% $(COCl)_2$ solution in benzene. The reaction was carried out for 30 min in an oil bath at 70°C. After the reaction, the solvent was removed at reduced pressure. NA solution (0.1 ml) and TEA solution (0.01 ml) were added; the mixture was reacted at 30°C for 15 min to give the NA derivatives.

Methods for the extraction and the quantitative analysis of serum FFA

For the extraction of FFA from serum, we adopted the column extraction method which we had examined previously [7]. To 0.5 ml of serum was added 0.1 ml of methanol solution containing 10 μg of margaric acid as an internal standard, and then mixed with 1.4 ml of 1/15 *M* phosphate buffer (pH 7.0). After shaking sufficiently, the mixture was poured into a glass column (45 × 12 mm I.D.) packed with 1 g of Extrelut®. After adsorbing for 20 min, FFA was eluted out with 10 ml of chloroform. After removing the solvent at reduced pressure, the residue was redissolved in 0.6 ml of benzene. The reaction was carried out according to the derivatizing method and then 40 μl of the reaction mixture were injected directly into the HPLC apparatus. From the chromatogram obtained, the ratio of the peak height for each FFA to that fo the internal standard was measured, and the amount of each FFA was calculated from the calibration curves previously made for each FFA.

Experiments on the recovery of the FFA added to human serum

Into 0.5 ml of the serum separated immediately after blood sampling were added 0.1 ml of methanol solution containing the internal standard, margaric acid ($C_{17:0}$) 10 μg, and FFA of the following composition: $C_{14:0}$ 2 μg, $C_{16:0}$

20 μg, $C_{16:1}$ 5 μg, $C_{18:0}$ 4 μg, $C_{18:1}$ 20 μg, $C_{18:2}$ 10 μg. Separation and assay of FFA were performed according to the methods described above. From the values obtained, the amount of each FFA contained in the untreated fresh serum was subtracted, which was divided by the added amount to give the recovery ratio.

RESULTS AND DISCUSSION

Introduction of NA into FFA

As a result of the determination of the structure of the products of the reaction of NA with Ll · Cl, the acid chloride of Ll, it was confirmed that the structure was N-linoleoyl-1-naphthylamine (Ll · NA), which was formed by the binding of NA to the carboxyl group of Ll. In addition, UV spectra were determined using the methanol solution of Ll · NA obtained herein; as a result, as in the case of PT · NA shown in the previous report [6], strong absorption was found around 280—290 nm, which was not observed with NA (Fig. 1). These results indicate that NA which contains a primary amine can easily be introduced into saturated and unsaturated fatty acids by derivatizing them into acid chlorides. Therefore, the optimal condition when introducing NA into FFA was examined. The derivatizing method performed in the present study consisted of two reactions; derivatization of FFA into acid chlorides, and reaction of acid chlorides with NA. Thus, these two methods were examined separately.

(1) Derivatization of FFA into acid chlorides. Since the condition for the reaction to make acid chlorides of the saturated fatty acids was already examined in the previous report [6], the examination was made on the unsaturated fatty acids in the presents study. Ll was selected as the unsaturated

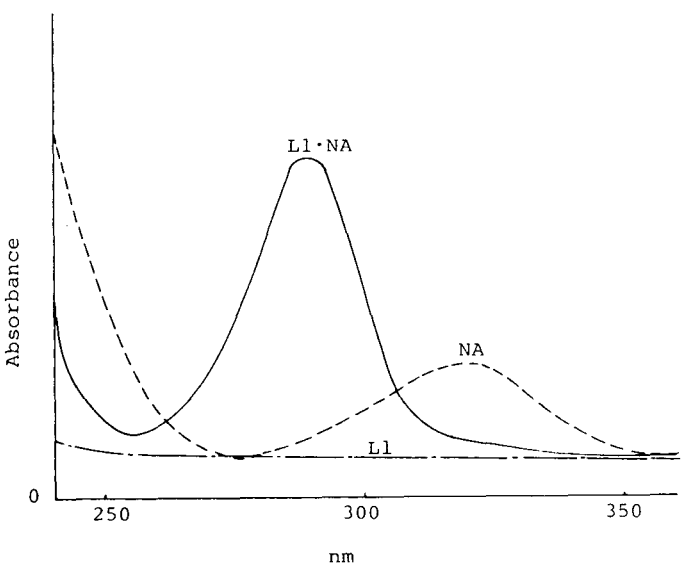

Fig. 1. Absorption spectra of Ll · NA and NA in methanol solution. Ll · NA = N-linoleoyl-1-naphthylamine, NA = naphthylamine, Ll = linoleic acid.

fatty acid, and the reaction was carried out utilizing a benzene solution of 50% $SOCl_2$ [8, 9] as the derivatizing reagent. The Ll · Cl produced was transformed to its NA derivative, and measured according to the method described in the Experimental section. As shown in Fig. 2a, the reaction ratio from Ll to Ll · Cl was low (about 17%), and several peaks other than that corresponding to Ll · Cl were observed. In the case of saturated fatty acids, their acid chlorides were produced by reaction with $SOCl_2$ at the yield of almost 100%. Therefore, in contrast to saturated fatty acids, it was found that the reaction ratio from unsaturated fatty acids to their acid chlorides was low when using $SOCl_2$. Thus, in the next step, $(COCl)_2$ was investigated as the derivatizing reagent [10]. Reaction was carried out by adding a 50% benzene solution of $(COCl)_2$ to Ll. The yield of Ll · Cl was high (about 88%) and side-products were not formed (Fig. 2b). From these results, it was decided to use $(COCl)_2$ for derivatizing FFA, including unsaturated fatty acids, into their acid chlorides, and the optimal concentration of $(COCl)_2$ was therefore examined. $(COCl)_2$ was adjusted at six different concentrations with benzene: 50, 10, 5, 1, 0.5 and 0.1%, and each solution was reacted with PT or Ll. The acid chlorides produced were changed into NA derivatives and the measurements made; the amounts of acid chlorides were calculated on the basis of NA derivatives. The yield of the acid chlorides increased with increasing concentration of $(COCl)_2$ in the case of both PT and Ll. The maximum yields were obtained using 1% $(COCl)_2$ (PT, 100%; Ll, 92%); there was little change in the yield of acid chloride if the concentration of $(COCl)_2$ was further increased (Fig. 3). Therefore, the concentration of $(COCl)_2$ for derivatizing FFA into their acid chlorides was set at 1%.

In the next step, we examined the optimal reaction temperature and reaction time when leading FFA into their acid chlorides with using 1% $(COCl)_2$. PT and

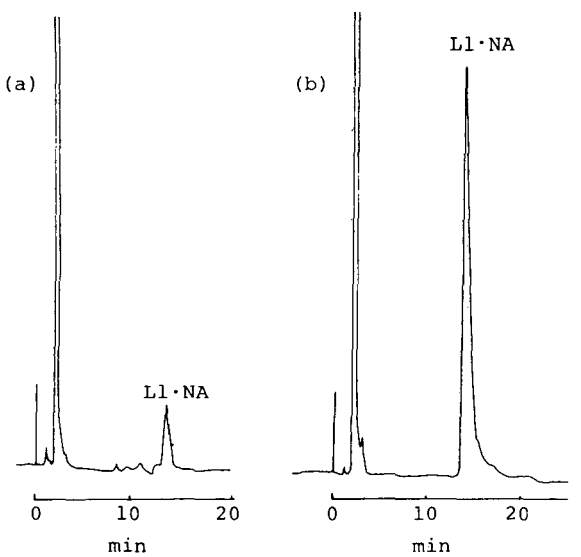

Fig. 2. Comparison of reagents to prepare Ll · Cl from Ll. (a) Ll (0.78 μmole) was reacted with 50% $SOCl_2$ in benzene; (b) Ll (0.78 μmole) was reacted with 50% $(COCl)_2$ in benzene. Ll · Cl produced was changed into the NA derivative for determination by HPLC.

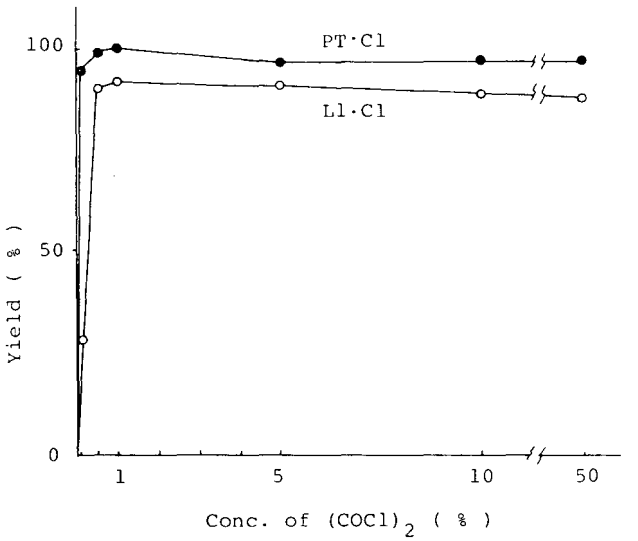

Fig. 3. Effect of concentration of $(COCl)_2$ on production of PT · Cl and Ll · Cl. Each 0.78 μmole of PT and Ll was dissolved in 0.2 ml of six different concentrations of $(COCl)_2$ solution. The mixture was heated at 70°C for 30 min. Each PT · Cl and Ll · Cl thus produced was changed into NA derivatives.

Ll were selected as the saturated and unsaturated fatty acids, respectively, and they were reacted in a 1% benzene solution of $(COCl)_2$ with varying temperatures and reaction times. The production of PT · Cl from PT increased with increasing reaction temperature: PT · Cl was produced at a yield of about 100% after the reaction at 70°C for 30 min. Further increase in temperature up to 90°C resulted in a decrease in the production of acid chlorides compared to the value at 70°C. Moreover, when the reaction was carried out at 90°C, the amount of acid chloride produced varied considerably depending on the reaction time. With respect to Ll, the production of Ll · Cl increased with increasing reaction temperature, and a maximum yield of 92% was obtained at 70°C. Thus, the yield of the reaction from Ll to its acid chloride was found to be low compared to PT. From these results, the temperature and time of reaction for converting FFA into acid chlorides were fixed at 70°C and 30 min, respectively.

(2) Reaction of acid chlorides with NA. With respect to the condition for the reaction of PT · Cl with NA, as described in the previous report [6], PT · NA was confirmed to be produced at a yield close to 100% after reaction at 30°C for 15 min. In the present study, the reaction with NA and Ll · Cl was examined. To the benzene solution of Ll · Cl was added the NA solution, and TEA solution to neutralize a side-product, HCl. The amount of Ll · NA produced was compared by varying the reaction time and temperature. The amounts of Ll · NA were calculated from the calibration curve which was constructed using the derivative prepared according to the method described in the experimental section as a standard and using chrysene as an internal standard. It was found that changes in reaction temperature and time hardly affected the amount of NA derivative produced, and that Ll · NA was obtained at a yield of almost 100% even at a temperature as low as 30°C and a short

reaction time (15 min). These results coincide with those for the reaction conditions for PT · Cl with NA; thus, the conditions for the reaction of acid chlorides with NA were decided to be 30°C and 15 min.

HPLC of NA derivatives of FFA

Seven NA derivatives were produced of the main FFA contained in human serum — $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ — and an internal standard, $C_{17:0}$. Examinations on the separation of these derivatives by HPLC indicated that good separation was obtained when using methanol—water (81:19) for the mobile phase and operating at the flow-rate of 2.0 ml/min (Fig. 4). Before applying the present derivatizing method to the quantitative analysis of human serum FFA with HPLC, calibration curves were made from the chromatogram obtained by injecting the following amounts of FFA for HPLC: $C_{14:0}$, 0.73—2.91 μg; $C_{16:0}$, 3.64—14.56 μg; $C_{16:1}$, 0.73—2.91 μg; $C_{18:0}$, 0.73—2.91 μg; $C_{18:1}$, 3.64—14.56 μg; $C_{18:2}$, 1.46—5.28 μg. The regression lines between the amount injected into the column (X) and the peak height ratio (Y) to the internal standard ($C_{17:0}$, 3.64 μg) were: $C_{14:0}$, $Y = 0.788X - 0.080$ ($R = 0.998$); $C_{16:0}$, $Y = 0.382X + 0.125$ ($R = 0.999$); $C_{16:1}$, $Y = 0.589X + 0.05$ ($R = 0.999$); $C_{18:0}$, $Y = 0.192X + 0.04$ ($R = 0.998$); $C_{18:1}$, $Y = 0.320X + 0.115$ ($R = 0.999$): $C_{18:2}$, $Y = 0.421X + 0.130$ ($R = 0.999$). The detection limit for $C_{16:0}$ was 4 ng, assuming a signal-to-noise ratio of 3.

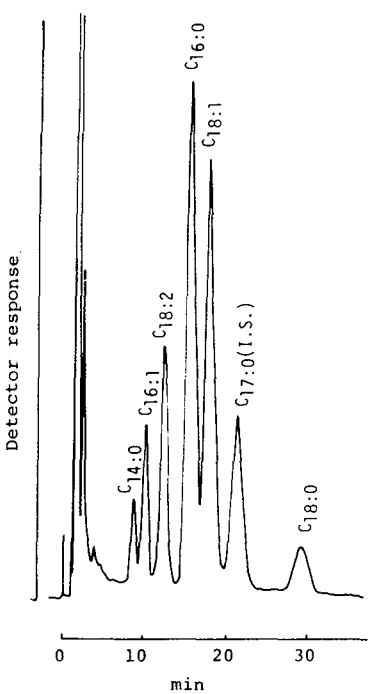

Fig. 4. HPLC of some NA-derivatized FFA. Column: μBondapak C_{18}. Mobile phase: methanol—water (81:19). Flow-rate: 2.0 ml/min. Detector: 280 nm. Temperature: 40°C.

Amount of human serum FFA determined by the present method

Before determining the amount of human serum FFA with the present derivatizing method, we performed the recovery experiments of FFA added to human serum. Six FFA were selected, which are the main FFA contained in human serum. The amount of each FFA added was close to that naturally contained in 0.5 ml of human serum. The percentage recovery of each FFA was calculated, and was found to fall in the range 94—106% for all six FFA (coefficient of variation = 0.5—4.1%, n = 4). Thus, it was confirmed that the present derivatizing method can be applied to the quantitative analysis of serum FFA (Table I).

TABLE I

ANALYTICAL RECOVERY OF FATTY ACIDS ADDED TO HEALTHY HUMAN SERUM

Fatty acid	Added* (μg)	Found** (μg)	Recovery** (%)	C.V. (%)
$C_{14:0}$	2.0	2.0 ± 0.1	100.0 ± 4.1	4.1
$C_{16:0}$	20.0	21.0 ± 0.3	105.0 ± 1.4	1.3
$C_{16:1}$	5.0	4.9 ± 0.1	98.0 ± 1.6	1.6
$C_{18:0}$	4.0	3.8 ± 0.1	94.0 ± 3.4	3.6
$C_{18:1}$	20.0	21.2 ± 0.3	105.8 ± 1.5	1.4
$C_{18:2}$	10.0	10.3 ± 0.1	103.3 ± 0.5	0.5

*Each FFA was added to 0.5 ml of serum.
**Mean ± S.D., n = 4.

In the next step, blood was collected from five volunteers (adult, either sex), and the serum was immediately separated. The amount of FFA in 0.5 ml of each serum was determined with the present method (Table II). Values obtained were in good agreement with the amount of each FFA in normal human serum reported previously [11, 12]. Moreover, the amount of fatty acid was repeatedly determined in a particular person, and the amount of each fatty acid can be determined accurately with a coefficient of variation of 1.4—5.9%, as shown in Table III. Especially, when compared with the results of repeated determinations of serum FFA with gas chromatography as reported by Haan et al. [13], $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ were found to be determined more accurately with the present method.

TABLE II

FFA CONCENTRATION IN HEALTHY HUMAN SERUM DETERMINED BY HPLC

Volunteer	FFA (μg/ml)					
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
M.N.	3.4	18.8	2.0	3.0	21.0	18.4
Y.I.	3.2	16.4	1.4	2.8	12.4	7.4
K.S.	4.0	21.0	2.2	2.2	25.4	22.2
M.S.	1.8	7.8	1.2	0.8	8.2	5.2
M.I.	4.0	19.6	2.4	6.4	27.6	24.6

TABLE III

REPRODUCIBILITY OF FOUR ANALYSES OF THE SAME HEALTHY HUMAN SERUM

	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Mean (μg per 0.5 ml)	1.7	8.5	1.4	1.8	9.2	7.4
S.D.	0.1	0.4	0.1	0.1	0.3	0.1
C.V. (%)	5.9	4.7	7.1	5.6	3.3	1.4

From the experimental results described, the following conclusion can be drawn. In the present derivatizing method, simply by converting fatty acids into their acid chlorides, the primary amine which has rarely been utilized as the UV- or fluorescence-labeling reagent in the past was found to be applicable to the determination of fatty acids. Thus, the present method is considered to be widely applicable as a labeling method for fatty acids because there are many reagents containing primary amines which exhibit UV absorption or fluorescence. Moreover, quantitative analysis of FFA in healthy human serum was performed with the present method, and it was confirmed that the method can be applied to the clinical fields.

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CHROMBIO. 1503

IMMOBILIZED 3α-HYDROXYSTEROID DEHYDROGENASE AND DANSYL HYDRAZINE AS A PRE-LABELING REAGENT FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION OF BILE ACIDS

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SUMMARY

A high-performance liquid chromatographic method with fluorescence detection is described for the determination of bile acids and their conjugates. After enzymatic conversion of bile acids to 3-oxo-bile acids using the immobilized 3α-hydroxysteroid dehydrogenase reactor column, 3-oxo-bile acids were extracted with a Sep-Pak C_{18} cartridge, labelled with dansyl hydrazine and then separated by high-performance liquid chromatography on a reversed-phase column. The eluate is monitored by a fluorophotometer at 365 nm (excitation) and 520 nm (emission). Reactions proceed quickly under mild conditions to give fluorescent derivatives. Linearity of the fluorescence intensity (peak height) with the amounts of various bile acids and their conjugates was obtained above 0.5—1.0 pmol. The method is sensitive, reliable and useful for the simultaneous determination of bile acids in biological samples.

INTRODUCTION

The identification and determination of various bile acids may be of value in the diagnosis of liver disease. Many methods have been reported for the simultaneous determination of individual unconjugated and conjugated bile acids, including thin-layer chromatography [1, 2], gas—liquid chromatography [3] and gas chromatography—mass spectrometry [4, 5]. Recently, high-performance liquid chromatographic (HPLC) methods [6—9] have been developed, combining the advantages of mild separation conditions. However, the sensitivity of HPLC is low due to the use of a refractive index or UV detector, because most common bile acids have no strong UV-absorbing groups in their molecules. Therefore, bile acids have been derivatized before column separation

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by UV-absorbing reagents [10—13] and fluorescent derivatizing reagents [14, 15] in order to improve their detectability. However, taurine-conjugated bile acids could not be determined using these reagents because they react only with carboxyl groups. Baba et al. [16] reported a highly sensitive and selective fluorescence HPLC method for bile acids using 3α-hydroxysteroid dehydrogenase (3α-HSD) and cofactor (NAD^+). Recently, modified methods have been developed using an immobilized enzyme reactor column instead of enzyme solution by Okuyama et al. [17] and Arisue et al. [18] with a fluorescence detector and by Kamada et al. [19] with an electrochemical detector.

Dansyl hydrazine is a favorable reagent for the formation of hydrazones because of its high reactivity under mild conditions. We used it as a fluorescent labeling reagent in the HPLC determination of various oxosteroids [20—22]. In this paper, we have attempted to develop a new fluorescence HPLC method for the determination of bile acids and their conjugates using immobilized 3α-HSD reactor and dansyl hydrazine as a pre-labeling reagent.

EXPERIMENTAL

Reagents and materials

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA) and their glycine (G) and taurine (T) conjugates were obtained from Sigma or PL Biochemical Co. β-Nicotinamide-adenine dinucleotide (NAD^+, Grade I) was from Boehringer Mannheim-Yamanouchi Co. 3α-Hydroxysteroid dehydrogenase (Grade II) and dansyl hydrazine (Grade II) were from Sigma. Amino glass beads used as the solid phase of immobilized enzyme were Amino Propyl-CPG 180 Å of Electro-Nucleonics and the Sep-Pak C_{18} cartridge was from Waters. All other chemicals were obtained commercially.

Bile acid stock solution: each bile acid was dissolved in methanol and made up to 10 μmol/ml.

NAD^+ solution: 0.5 m*M* NAD^+ solution was prepared by dissolving NAD^+ in 0.1 *M* pyrophosphate buffer (pH 9.0).

Immobilized 3α-HSD reactor column: 3α-HSD was coupled to amino glass beads (120—200 mesh) by the glutaraldehyde method [23] and packed in a glass syringe (35 × 6 mm I.D.).

Dansyl hydrazine solution: a 0.2% (w/v) solution was prepared by dissolving 20 mg of dansyl hydrazine in 10 ml of benzene; it was stored in a refrigerator until use.

Trichloroacetic acid (TCA) was in benzene solution (0.1%, w/v).

Instrumentation

We used a Hitachi Model 635 high-performance liquid chromatograph equipped with a Kyowa Seimitsu KHP-UI-130 injection valve and a Jasco Model FP-110 fluorescence spectrophotometer. A Radial-Pak A column (5 μm, Waters), a μBondapak Phenyl column (10 μm, 300 × 6 mm, Waters) and a Zorbax ODS column (5—6 μm, 250 × 4.6 mm, DuPont) were used. The detector wavelengths were 365 nm for excitation and 520 nm for emission.

Analytical procedure

The bile acids were dissolved by adding 100 μl of methanol, transferred onto the 3α-HSD reactor column and eluted from the column with 2 ml of NAD^+ solution at a flow-rate of 0.5 ml/min. To the eluent were added 2 ml of 0.5 *M* phosphate buffer (pH 6.0), the solution was then poured into the syringe attached to the Sep-Pak C_{18} cartridge. The cartridge was washed with 4 ml of water, followed by elution with 2 ml of methanol and the methanol eluent was evaporated to dryness under reduced pressure. The residue was dissolved by adding 0.2 ml of TCA—benzene solution, left to stand for 10 min at 30°C and evaporated to dryness under a stream of nitrogen gas. The labelled residue was dissolved in 500 μl of methanol and an aliquot of the solution was injected into the chromatograph.

Extraction of bile acids from serum

A 0.1-ml serum sample was mixed with 0.5 ml of methanol and ultrasonicated for 15 min. Then 0.3 ml of the supernatant was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 1.0 ml of 0.05 *M* phosphate buffer (pH 7.0) and applied onto a Sep-Pak C_{18} cartridge, and washed with 2 ml of 2% methanol. Bile acids were eluted with 4 ml of 80% methanol and the eluent was then evaporated under reduced pressure at 40°C.

Fractionation of bile acids

The fractionation of three different groups (unconjugated, glycine-conjugated and taurine-conjugated) was carried out using a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) column (13 × 7.5 mm I.D.) as reported by Goto et al. [24]. The resultant residue of each fraction was assayed by the analytical procedure.

Preparation of 3-oxo-bile acids and dansyl hydrazones of bile acids

3-Oxo-bile acids were prepared from bile acids by selective oxidation of the hydroxyl group at carbon-3 using silver carbonate adsorbed on Celite as a mild oxidizing reagent according to the descriptions of Fetizon and Golfier [25] and Dayal et al. [26]. For example, cholic acid was oxidized to 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid. (Anal. calc. for $C_{24}H_{38}O_5$: C, 70.90; H, 9.42. Found: C, 70.58, H, 9.48.)

Dansyl hydrazones of bile acids were synthesized from 3-oxo-bile acids and dansyl hydrazine in TCA—benzene solution under the same conditions as described in the analytical procedure.

Calculation

The enzymatic conversion ratio from bile acids to 3-oxo-bile acids with immobilized 3α-HSD column, the recoveries of 3-oxo-bile acids from Sep-Pak C_{18} cartridge extraction and the yields of dansyl hydrazones of 3-oxo-bile acids were calculated as follows: percentage enzymatic conversion = $H_1/H_2 \times 100$, percentage Sep-Pak extraction = $H_2/H_3 \times 100$, percentage dansylation yield = $H_3/H_4 \times 100$, where H_1 = peak height of bile acid in chromatogram obtained according to the analytical procedure, H_2 = peak height of 3-oxo-bile

acid obtained by the analytical procedure without the enzymatic reaction step, H_3 = peak height of 3-oxo-bile acid in chromatogram obtained by dansylation step, H_4 = peak height of synthesized dansyl hydrazone of 3-oxo-bile acid.

Recovery test

A synthetic mixture of 1.0 nmol of each bile acid was added to 0.1 ml of normal human serum and then assayed by the present method. Recoveries were calculated against a pure standard bile acid mixture carried through the procedure.

RESULTS AND DISCUSSION

The assay procedure is illustrated schematically in Fig. 1. The following parameters were examined in order to obtain the optimum conditions for the assay procedure such as enzymatic reaction, extraction of 3-oxo-bile acids and labelling reaction.

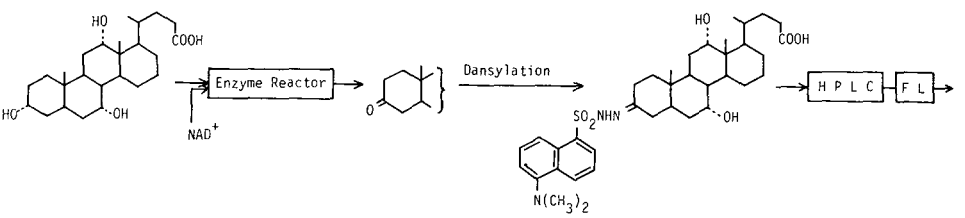

Fig. 1. Flow diagram of the fluorescence HPLC method. Conditions: enzyme reactor, immobilized 3α-HSD (35 × 6 mm I.D.); 0.5 m*M* NAD^+ in 0.1 *M* pyrophosphate buffer (pH 9.0); fluorophotometer (excitation 365 nm, emission 520 nm).

The first step of this method is the enzymatic oxidation of bile acids to 3-oxo-bile acids with immobilized 3α-HSD reactor column. Fig. 2 shows the effects of type and pH of buffer solution in enzymatic reaction on the fluorescence intensity (peak height in chromatogram). The pyrophosphate buffer (pH 9.0) gave the highest peaks and the peak height increased with increasing concentration of pyrophosphate up to 0.1 *M*, it reached a constant

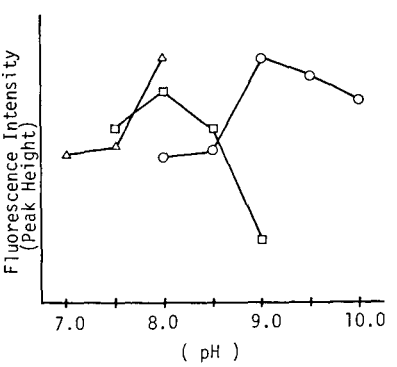

Fig. 2. Effects of type and pH of buffer in enzymatic reaction of fluorescence intensity. (○) Pyrophosphate buffer, (□) Tris--HCl buffer, (△) phosphate buffer.

at 0.2 *M* but sodium pyrophosphate was deposited occasionally at this concentration. Therefore, 2 ml of 0.1 *M* pyrophosphate buffer (pH 9.0) containing 0.5 m*M* NAD was used as the enzyme reaction medium because the peak height reached a plateau at 0.2 m*M* NAD. Under these conditions, the percentage conversion of bile acids (cholic acid, deoxycholic acid, lithocholic acid) to 3-oxo-bile acids ranged from 75% to 95%.

3-Oxo-bile acids, formed by enzymatic oxidation, were extracted successfully by the Sep-Pak C_{18} cartridge. Recoveries of unconjugated, glycine- and taurine-conjugated bile acids in the extraction step using the Sep-Pak C_{18} cartridge were approximately 96% and the coefficient of variation was 2--4%.

The derivatization conditions of 3-oxo-bile acids with dansyl hydrazine were examined. Fig. 3 shows the effects of the concentration of TCA solution on the fluorescence intensity (peak height in the chromatogram). The peak height reached a maximum with 0.1% TCA—benzene solution, and decreased slightly with increasing TCA concentration in benzene. Though the peak height reached a constant value within 5 min with this TCA solution, the reaction time was held for 10 min. The reaction temperature was set at 30°C, because the reaction rate was independent of temperature between 20 and 50°C. Under these conditions, the yield of dansyl hydrazone of each bile acid was about 98%.

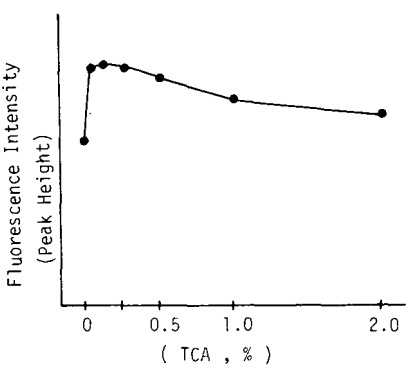

Fig. 3. Effect of TCA concentration in dansylation on fluorescence intensity.

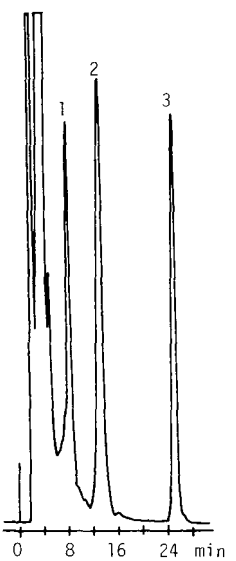

Fig. 4. Chromatogram of standard mixture of unconjugated bile acids. Peaks: 1 = cholic acid, 2 = deoxycholic acid, 3 = lithocholic acid. Column: Zorbax ODS (250 × 4.6 mm I.D.). Mobile phase: methanol—acetonitrile—0.03 *M* phosphate buffer (pH 3.2) (68:15:17), 1 ml/min.

Fig. 4 shows a typical chromatogram of unconjugated bile acids obtained using a Zorbax ODS column with a mixed solvent system of methanol—phosphate buffer. Under these chromatographic conditions, deoxycholic acid and

chenodeoxycholic acid could not be separated. Linearity of the relationship between fluorescence intensity (peak height) and amounts of bile acids was obtained between 3 and 40 pmol, and the detection limit for bile acids was about 0.5 pmol (signal-to-noise ratio = 3.5). The sensitivity of this method was superior to that of other HPLC methods using fluorescence derivatization. As shown in Table I, the precision of this method was satisfactory. In order to obtain good separation, many chromatographic conditions were examined. Shimada et al. [6] used 0.3% ammonium carbonate (pH 7.8)—acetonitrile, but in this method the peaks of the bile acids were interfered with by excess dansyl hydrazine under the alkaline conditions. As shown in Table II, complete separation could not be obtained with methanol—phosphate buffer. Ion-pair chromatography with tetrabutylammonium phosphate was suitable for the separation of all unconjugated and taurine-conjugated bile acids (Table II, Fig. 5).

TABLE I

REPRODUCIBILITY OF PEAK HEIGHTS OF BILE ACIDS*

nmol	C.V. (%) (n = 5)					
	Unconjugated			Taurine-conjugated		
	CA	DCA	LCA	CA	DCA	LCA
10.0	1.5	2.5	5.1	1.9	2.0	3.4
5.0	3.9	2.5	2.3	2.1	1.8	2.9
2.5	2.2	2.3	2.4	2.5	2.3	2.2
1.25	2.9	3.1	5.0	3.2	3.0	3.6

*For abbreviations see Reagents and materials.

TABLE II

CAPACITY FACTORS OF UNCONJUGATED BILE ACIDS AS ANALYSED ON THE ZORBAX ODS, RADIAL-PAK A AND μBONDAPAK PHENYL COLUMNS WITH VARIOUS SOLVENT SYSTEMS*

	Capacity factor							
	Zorbax ODS			Radial-Pak A		μBondapak Phenyl		
	I	II	III	IV	III	IV	V	III
Ursodeoxycholic acid	6.4	4.8	3.7	9.0	3.2	4.7	3.5	2.0
Cholic acid	7.8	5.4	4.1	9.6	3.7	5.2	3.6	2.3
Chenodeoxycholic acid	12.0	8.7	7.3	19.2	6.1	8.5	5.5	3.1
Deoxycholic acid	12.4	8.8	8.3	19.4	6.9	8.5	6.5	3.4
Lithocholic acid		22.8	14.8	45.2	12.2	16.0	10.4	4.8

*I = methanol—acetonitrole—water—acetic acid (68:15:17:1); II = methanol—0.03 M phosphate buffer (pH 2.5) (75:25); III = 0.05 M tetrabutylammonium phosphate in methanol—water (75:25); IV = methanol—0.03 M phosphate buffer (pH 2.0) (75:25); V = methanol—0.03 M phosphate buffer (pH 4.5) (75:25).

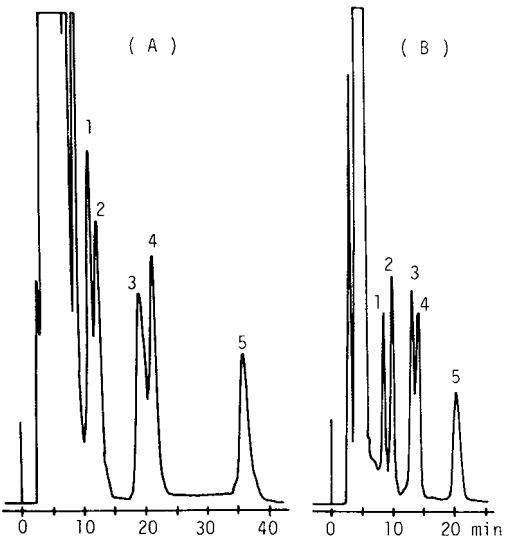

Fig. 5. Chromatograms of standard mixture of bile acids. (A) Unconjugated and taurine-conjugated bile acids. Peaks: 1 = ursodeoxycholic acid, 2 = cholic acid, 3 = chenodeoxycholic acid, 4 = deoxycholic acid, 5 = lithocholic acid. Column: Radial-Pak A. Mobile phase: 0.05 *M* tetrabutylammonium phosphate in methanol—water (75:25), 0.8 ml/min. (B) Glycine-conjugated bile acids. Peaks: 1 = urosdeoxycholic acid, 2 = cholic acid, 3 = chenodeoxycholic acid, 4 = deoxycholic acid, 5 = lithocholic acid. Column: μBondapak Phenyl (300 × 6 mm I.D.). Mobile phase: 0.03 *M* KH_2PO_4 (pH 2.0)—methanol (25:75), 1 ml/min.

TABLE III

RECOVERY AND REPRODUCIBILITY OF BILE ACIDS ADDED TO HUMAN SERUM

Normal human serum (0.1 ml) was used to which 1 nmol of each of nine bile acids was added $n = 5$.

	Unconjugated		Taurine-conjugated		Glycine-conjugated	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Cholic acid	98.1	3.6	94.8	3.8	92.6	7.0
Deoxycholic acid	86.4	4.2	89.2	4.4	90.0	5.1
Lithocholic acid	92.0	5.1	88.6	4.8	94.1	4.1

Reproducibility was tested by measuring simultaneously the recoveries of bile acids (1 nmol) in a standard mixture in normal human serum (0.1 ml). The bile acids in serum were extracted with the Sep-Pak C_{18} cartridge and then fractionated to three different groups (unconjugated, glycine-conjugated and taurine-conjugated) using the PHP-LH-20 column according to the procedure reported by Goto et al. [24]. Bile acids in each fraction were assayed by the present fluorescence HPLC method. The recovery of each bile acid was calculated from the peak height ratio of each bile acid against the peak

height of pure standard bile acid mixture carried through the analytical procedure. As illustrated in Table III, the recoveries ranged from 86.4% to 98.1% with the coefficients of variation (C.V.) ranging from 3.6% to 7.0%. Application of this method to the assay of serum bile acids or other physiologically important substances such as 3α-hydroxysteroids is being conducted in our laboratory; the details will be reported elsewhere in the near future.

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CHROMBIO. 1514

SEPARATION AND ESTIMATION OF RETINYL FATTY ACYL ESTERS IN TISSUES OF NORMAL RAT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatography system for the complete separation of naturally occurring retinyl fatty acyl esters (RFAE) is described. The sensitivity of the method allows the detection of as little as 40 pmol of the various RFAE. The procedure was applied to the separation and estimation of endogenous RFAE present in tissues of normal rats; in addition, the incorporation of [^{3}H]retinyl acetate into RFAE was also investigated. Retinyl palmitate is the major fatty acyl ester (79%) present endogenously in various tissues. However, eight other RFAE were also present in some tissues. At 24 hours after the injection of the label, radioactivity present in retinol and its metabolites was recovered mainly in liver tissue followed by kidneys, adrenals, lungs, intestine, trachea, testis, blood, heart and spleen. However, it was found that, in liver tissue, the specific radioactivity (dpm/nmol) of several RFAE was greater than that of retinyl palmitate (retinyl laurate 66-fold, retinyl pentadecanoate 5-fold, retinyl palmitoleate 4-fold).

INTRODUCTION

Retinol is stored in large quantities in liver as fatty acyl esters [1] and is released from the esters as and when it is required for its action. To study the biosynthesis and turnover of the various retinyl fatty acyl esters (RFAE), an effective and sensitive technique to separate and estimate the RFAE is required. In the past, the separation of RFAE was achieved either by reversed-phase paper chromatography [2] or by thin layers of silica gel [3, 4], and was quantitated by fluorometry [5]. However, the recoveries of RFAE by these techniques are poor, and losses up to 50% were reported [5]. De Ruyter and De Leenheer [6] developed a reversed-phase high-performance liquid chromatographic (HPLC) method for the simultaneous determination of retinol and

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retinyl esters and later modified the method to separate retinyl oleate from palmitate using silver ions in the mobile phase [7]. In the present paper we describe a fast and reliable HPLC technique for the separation and estimation of naturally occurring RFAE. The method has been applied for the determination of endogenous RFAE present in various tissues of normal rat.

MATERIALS AND METHODS

Chemicals

All-*trans*-retinol was a gift from Hoffmann-La Roche (Nutley, NJ, U.S.A.). The fatty acyl chlorides (99% pure) were purchased from Sigma (St. Louis, MO, U.S.A.). [11-^{3}H]Retinyl acetate (specific activity 2.9 Ci/mmol) was a gift from Dr L. De Luca, NIH and was purified by HPLC using an ODS-1 column [8] and found to be greater than 95% pure. Neutral alumina (Brockmann activity 1; 80—200 mesh) and HPLC grade solvents were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Chemical synthesis of RFAE

Retinyl laurate, myristate, palmitoleate, linoleate, pentadecanoate, palmitate, oleate, heptadecanoate, and stearate were synthesized by reaction of all-*trans*-retinol with the corresponding acyl chlorides as described by Huang and Goodman [4], with slight modifications. To 10 μg of all-*trans*-retinol in a test tube were added 500 μl of anhydrous pyridine and 15 μg of the appropriate fatty acyl chloride. The test tube was warmed to 50—55°C under nitrogen for 1 h. Then the pyridine in the tube was completely evaporated and the contents were extracted with hexane. The hexane extract was concentrated to a small volume and directly applied to a 10% (v/w) water deactivated alumina column (10 cm × 1.5 cm I.D.). The column was first washed with hexane (20 ml). The fatty acyl esters were then eluted from the column using 2% acetone in hexane as the eluting solvent. Retinyl esters obtained from alumina column chromatography were further purified by HPLC on a Partisil ODS-1 column [8]. The yields of different esters of retinol varied between 75 and 95%.

Metabolic studies in normal rat tissues with [11-^{3}H] retinyl acetate

Sprague—Dawley rats (200—250 g) maintained on normal laboratory chow were injected intraperitoneally with 25 μCi (2.83 μg) of [11-^{3}H]retinyl acetate in 50 μl ethanol. Rats were sacrificed by cervical dislocation 24 h after the injection of the label. The tissues were immediately removed, washed with cold phosphate-buffered saline and lyophylized overnight.

Extraction of tissues

The lyophilized tissues were ground to powder with a pestle and mortar and extracted successively with 20 ml of 99% methanol per gram of tissue and with 50 ml of hexane. The combination of 99% methanol and hexane was found to be effective in extracting more than 90% of the polar metabolites and the fatty acyl esters of retinol. The 99% methanol and hexane extracts were evaporated

separately and the residues were combined and redissolved in chloroform—methanol (1:1). An aliquot was counted and used for HPLC.

High-performance liquid chromatography

HPLC was carried out on a Beckman Model 322 MP programmable liquid chromatographic system. The UV spectrophotometer was a Hitachi Model 100-40 equipped with a wavelength variable between 195 and 850 nm (maximal sensitivity, 0.01 a.u.f.s.). The HPLC column, Partisil 10 ODS-1 (25 cm × 4.6 mm I.D.) was obtained from Whatman (Clifton, NJ, U.S.A.). Ultrasphere ODS (5-μm particle size, 25 cm × 4.6 mm I.D.) was purchased from Beckman Instruments (Toronto, Canada). The Ultrasphere column was found to be the column of choice for the separation of RFAE. In a typical separation the column was eluted with methanol—water (98:2) at a flow-rate of 1.5 ml/min for the first 62 min to elute retinyl laurate, myristate, palmitoleate, linoleate, pentadecanoate, palmitate and oleate, and 2.0 ml for the following 28 min to elute retinyl heptadecanoate and stearate.

Determination of extinction coefficient ($\epsilon^{1\%}_{1\,cm}$ in ethanol) of standard RFAE

$\epsilon^{1\%}_{1\,cm}$ of various RFAE were measured at 325 nm in ethanol and the following values were obtained: retinyl laurate 1052, myristate 990, pentadecanoate 970, palmitoleate 944, palmitate 948, linoleate 900, oleate 890, heptadecanoate 916 and stearate 870. These values are in close agreement with the theoretical values calculated from the reported $\epsilon^{1\%}_{1\,cm}$ of retinyl palmitate, which is 940 at 325 nm [9].

Quantitation of RFAE

The procedure is standardized by adding known amounts of a mixture of standard RFAE to 1 g of liver tissue from a vitamin A deficient rat and taking these through the entire procedure of lyophilization, extraction and separation by HPLC. Peak areas of different RFAE were measured at 325 nm by triangulation [10] and plotted against the amounts. The losses (usually 5—10%) due to extraction and evaporation were corrected for the exact amount. The amounts of RFAE in the unknown samples were determined by measurement of the peak area and by use of the standard curve.

Hydrolysis of RFAE

Either the UV or radioactive RFAE peaks were collected in large amounts by repeated injections and were saponified at 55°C for 90 min in 200 μl of 0.4 *M* ethanolic potassium hydroxide under nitrogen. The mixture was then diluted with 500 μl of water and extracted 3—4 times with 2 ml of petroleum ether in order to remove retinol. The petroleum ether fraction was then concentrated and directly injected onto an ODS-1 column to detect retinol [8]. The hydrolysis and recovery of retinol from RFAE, based on radioactivity, was greater than 90%.

Determination of radioactivity

Samples from the column were collected at 0.5- or 1.0-min intervals and were counted after adding 7.5 ml of phase combining system (Amersham).

Samples were counted in a Beckman Model LS-230 liquid scintillation counter. The efficiency of counting for ^3H was 31%.

RESULTS

Separation of RFAE by HPLC

Fig. 1 illustrates the chromatography profile from a reversed-phase HPLC system that separates standard fatty acyl esters of retinol. In this system, an Ultrasphere ODS column (5 μm) was used and developed with a solvent system of methanol—water (98:2). An effective separation of known naturally occurring RFAE was achieved, including the separation of retinyl myristate and palmitoleate and of retinyl palmitate and oleate. Peak areas of various RFAE were measured at 0.01 a.u.f.s. setting as described in Materials and methods. A linear relationship between the peak areas of different RFAE and their amounts (40—120 pmol) was observed.

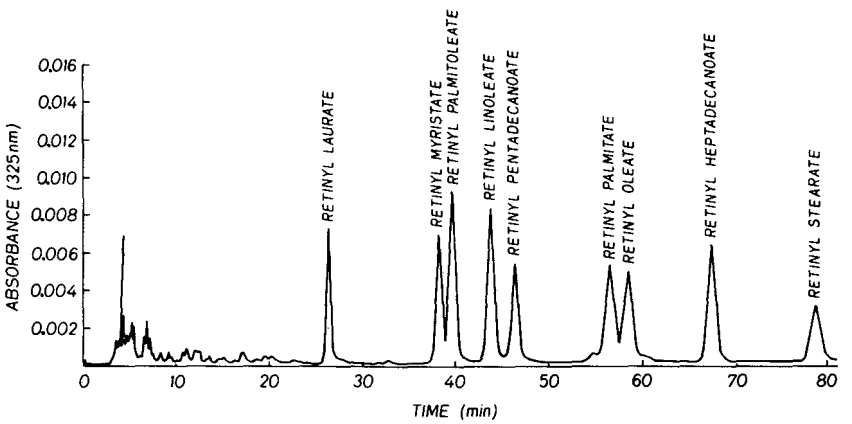

Fig. 1. Separation of a mixture of nine standard RFAE on an Ultrasphere ODS (5 μm) column. Conditions of elution are given in Materials and methods. The quantity of each standard RFAE was 150—200 ng.

The recovery of all standard RFAE from the column based on UV spectrophotometry was greater than 95%. Known amounts of individual RFAE were mixed with 1 g of liver tissue from a vitamin A deficient rat, extracted and estimated as described in Materials and methods. The recoveries of various esters of retinol varied between 85 and 95%.

Tissue distribution of radioactivity present in retinol and its metabolites 24 h after the injection of [11-^3H] retinyl acetate

The distribution of radioactivity (dpm/g or dpm/ml) as well as the percentage of injected radioactivity in different tissues of a normal rat is presented in Table I. The highest concentration of radioactivity was observed in liver. This was followed in descending order by kidneys, adrenals, lungs, intestine, trachea, testis, blood, heart and spleen tissues. The radioactivity, expressed as the percentage of injected dose was greater in liver (29.2%) followed by kidneys, intestine and lungs.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN TISSUES OF A NORMAL RAT, INJECTED WITH [11-^{3}H]RETINYL ACETATE

Normal rat was injected intraperitoneally with 25 μCi (2.83 μg) of [11-^{3}H]retinyl acetate and sacrificed after 24 h. Retinol and its metabolites were extracted from the tissues as described in Materials and methods.

Tissue	^{3}H (dpm/g or dpm/ml $\cdot$ 10^{-6})	Percentage of injected dose
Liver	2.033600	29.20
Kidney	1.056971	3.16
Adrenals	0.435429	0.04
Lungs	0.274784	0.74
Intestine	0.187210	1.06
Trachea	0.154280	0.01
Testis	0.057220	0.24
Blood	0.056686	0.51
Heart	0.054026	0.09
Spleen	0.044505	0.04

Separation of RFAE in normal rat tissues

Fig. 2A shows the chromatograms of the lipid extract of normal rat liver tissue. Several peaks (4--11) were positively identified as fatty acyl esters of retinol by UV monitoring at 325 nm and also by co-chromatography with standard RFAE. Furthermore the hydrolysis of each of these fractions yielded retinol as the only material absorbing at 325 nm. The labelling of RFAE by [11-^{3}H]retinyl acetate is shown in Fig. 2B. Hydrolysis of each of the radioactive peaks (3--11) yielded [^{3}H]retinol. The polar metabolites of retinol, such as retinyl phosphate, mannosyl retinyl phosphate, retinyl glucuronide, retinoic acid, are eluted in the area of radioactive peak 1 and hence constitute a mixture of polar metabolites of retinol. The radioactive peak 2 in Fig. 2B was identified as being [^{3}H]retinol, as judged by its retention time with standard retinol when separated on an ODS-1 column with acetonitrile—water mixtures [8]. Radioactive peak 3 appears to be an ester of retinol, but was not identified further and was not found to be present in other tissues.

Since retinyl palmitate was present in large quantities in liver tissue, a broad peak of retinyl palmitate can be seen in Fig. 2A as peak 9. Retinyl oleate which elutes after retinyl palmitate, therefore was not resolved properly. For this reason, the lipid extract was further diluted 20-fold and injected into the chromatograph to resolve retinyl oleate from palmitate. Fig. 3A illustrates such a chromatogram which indicates that retinyl oleate is not present as one of the major RFAE in the liver tissue. This was further confirmed in the labelling of the RFAE (Fig. 3B).

Quantitation of RFAE in normal rat tissues

The amounts of endogenous RFAE expressed as nmol per g or per ml in various tissues are presented in Table II. Retinyl palmitate and stearate were

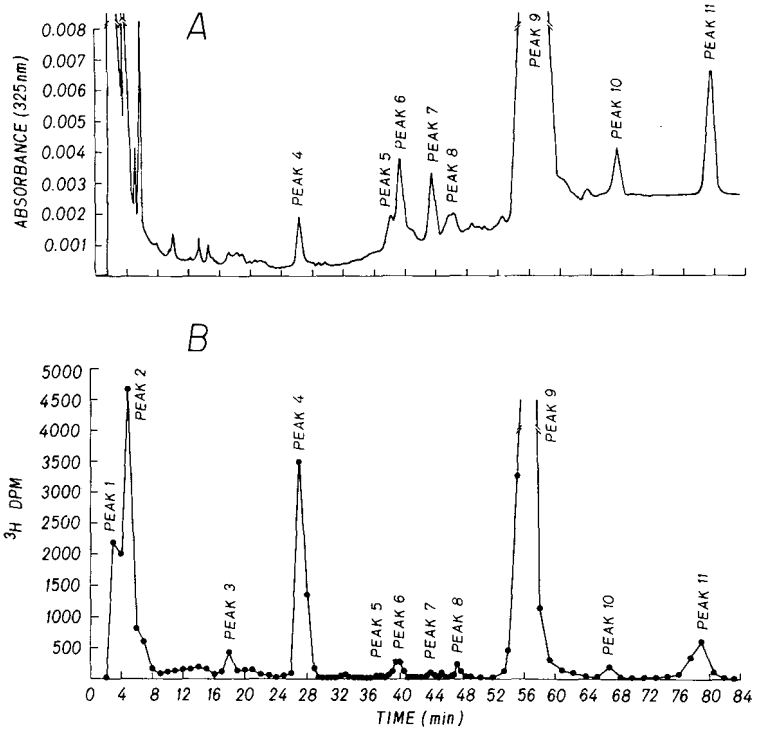

Fig. 2. HPLC of lipid extract of normal rat liver. One rat was injected with 25 μCi of [11-^{3}H]retinyl acetate intraperitoneally and sacrificed after 24 h. An aliquot of the lipid extract (20 μl) was injected in chloroform—methanol (1:1) onto an Ultrasphere column and eluted with methanol—water mixtures as described in Materials and methods. The UV was monitored at 325 nm to detect RFAE (A) and 0.5- or 1.0-min fractions were collected and counted (B) as described in Materials and methods. Peaks: 4 = retinyl laurate, 5 = myristate, 6 = palmitoleate, 7 = linoleate, 8 = pentadecanoate, 9 = palmitate, 10 = heptadecanoate and 11 = stearate, identified by co-chromatography with standard RFAE. Radioactive peaks: 3 = an unidentified ester of retinol, 2 = retinol and 1 = a mixture of polar metabolites of retinol.

present in all the tissues examined. Retinyl oleate was present in small amounts in intestine and kidney and could not be detected in liver tissue.

In liver tissue, retinyl palmitate constituted 79% of the total RFAE, followed by stearate (7%), palmitoleate (3.7%), linoleate (2.9%), heptadecanoate (2.2%), myristate (2.1%), pentadecanoate (1.6%), and laurate (1.2%). However, the labelling of these esters by [11-^{3}H]retinyl acetate indicated an entirely different pattern (Table II). Retinyl laurate was found to be highly labelled and its specific radioactivity (S.A.) (dpm/nmol) was 66-fold greater than that of retinyl palmitate. In addition to retinyl laurate, the S.A. of several other RFAE were also found to be greater than that of retinyl palmitate, retinyl pentadecanoate (5-fold), and palmitoleate (4-fold).

DISCUSSION

The separation of naturally occurring RFAE by HPLC has been previously

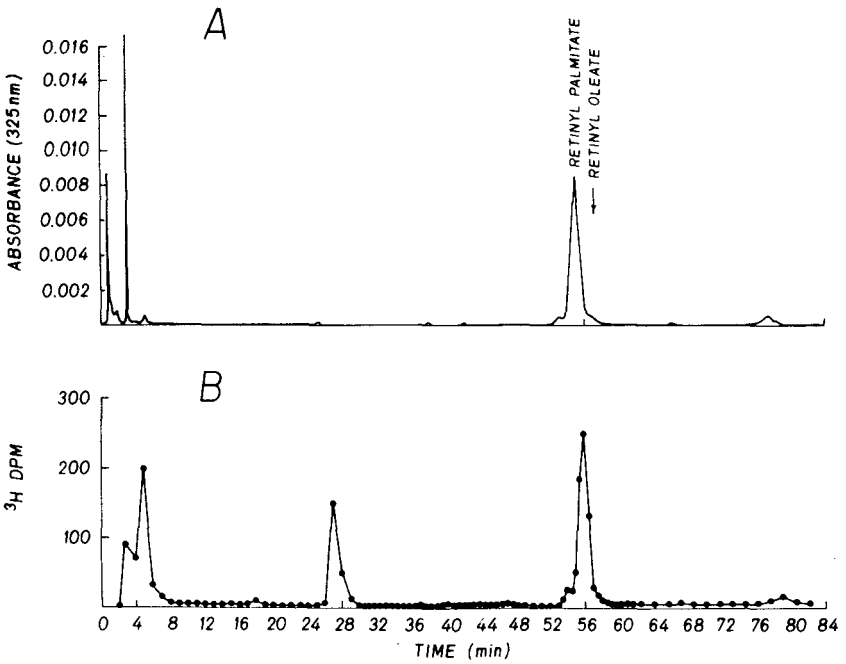

Fig. 3. HPLC of lipid extract of normal rat liver. The lipid extracts were prepared from liver tissue as described in Materials and methods and diluted 20-fold to resolve retinyl oleate from retinyl palmitate. (A) represents the detection by UV and (B) is the tracing of radioactive peaks. The conditions of elution were as described in Materials and methods.

reported by other workers [6, 7, 11]. De Ruyter and De Leenheer [7] obtained reasonably good separation of RFAE using silver ions in the mobile phase. However, in their study, no attention has been given to the effect of silver ions on the retention of polar metabolites of retinol. Moreover, the applicability of the method for the separation of complex RFAE present in tissues like liver has not been reported. In our method, we have shown that polar metabolites of retinol are clearly separated from RFAE. It can be seen from Fig. 1, that the effective separation of a variety of esters of retinol was achieved by an Ultrasphere ODS column with methanol—water as the eluting solvent. Using this solvent system the polar metabolites of retinol are eluted in the void volume of the column.

Futterman and Andrews [3] reported the presence of at least eight RFAE in rat liver: retinyl palmitate, stearate and oleate being the major components. In our studies, retinyl oleate could not be detected in liver tissue. This discrepancy could be due to the method used by Futterman and Andrews [3] for the separation and identification of RFAE. They separated RFAE by thin layers of silica gel and identified them by determining the fatty acids using gas—liquid chromatography. Although they were able to separate various RFAE by thin-layer chromatography, it is not certain that they have separated RFAE from other lipids present in liver tissue. Therefore separation and identification of RFAE by these techniques might have given misleading results. Since retinyl palmitate and oleate are eluted closely in our system, it is possible

TABLE II

COMPOSITION OF RETINYL FATTY ACYL ESTERS IN TISSUES OF A NORMAL RAT

RFAE	Liver		Kidneys		Lungs		Intestine		Testis	
	nmol/g	S.A.*	nmol/g	S.A.	nmol/g	S.A.	nmol/g	S.A.	nmol/g	S.A.
Retinyl laurate	9.9	12952	N.D.**		N.D.		N.D.		N.D.	
Retinyl myristate	17.4	302	N.D.		N.D.		N.D.		N.D.	
Retinyl palmitoleate	30.7	740	0.4	3242	N.D.		N.D.		0.069	9244
Retinyl linoleate	24.1	234	0.3	8500	N.D.		3.50	600	0.034	10900
Retinyl pentadecanoate	13.1	1038	N.D.		N.D.		N.D.		N.D.	
Retinyl palmitate	651	195	38.5	606	15.5	498	7.43	248	20.1	478
Retinyl oleate	N.D.		N.D.		N.D.		2.3	416	N.D.	
Retinyl heptadecanoate	18.4	454	N.D.		N.D.		N.D.		N.D.	
Retinyl stearate	57.10	529	1.57	3931	5.99	508	N.D.		0.14	2753

*S.A. = specific radioactivity, expressed as dpm/nmol.
**N.D. = not detected endogenously.

that small amounts of retinyl oleate, if present, might have been masked by the presence of large amounts of retinyl palmitate. Nevertheless, the results indicate that retinyl oleate is not one of the major RFAE present in the liver tissue as claimed by other workers [3, 12].

At 24 h after the injection of [11-^{3}H]retinyl acetate to the normal rat, the highest radioactivity was observed in liver and kidney tissues. This confirms the previous observations of Goodman et al. [12] who reported similar radioactivity distribution in liver and kidney tissues after intravenous administration of [15-^{14}C]retinol in chylomicrons. In addition, we also observed a considerably high concentration of radioactivity in adrenals. This is in general agreement with previous studies by Sundaresan and Sundaresan [13] and Willmer and Laughland [14].

The study of the labelling of various esters of retinol in liver tissue yielded interesting results. The specific radioactivity (dpm/nmol) of some RFAE such as retinyl laurate, pentadecanoate and palmitoleate was found to be higher than that of retinyl palmitate 24 h after the injection of the label. According to the reports of Reitz et al. [15] and Varma and Beaton [16] it is unlikely that newly administered labelled vitamin A mixes uniformly with the endogenous pool of RFAE within 24 h after injection. However, recent reports [17, 18] indicate that a portion of newly absorbed vitamin A is rapidly metabolized in the liver possibly in a separate compartment (pool 1) and that the excess would go for storage (pool 2). Labelling of RFAE at 24 h may represent the metabolism of retinol in pool 1 of liver. This is supported by the finding that the specific radioactivity of certain RFAE increase rapidly, possibly due to the rapid turnover of these esters in pool 1.

In conclusion, it is believed that the HPLC technique described in this paper will provide an excellent tool to study further the distribution, turnover and metabolism of natural RFAE in tissues.

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DETERMINATION OF POLYAMINES IN HYDROLYSATES OF UREMIC PLASMA BY HIGH-PERFORMANCE CATION-EXCHANGE COLUMN CHROMATOGRAPHY

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SUMMARY

The levels of putrescine, cadaverine, spermidine and spermine in uremic plasma were determined with an automatic polyamine analyzer with a 7.5 × 0.2 cm I.D. cation-exchange column using a stepwise sodium chloride gradient. All four polyamines were higher in ten patients with chronic renal failure than in eight normal subjects. The total polyamine content was also measured in the patients' plasma before and after maintenance dialysis; putrescine and spermidine levels were significantly lowered by the procedure.

INTRODUCTION

Among the methods reported for the separation and quantitation of polyamines [1], various amino acid analyzers coupled with continuous fluorescence monitoring showed good separations [2–6]. A number of papers have been published comparing the polyamine levels in the urine and plasma of cancer patients. To date, however, only a few [7, 8] have reported clinical applications of polyamine determinations in uremic patients. Numerous compounds are known to accumulate in the body fluids of uremic patients, among them polyamines have been proposed as one type of uremic toxin [9]. Recently, elevated polyamine levels in erythrocytes of uremic patients were described [10], and spermine was identified as an inhibitor of erythropoiesis [11]. But the relationship between uremic symptoms and accumulation of uremic toxins is not yet definitely established.

In this paper, a simple and rapid method for the simultaneous determination of putrescine, cadaverine, spermidine and spermine in the plasma of uremic patients is described.

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EXPERIMENTAL

Chemicals

Putrescine, cadaverine, spermidine and spermine were obtained in the form of hydrochlorides from Sigma (St. Louis, MO, U.S.A.). *o*-Phthalaldehyde (OPA) was purchased from Wako Pure Chemicals (Osaka, Japan).

Chromatographic system

Fig. 1 is a flow diagram of the automatic polyamine analyzer which was constructed in our laboratory. Two high-pressure piston pumps (Mitsumi Scientific Industry Co., Tokyo, Japan) served to pump the eluent and OPA solution. The column eluent was pumped through a six-port sample injection valve with a 100-μl sample loop. A jacketed, stainless-steel column, 7.5 × 0.2 cm I.D., was used, slurry-packed with CK-10S cation-exchange resin with a mean particle size of 11.5 μm (Mitsubishi Kasei Co., Tokyo, Japan). The column temperature was kept at 60°C with a Haake constant-temperature circulator (Haake Co., Berlin, G.F.R.). The reservoir of the OPA solution was kept in a refrigerator at 4°C to prevent degradation of the reagent. All buffer solutions were delivered by an automated buffer exchanger (Hijiri Seiko Co., Tokyo, Japan). A JASCO FP-550 fluorescence spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan) was used to detect the fluorescent polyamine derivatives produced by the reaction with OPA—2-mercaptoethanol. The fluorescence setting was activated at 340 nm and emission was measured at 455 nm. The fluorometric

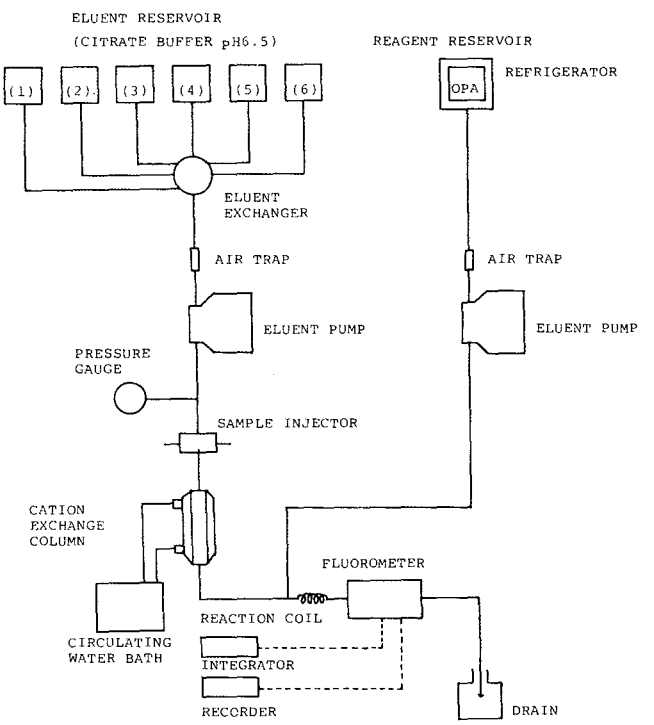

Fig. 1. Flow diagram of the polyamine analyzer.

signal was recorded using a JASCO RC-100 recorder at a chart speed of 12 cm/h. Peak areas were determined with a digital integrator (Chromatopac C-R1A, Shimadzu Seisakusho Co., Kyoto, Japan).

Eluent and reagent solutions

All reagents were of analytical grade, made up in double glass-distilled water. All aqueous reagents were filtered through a 0.45-μm microfilter (Fuji Photo Film Co., Tokyo, Japan), and degassed. Standard polyamine solutions were made in 0.5 *N* hydrochloric acid with putrescine, cadaverine, spermidine and spermine each at 0.5 nmol per 100 μl; these solutions could be kept at 4°C for a month without any degradation. The OPA solution was made with 0.8 g/l OPA dissolved in a 0.4 mol/l potassium borate solution, pH 10.5, and 2 ml/l 2-mercaptoethanol. Six buffers, each prepared with 0.25 *M* sodium citrate (pH 6.5) and sodium chloride, were used to elute polyamines with a stepwise gradient of 0, 0.2, 0.5, 1.5, 2.0 and 3.0 *M* sodium chloride. The first buffer was pumped through the column for 16 min after the introduction of the sample, the second for 2 min, the third for 4 min, the fourth for 7 min, the fifth for 8 min and the sixth for 33 min. The column was operated at a flow-rate of 12 ml/h and a column inlet pressure of 20 kg/cm^2. The OPA solution was pumped into the reaction coil at a flow-rate of 12 ml/h.

Preparation of plasma samples

Whole blood was centrifuged at 1000 *g* for 15 min immediately after removal from the patient. Plasma was mixed thoroughly for 3 min with 50% trichloroacetic acid to a final concentration of 5% trichloroacetic acid [12]. After standing at 4°C for 30 min and centrifugation at 2000 *g* for 20 min, the supernatant was lyophilized. The residue was hydrolysed in 3 ml of 6 *N* hydrochloric acid for 16 h at 110°C. The hydrolysate was evaporated in vacuo. The residue was dissolved in 0.5 ml of 0.5 *N* hydrochloric acid and centrifuged; 100-μl aliquots were separated. Treatment of standard polyamine solutions by this method resulted in recoveries of putrescine, cadaverine, spermidine and spermine of 94%, 88%, 93% and 91%, respectively.

RESULTS

A chromatogram of a standard mixture of polyamines, each at a concentration of 0.5 nmol per 100 μl, is shown in Fig. 2. The reproducibility of the analytical system was evaluated from ten chromatograms of standard samples. The retention times were: putrescine, 43.8 ± 0.2 min; cadaverine, 51.1 ± 0.3 min; spermidine, 55.5 ± 0.3 min; and spermine, 63.7 ± 0.4 min. The retention time of each polyamine compound showed a relative standard deviation of $\leqslant$ 0.6%. The deviation of the peak areas was within 4.4% for all the compounds.

Calibration curves for the polyamines in the range 100 pmol to 20 nmol are shown in Fig. 3. The minimum detectable quantities were 7.9 pmol of putrescine, 7.1 pmol of cadaverine, 6.0 pmol of spermidine and 10.0 pmol of spermine. The limit of detection was defined as the peak height twice that of the noise level. Typical chromatograms of total polyamines in a plasma sample of a normal subject and in a uremic patient are shown in Fig. 4A and B, respective-

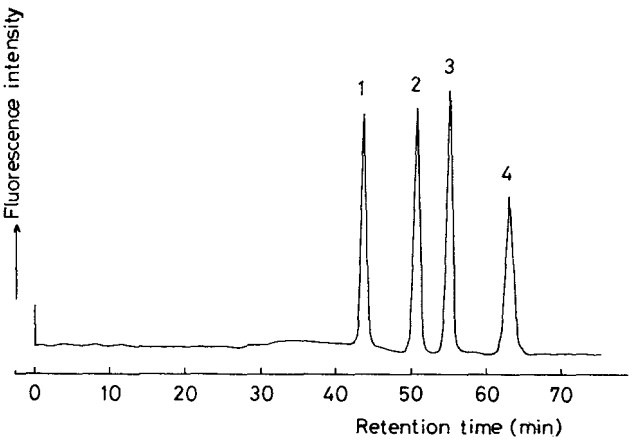

Fig. 2. Chromatogram of a standard mixture of polyamines, each at a concentration of 0.5 nmol per 100 μl. Chromatographic conditions: stationary phase, CK-10S (average particle size 11.5 μm) slurry-packed in a 7.5 × 0.2 cm I.D. stainless-steel column; mobile phase, 0.25 *M* citrate buffer (pH 6.5) with a stepwise sodium chloride gradient; flow-rate, 12 ml/h; detector, fluorescence was activated at 340 nm and emission was at 455 nm; recorder chart speed, 12 cm/h. Peaks: 1 = putrescine; 2 = cadaverine; 3 = spermidine; 4 = spermine.

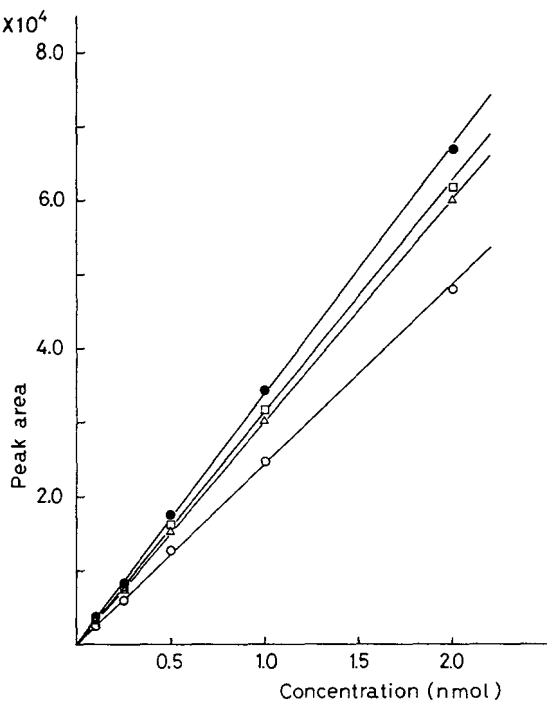

Fig. 3. Calibration curves of polyamines in high-performance liquid chromatography with fluorescence detection. One hundred microliters of a sample containing various amounts (100 pmol to 20 nmol) of polyamines were injected into the column and detected by a fluorescence detector. The conditions are described under Experimental procedures. (○) Putrescine; (△) cadaverine; (●) spermidine; (□) spermine.

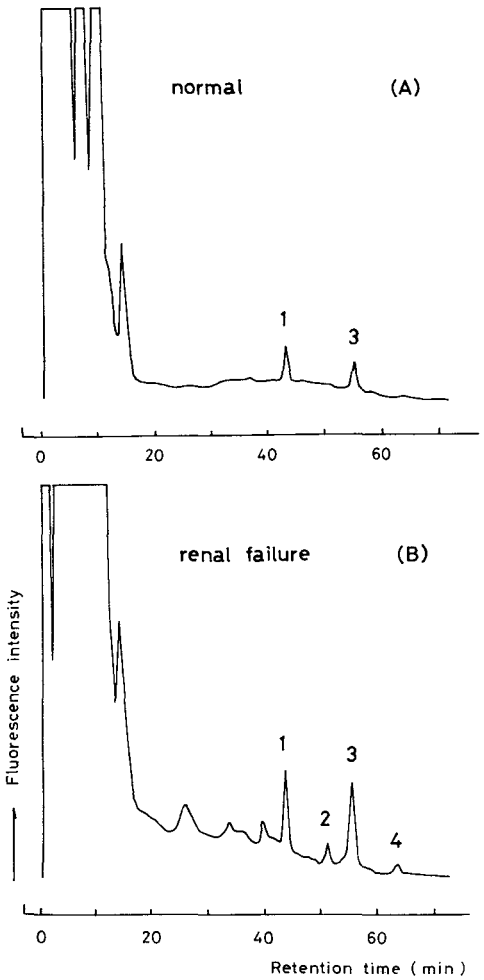

Fig. 4. Chromatograms of the hydrolysates of plasma samples. The conditions are described under Experimental procedures. (A) The hydrolysate of plasma sample from a normal subject. (B) The hydrolysate of plasma sample from a uremic patient. The polyamine peaks are numbered as in Fig. 2.

ly. In normal plasma the concentrations of putrescine and spermidine were 0.24 ± 0.10 (mean ± S.D.) nmol/ml and 0.23 ± 0.06 nmol/ml, respectively. Cadaverine and spermine were below detectable levels.

Table I compares the polyamine levels in patients with chronic renal failure before and after dialysis. The concentrations of all plasma polyamines were lower after the dialysis. In the present experiments we measured the concentration of total polyamines in normal and uremic plasma, and found that all polyamines were significantly elevated in uremic plasma. In ten patients undergoing maintenance hemodialysis, the total polyamine content was measured in plasma before and after dialysis. The mean values before dialysis for both putrescine and spermidine were significantly higher than those in normal subjects, while the mean cadaverine and spermine values were slightly but not

TABLE I

PLASMA LEVELS OF TOTAL POLYAMINES IN NORMAL SUBJECTS AND IN PATIENTS WITH CHRONIC RENAL FAILURE BEFORE AND AFTER DIALYSIS

Compound	Normal plasma (nmol/ml)*	Dialysis plasma (nmol/ml)**	
		Before	After
Putrescine	0.24 ± 0.10 (mean ± S.D.)	0.91 ± 0.43	0.42 ± 0.12
Cadaverine	N.D.***	0.22 ± 0.11	0.16 ± 0.09
Spermidine	0.23 ± 0.06	1.03 ± 0.64	0.45 ± 0.10
Spermine	N.D.	0.07 ± 0.03	0.04 ± 0.04

*n = 8.
**n = 10.
***N.D. = not detected.

significantly elevated. The values after dialysis for both putrescine and spermidine were almost the same as those prior to dialysis.

DISCUSSION

High-performance liquid chromatography with fluorometric detection has proved to be a useful and sensitive method for the determination of polyamines in body fluids [13—16]. The method selected for this study was a high-performance cation-exchange chromatographic method which efficiently separated a series of polyamines with a stepwise sodium chloride gradient. The reaction with OPA—2-mercaptoethanol was utilized for fluorescence detection. Analytical methods so far described using a modified automatic amino-acid analyzer were time-consuming. In our method, automatic quantitative determination was performed within 65 min and the column was regenerated repeatedly by washing with sodium hydroxide.

The method was successfully applied to the simultaneous determination of polyamines in uremic plasma. Quite a number of different amines accumulate in uremic serum [17—19]. We have already reported that the concentrations of aromatic and aliphatic amines were higher in uremic plasma than in normal plasma [20]. Since polyamines are present either free or in conjugated form in plasma [21], urine [22—24], and other body fluids [25], the samples have to be hydrolysed with 6 N hydrochloric acid. Campbell et al. [7] reported that the level of free polyamine in the serum, expressed in spermine equivalent, is elevated in children with uremia. In their report, serum polyamines were determined by a radioimmunoassay method using antispermidine and antispermine antibody. The main disadvantage was the lack of specificity of the method for spermidine and spermine. Swendseid et al. [10] measured free spermidine and spermine in the erythrocytes and putrescine in the urine of patients with renal failure using an amino acid analyzer. In our results, all four polyamines of plasma were higher in patients with chronic renal failure than in normal subjects. After maintenance dialysis, putrescine and spermidine levels were significantly lowered by the procedure.

A large number of samples of plasma and erythrocytes from normal subjects

and uremic patients are currently being analysed by our method, and the results will be the subject of another report.

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DETERMINATION OF URINARY 5-S-CYSTEINYLDOPA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance ion-pair liquid chromatographic method with electrochemical detection is described, which is suitable for routine determination of urinary 5-S-cysteinyldopa. The clean-up procedure includes a first purification step on the cation exchanger AG 50 W (H^+). After desorption from the resin at moderately raised pH the catecholic amino acid is adsorbed on alumina at pH 8.6, washed and finally desorbed by elution with perchloric acid. By the combined clean-up procedures, easily oxidized compounds are eliminated, which otherwise cause a number of interfering peaks in the chromatography. The synthesis of 5-S-cysteinyl-L-3,4-dihydroxyphenyl [2,3-^{3}H]alanine is described, and this tritium-labelled 5-S-cysteinyldopa is used to determine the recovery in the sample. The precision (C.V. = 5.7% at low and C.V. = 4.9% at high 5-S-cysteinyldopa concentration) and recovery (105.0 ± 8.6%) were satisfactory. The mean urinary excretion was 0.34 ± 0.13 (S.D.) μmol per 24 h (range 0.02—0.58 μmol per 24 h) in healthy subjects (n = 24) and in patients with melanoma metastates (n = 13) the excretion ranged from 0.9 to 4.8 μmol per 24 h.

INTRODUCTION

Malignant melanoma is one of those carcinomas that is increasing most rapidly in white-skinned populations and causes a substantial number of deaths [1—3]. This cancer produces a number of substances which have been suggested as tumor markers [4—7]. As measured by a fluorimetric method [8] the urinary excretion of 5-S-cysteinyldopa was recently evaluated for that purpose and found to be a good estimate of the increased tumor burden in patients with metastases [9].

Recently a method with increased sensitivity and selectivity was described [10, 11] for the determination of 5-S-cysteinyldopa in urine. After precipitation of urinary proteins with perchloric acid, the sample, spiked with an

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internal standard, was treated with activated alumina which adsorbs the catechols at alkaline pH. After washing, 5-S-cysteinyldopa and other catechols were eluted with perchloric acid. The sample was then subjected to ion-pair high-performance liquid chromatography (HPLC) with electrochemical detection.

When we tried to apply and validate this method for routine clinical chemical determinations of urinary 5-S-cysteinyldopa, we found a number of drawbacks such as peaks interfering with the 5-S-cysteinyldopa peak and the occurrence of peaks with long retention times. The latter fact made the method unsuitable for routine determinations because of long chromatographic runs. Instability of the alumina-treated sample precluded storage until the next day. Furthermore HPLC with electrochemical detection gave a high baseline current and a variable detector response to 5-S-cysteinyldopa. Although we soon found that addition of disodium ethylenediaminetetraacetate (Na_2EDTA) both in the final sample eluate and in the moble phase improved the stability, the remaining drawbacks hampered the usability of the method in routine clinical work. We therefore conducted systematic studies, especially on the clean-up procedures for 5-S-cysteinyldopa. Further, some modifications of the HPLC procedure are reported.

EXPERIMENTAL

Chemicals

Tyrosinase from mushroom and cysteine as free base were obtained from Sigma (St. Louis, MO, U.S.A.) and 3,4-dihydroxy-L-phenylalanine (L-DOPA) was from Merck (Darmstadt, G.F.R.). L-3,4-Dihydroxyphenyl[2,3-^{3}H]alanine (specific activity 77.7 GBq/mmol) was obtained from The Radiochemical Centre (Amersham, Great Britain). Cation-exchange chromatography was performed with AG 50 W-X4, 200—400 mesh, and with AG 50 W-X8, 100—200 mesh, from Bio-Rad Labs. (Richmond, CA, U.S.A.). Aluminium oxide, Brockman activity II, from BDH Chemicals (Poole, Great Britain) was pretreated according to the procedure of Anton and Sayre [12].

Methanesulphonic acid (puriss.) was from Fluka (Buchs, Switzerland). Phosphoric acid (85%) and Na_2EDTA were from Merck and all other reagents were of reagent grade quality.

Synthesis of 5-S-cysteinyldopa

5-S-Cysteinyldopa was synthesized from cysteine and L-DOPA by the tyrosinase reaction according to the description of Agrup et al. [13]. They performed mass spectrometric analysis of the compound synthesized. The identity of the substance we obtained was checked by comparison of the retention time in HPLC with that obtained with an authentic sample of 5-S-cysteinyldopa obtained from these authors. Furthermore, we obtained similar R_F values on cellulose thin-layer chromatography and similar UV-absorption curves [13]. The concentration of a standard solution was determined with use of the molar extinction coefficient at 292 ($\log \epsilon = 3.47$) and 255 nm ($\log \epsilon = 3.58$) as given by Prota et al. [14], and checked by comparison with the total sulphur content determined by a wet combustion technique

[15] followed by sulphate determination [16]. Good agreement was obtained with these methods. Stock solution of 5-S-cysteinyldopa, 2.75 mmol/l, was prepared in hydrochloric acid, 2 mol/l. This stock solution of 5-S-cysteinyldopa was found to be stable for two years as it gave correct results when UV absorbance and HPLC peaks were compared with a newly synthesized preparation. The working standard solution (275 nmol/l) of 5-S-cysteinyldopa was prepared in perchloric acid, 1.0 mol/l, containing Na_2EDTA, 20 mmol/l.

Synthesis of 5-S-cysteinyl-L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine

For synthesis of tritium-labelled 5-S-cysteinyldopa, 1000 µl of tritium labelled L-DOPA were mixed with 40 µl of a solution containing 0.55 mg of cysteine and 0.55 mg of L-DOPA in phosphate buffer, 0.5 mol/l, pH 6.5. The reaction was started by addition of 150 µg of tyrosinase dissolved in 20 µl of phosphate buffer. After 13 min the enzyme reaction was stopped by the addition of 120 µl of hydrochloric acid, 6.0 mol/l and 160 µl of sodium metabisulphite, 50 g/l. The product obtained was then transferred to a 180 × 10 mm column of AG 50 W-X4, 200—400 mesh, equilibrated with hydrochloric acid, 1.0 mol/l. After washing with 50 ml of hydrochloric acid, 1.0 mol/l, elution was performed with hydrochloric acid, 2.0 mol/l.

Urine collection

Urine was collected for 24 h in plastic bottles containing 5 ml of thymol, 0.7 mol/l in isopropanol, added as preservative [17], or containing 50 ml of glacial acetic acid and 1 g of sodium metabisulphite [9], and stored at −18°C until processed.

Procedure for purification of urinary 5-S-cysteinyldopa

To 6 ml of urine, 50 µl of tritium-labelled 5-S-cysteinyldopa and 2 ml of Na_2EDTA, 0.27 mol/l, were added followed by 0.8 ml of perchloric acid, 4 mol/l. After mixing, the precipitate was removed by centrifugation, and 0.1 ml was taken off for radioactivity determination. From the supernatant 4 ml were transferred to a 4.0 × 0.5 cm column (Econo-Column, Bio-Rad Labs.) of AG-50 W-X8 (100—200 mesh) which was equilibrated with hydrochloric acid, 1.0 mol/l. After washing with 2 ml of hydrochloric acid, 1 mol/l, elution was performed with citrate–phosphate buffer, 0.5 mol/l and pH 4.0 which was prepared by mixing 380 ml of citric acid, 0.5 mol/l, with 500 ml of disodium hydrogen phosphate, 0.5 mol/l. The first 3 ml of the eluate were discarded and the following 7 ml were transferred to a beaker containing 400 mg of activated alumina and 10 mg of sodium metabisulphite. Then the pH was raised by adding about 1.0 ml of sodium hydroxide, 4.0 mol/l, until pH 8.0 followed by final adjustment to pH 8.6 with sodium hydroxide, 0.4 mol/l. After stirring, the alumina was quantitatively transferred to a 100 × 20 mm I.D. separating funnel with glass filter (porosity 2), the liquid sucked off and the alumina washed four times with 10-ml portions of water. Desorption was effected by shaking for 15 min with 1.5 ml of perchloric acid, 1.0 mol/l containing Na_2EDTA, 20 mmol/l. The amount of 5-S-cysteinyldopa in the eluate was then determined by HPLC by comparison with standard solutions, and 0.1 ml was taken from the eluate for radioactivity counting and recovery determination.

High-performance liquid chromatography

We used a Constametric III pump from LDC (Riviera Beach, FL, U.S.A.) and a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 100-μl sample loop. The electrochemical detector consisted of a TL-5A electrochemical cell from Bioanalytical Systems (West Lafayette, IN, U.S.A.). Working and auxiliary electrodes were made of glassy carbon. The detector was operated at +0.75 V vs. an Ag/AgCl reference electrode. The detector cell, placed in a Faraday cage to minimize electrical disturbances, was connected to an electrochemical controller Model LC-2A, also from Bioanalytical Systems.

The HPLC column was a Supelcosil LC-8 column (150 × 4.6 mm), particle size 5 μm, from Supelco (Bellefonte, PA, U.S.A.). In order to protect the analytical column, a precolumn packed with Solvecon Silica, particle size 37–53 μm (Pierce, Rockford, NJ, U.S.A.) was put in line between the pump and injector. The mobile phase was a water solution containing phosphoric acid, 30 mmol/l, methanesulphonic acid, 42 mmol/l, and Na_2EDTA, 0.1 mmol/l. The Na_2EDTA was dissolved in water before adding the other reagents, and final adjustment to pH 2.55 was obtained by adding sodium hydroxide, 5 mol/l, about 65 ml per 5000 ml final volume. The mobile phase was degassed by vigorous helium bubbling and, furthermore, a low helium flow-rate was maintained through the mobile phase bottle during chromatography.

Radioactivity determinations

From the supernatant obtained after perchloric acid precipitation 0.1 ml was transferred to a glass scintillation vial containing 10 ml of the liquid scintillator (Riafluor, New England Nuclear, Boston, MA, U.S.A.). Similarly the radioactivity was determined in a 0.1-ml aliquot from the final eluate. The β-liquid scintillation counter was an instrument from Intertechnique (Model SL-4001).

Calculations

The amount of 5-S-cysteinyldopa in 0.1 ml of the final eluate (A_E) was determined by comparing the sample peak height (H_{sample}) with the peak height ($H_{standard}$) of the standard solution (27.5 pmol per 100 μl) according to the formula

$$A_E = \frac{H_{sample}}{H_{standard}} \times 27.5 \text{ pmol}$$

The amount of 5-S-cysteinyldopa in 0.1 ml of the supernatant after perchloric acid precipitation (A_P) was determined according to the formula

$$A_P = A_E \times \frac{cpm_P}{cpm_E} \text{ pmol}$$

where cpm_P and cpm_E are the radioactivity counts in the supernatant and eluate, respectively.

The total amount of 5-S-cysteinyldopa in the mixed sample (volume 8.85 ml) is then $A_P \times 88.5$ pmol. From this, the added chemical amount of tritium-labelled 5-S-cysteinyldopa (493 pmol) was subtracted. The original concentration in the urinary sample was then finally determined as

$$\frac{(A_P \times 88.5) - 493}{6 \times 1000}\ \mu mol/l.$$

RESULTS

Synthesis of tritium-labelled 5-S-cysteinyldopa

5-S-Cysteinyl-L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine was synthesized by scaling down the amounts and volumes used in the macroscale method for 5-S-cysteinyldopa synthesis. It should be noted that a number of side reaction products may be obtained, especially if the tyrosinase reaction is continued for too long. Fig. 1 shows the chromatography of a successful synthesis of tritium-labelled 5-S-cysteinyldopa on AG 50 W-X4. A major radioactivity peak was identified by HPLC as 5-S-cysteinyldopa. To check the radiochemical purity of the labelled 5-S-cysteinyldopa, samples from different parts of the radioactivity peak were analyzed by HPLC. The effluent from the liquid chromatograph was collected after the electrochemical cell, and the radioactivity was determined. Good agreement was obtained between the electrochemical detection and the radioactivity determinations, except for the first few fractions of the 5-S-cysteinyldopa peak (Fig. 1) which contained a small radioactive contaminant. Otherwise the fractions contained pure 5-S-cysteinyldopa, and after pooling fractions 55—73, 90 ml of tritium-labelled 5-S-cysteinyldopa, 9.86 μmol/l, were obtained with a specific activity of 188 mCi/mmol (7.0 GBq/mmol).

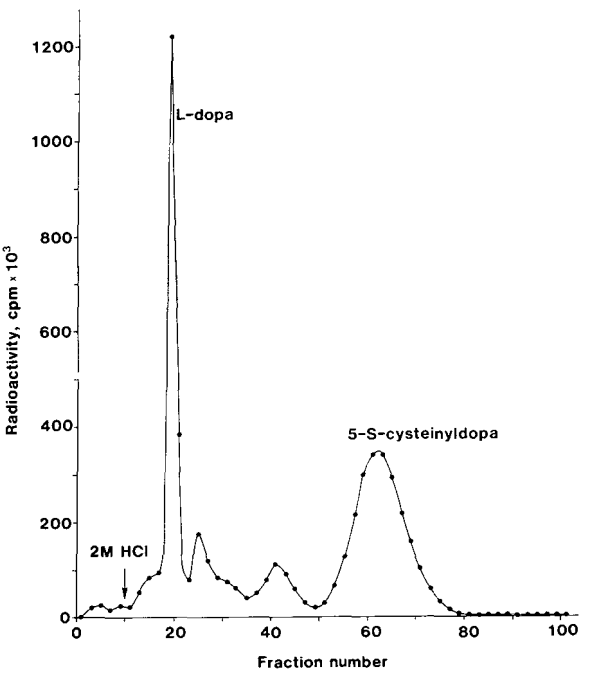

Fig. 1. Purification of 5-S-cysteinyl-L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine synthesized from L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine and cysteine by chromatographic cation exchange (AG 50 W-X4, bed dimensions 180 × 10 mm). Tritium activity was determined in aliquots from the fractions. The L-DOPA and 5-S-cysteinyldopa were identified by HPLC.

Optimization of HPLC conditions

We first conducted the HPLC in a manner similar to that described by Hansson et al. [10]. However, when analyzing urine samples we found that several other peaks with retention times up to 2 h were obtained. Various procedures were then tried in order to select a procedure which gave a well cleaned-up sample. Preliminary investigations with a combined treatment of the sample with a cation exchanger and alumina gave promising results and showed that the HPLC conditions could favourably be changed to give shorter retention times for 5-S-cysteinyldopa. Therefore, we changed to a Supelcosil LC-8 column which gives a shorter retention time and in order to spare the HPLC equipment the pH of the mobile phase was increased to 2.55, which also gives a lower capacity factor [11].

Initially we found high background currents (usually 5—22 nA) with the original composition of the mobile phase. The effects of including Na_2EDTA in the mobile phase were therefore studied and we found that a concentration of 0.1 mmol/l gave a satisfactory baseline current (<1 nA).

Purification of urinary 5-S-cysteinyldopa

In preliminary experiments using the strong cation exchanger AG 50 W-X8 the 5-S-cysteinyldopa was desorbed from the ion exchanger by elution with hydrochloric acid, 2 mol/l [13]. Although an improved clean-up of the sample was obtained, the 5-S-cysteinyldopa was eluted from the column in a large volume. However, it was found that also when 5-S-cysteinyldopa was eluted with citrate—phosphate buffer, 0.5 mol/l, pH 4.0, an improvement of the purification was obtained. An elution diagram of 5-S-cysteinyldopa from AG 50 W-X8 is shown in Fig. 2. It can be seen that most of the 5-S-cysteinyldopa could be collected in an eluate of 7 ml. Fig. 3 shows the HPLC results after purification of a urinary sample from a patient with malignant melanoma on AG 50 W-X8, and for comparison purposes Fig. 4 shows the results obtained when the clean-up was performed with alumina as in the original procedure. A number of peaks were obtained in both chromatograms and some of the peaks interfered with the 5-S-cysteinyldopa peak. However, when the two procedures were combined a marked improvement was found (Figs. 5 and 6). This combined clean-up procedure was therefore taken as the final procedure.

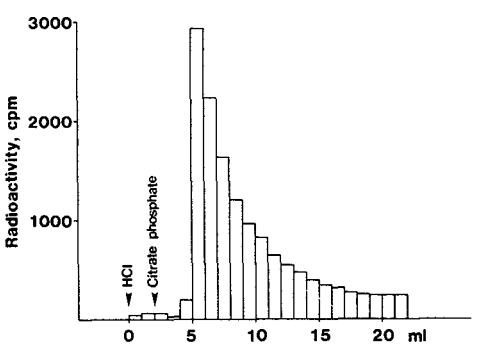

Fig. 2. Elution of 5-S-cysteinyl-L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine from AG 50 W-X8 (100—200 mesh) with citrate—phosphate buffer, 0.5 mol/l, pH 4.0.

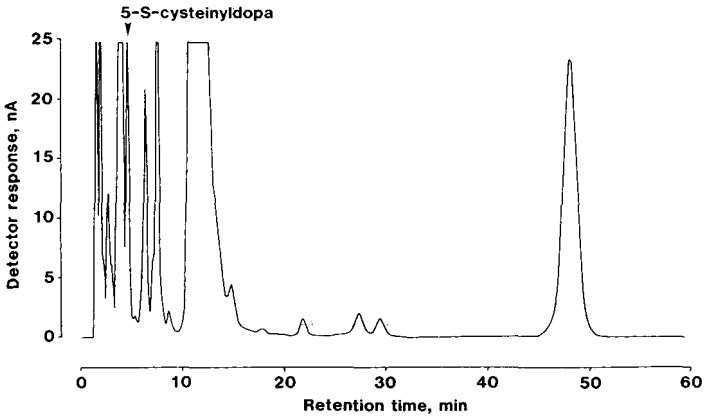

Fig. 3. HPLC of urine from a patient with malignant melanoma. The urine was pretreated on AG 50 W-X8 (cf. Fig. 2) and 7 ml of eluate were pooled.

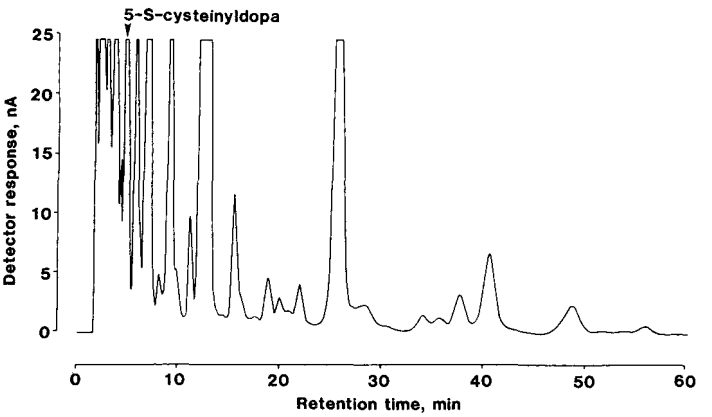

Fig. 4. HPLC of urine (same as in Fig. 3) adsorbed on alumina at pH 8.6 and desorbed with perchloric acid, 1.0 mol/l.

For the determination of urinary concentration the tritium-labelled 5-S-cysteinyldopa was added to the urine as described in the Experimental section.

Method evaluation

The standard curve was linear over the range 3.5—110 pmol of 5-S-cysteinyldopa injected, and no intercept was found. The precision of the HPLC was evaluated from peak height measurements after repeated injections (n = 10) of standard 5-S-cysteinyldopa (27.5 pmol per 100 μl) during 2 h. A coefficient of variation (C.V.) of 1.04% was found. When during ordinary working days the standard solution was injected with longer intervals the C.V. varied from 0.91 to 3.4%. This clearly showed that the determinations of 5-S-cysteinyldopa in the samples could well be done by comparison with an external 5-S-cysteinyldopa standard.

The intra-assay precision of the method for urinary determination was evaluated from duplicate analyses. For urines (n = 24) with 5-S-cysteinyldopa

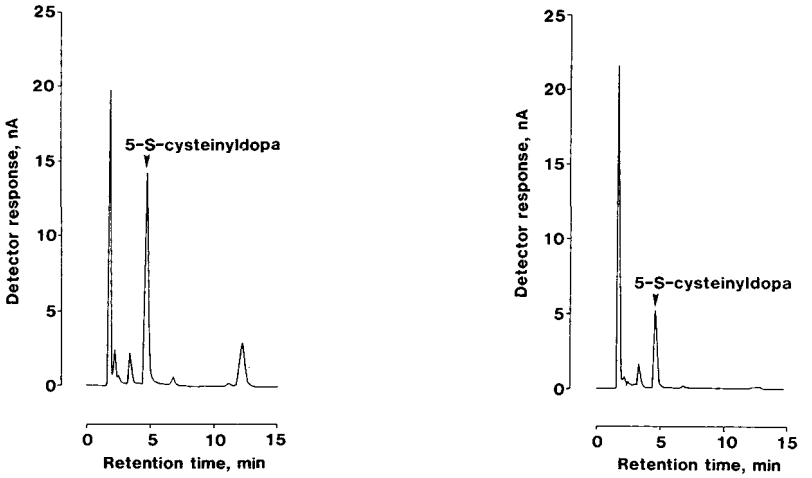

Fig. 5. HPLC of urine after combined treatment with AG 50 W-X8 and alumina. Same urine as in Figs. 3 and 4.

Fig. 6. HPLC of urine from a healthy subject. Urine clean-up as in Fig. 5.

concentrations between 0.02 and 0.6 μmol/l (mean 0.344 μmol/l) the standard deviation (S.D.) was calculated as 0.020 μmol/l which gave the C.V. as 5.7%. From the S.D. of 0.020 μmol/l the detection limit was calculated as 0.04 μmol/l. For urines with high 5-S-cysteinyldopa concentrations (n = 19) the resulting C.V. was 4.9% (mean = 2.60 μmol/l, S.D. = 0.128 μmol/l). The inter-assay variation was evaluated from repeated (n = 18) determination during a period of three months of a urinary sample stored frozen. From the mean of 0.521 μmol/l and an S.D. of 0.069 μmol/l the C.V. was found to be 13.3%. The recovery of 5-S-cysteinyldopa added to urines was satisfactory (Table I).

TABLE I

ANALYTICAL RECOVERY OF 5-S-CYSTEINYLDOPA ADDED TO SEVEN URINE SAMPLES, CORRESPONDING TO AN INCREASE IN CONCENTRATION OF 0.69 μmol/l

Initially present (μmol/l)	After addition (μmol/l)	Increase found (μmol/l)	Recovery (%)
0.24	0.97	0.73	105.8
0.50	1.27	0.77	111.6
0.29	1.02	0.73	105.8
0.44	1.20	0.76	110.1
0.14	0.91	0.77	111.6
0.24	0.95	0.71	102.9
0.49	1.09	0.60	87.0
	Mean ± S.D.		105.0 ± 8.6

Urinary excretion of 5-S-cysteinyldopa

The excretion of 5-S-cysteinyldopa was measured in 24 healthy subjects (12 men and 12 women) who collected urine during periods unexposed to sunshine. The mean (± S.D.) urinary excretion was 0.34 ± 0.13 μmol per 24 h (range 0.02—0.58 μmol per 24 h) and no sex difference was found (men 0.36 ± 0.13 μmol per 24 h, women 0.33 ± 0.13 μmol per 24 h). The distribution of the urinary excretion is illustrated in Fig. 7 together with the results from 13 patients with a diagnosis of malignant melanoma with metastases. The urinary excretion of 5-S-cysteinyldopa in the latter was highly increased, ranging from 0.9 to 4.8 μmol/l.

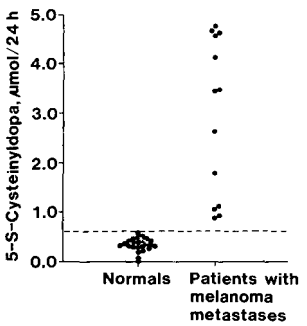

Fig. 7. Urinary excretion of 5-S-cysteinyldopa in healthy subjects and in patients with advanced malignant melanoma.

DISCUSSION

Classically catechols are purified from urine by alumina adsorption at alkaline pH and desorption with a strong mineral acid [12]. This method has also been used in the clean-up of urinary 5-S-cysteinyldopa [8, 10, 11], but in our hands did not give satisfactory results. However, since 5-S-cysteinyldopa in addition to the catechol moiety also contains an amino acid residue, we tried to use a clean-up procedure involving a strong cation exchanger. Also this procedure gave a number of peaks in the HPLC trace. However, when the two procedures were combined, the unrelated peaks were successfully eliminated indicating that different "impurities" were eliminated by the two clean-up procedures.

In contrast to earlier HPLC for 5-S-cysteinyldopa determination [10, 11] we included Na_2EDTA in the mobile phase. This markedly improved the unsteady baseline and lowered the baseline current from about 5—20 nA to less than 1 nA. We chose an EDTA concentration of 0.1 mmol/l in accordance with Moyer and Jiang [18]. In a recent paper Warsh et al. [19] stated that this is a rather high concentration, which causes the detector responses to deteriorate very rapidly. However, we have not noted such an effect with our mobile phase.

With the clean-up we describe here we had the goal to obtain a clean 5-S-cysteinyldopa eluate, and therefore other catechols were not recovered. With the procedure finally adopted the use of α-methyldopa or isoproterenol as internal standard [10] was therefore precluded. We conducted experiments to add these catechols to the final eluates for evaluation of the chromatograms.

We found however (data not shown) that evaluation of the chromatograms by peak height ratio did not improve the precision to any appreciable extent in comparison with peak height evaluation of 5-S-cysteinyldopa (C.V. = 1.04%). This was in accordance with the findings reported by Haefelfinger [20] who showed that in HPLC with automatic injection with complete loop filling (100 μl) the precision of the injection was not the limiting factor for the reproducibility and the internal standard brought no evident advantages.

For the quantitative determination of urinary 5-S-cysteinyldopa we had to determine the individual recovery. We solved this problem by adding tritium-labelled 5-S-cysteinyldopa to the sample and counting the radioactivity before and after the clean-up. This extra step may be considered as a drawback, especially since tritium-labelled 5-S-cysteinyldopa first had to be synthesized. However, we found that tritiated 5-S-cysteinyldopa could easily be synthesized for this purpose and it was stable for several months. The availability of such preparations was also very useful for the development of this method, and may be of importance in other, for example in vivo, studies.

In Table II the results we obtained for urinary excretion of 5-S-cysteinyldopa in healthy subjects can be compared with earlier studies [21—23] using the fluorimetric method [8]. We could not demonstrate any significant difference between men and women as in the earlier studies. This may depend on the rather limited number studied. The absolute amounts excreted, however, were in quite good accordance with earlier findings. This indicates that from the standpoint of evaluating patients' results, similar reference values may be used in clinical practice.

TABLE II

MEAN URINARY EXCRETION OF 5-S-CYSTEINYLDOPA IN HEALTHY SUBJECTS

Number of subjects in parentheses.

Reference	μmol per 24 h		
	Men	Women	Men and women
Agrup et al. [21]	0.32 (30)	0.25 (46)	0.27 (76)
Morishima and Hanawa [22]	0.36 (10)	0.25 (10)	0.30 (20)
Graef and Paul [23]	—	—	0.28 (45)
Present study	0.36 (12)	0.33 (12)	0.34 (24)

ACKNOWLEDGEMENT

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CHROMBIO. 1505

DOSAGE PLASMATIQUE ET URINAIRE DU 3,3,5-TRIMÉTHYLCYCLOHEXANOL-*cis* LIBRE ET CONJUGUÉ

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(Reçu le 11 juin 1982; manuscrit modifié reçu le 9 septembre 1982)

SUMMARY

Determination of free and conjugated cis-3,3,5-trimethylcyclohexanol in plasma and urine

A gas chromatographic procedure was developed to determine free and conjugated *cis*-3,3,5-trimethylcylcohexanol in plasma and urine.

The sample is extracted with dichloromethane when free *cis*-3,3,5-trimethylcyclohexanol is determined, or with hexane after enzymatic hydrolysis, when conjugated *cis*-3,3,5-trimethylcyclohexanol is determined. An aliquot of the organic extract is injected into a stainless-steel column (packed with Carbowax 20M, 15% on Chromosorb W AW 100—120 mesh) and detected with a flame ionization detector. Extraction recovery from plasma and urine was almost 100% and the limit of quantification was fixed at 100 ng/ml plasma or urine.

The procedure was evaluated in a pharmacokinetic study of cyclandelate and its metabolite *cis*-3,3,5-trimethylcyclohexanol.

INTRODUCTION

Le cyclandélate ou 3,3,5-triméthylcyclohexylmandélate est un vasodilatateur périphérique largement répandu en thérapeutique [1—5]. Au cours d'études portant sur la pharmacocinétique et le métabolisme de ce principe actif, une biotransformation rapide de l'ester a été mise en évidence, libérant dans l'organisme deux métabolites: l'acide mandélique et le 3,3,5-triméthylcyclohexanol-*cis* selon la Fig. 1. Dans une précédente publication [6], nous avions décrit le dosage plasmatique de la drogue active et d'un de ses métabolites: l'acide mandélique.

Le travail qui suit décrit la détermination quantitative de l'autre métabolite, le 3,3,5-triméthylcyclohexanol-*cis* libre et conjugué, dans le plasma et l'urine

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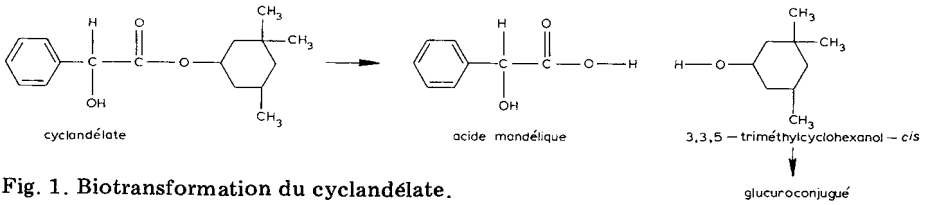

Fig. 1. Biotransformation du cyclandélate.

par chromatographie en phase gazeuse, après extraction de la substance dans le dichlorométhane (3,3,5-triméthylcyclohexanol-*cis* libre) ou dans l'hexane (3,3,5-triméthylcyclohexanol-*cis* total).

MÉTHODES EXPÉRIMENTALES

Réactifs

L'acide acétique, l'hydroxyde de sodium (de pureté analytique), l'hexane pour spectrographie ultraviolette sont des réactifs du commerce utilisés sans purification préliminaire. Le dichlorométhane doit obligatoirement être de qualité pour spectrographie ultraviolette et fourni par le Laboratoire Merck. La β-glucuronidase (Sigma Type B1 extrait du foie de boeuf) est utilisée en solution à environ 250 unités Fishman/ml de tampon pH 4.7, composé d'acide acétique en solution molaire et d'hydroxyde de sodium en solution molaire selon des proportions en volume 2:1 (1 unité Fishman représente la libération de 1 μg de phénolphthaléine par heure à partir du phénolphtaléine glucuronide à pH 4.7 et 37°C). Cette solution est préparée avant chaque analyse. Les solutions standards de 3,3,5-triméthylcyclohexanol-*cis* (I) (Veba-Chemie) et de cyclohexanol (II) (Merck) sont préparées dans de l'eau purifiée.

Instruments

Les différentes analyses ont été effectuées sur un chromatographe en phase gazeuse (Modèle Sigma 1, Perkin-Elmer) équipé d'un détecteur à ionisation de flamme et relié à un intégrateur automatique programmable (Sigma 10, Perkin-Elmer). La colonne de 2 m × 3.2 mm en acier inoxydable est remplie de Carbowax 20M fixée à 15% sur du Chromosorb W AW 100—120 mesh. Les températures de l'injecteur et du détecteur sont respectivement de 250°C et 280°C. La température initiale du four est fixée à 110°C avec une programmation prévoyant un palier initial de 2 min, une montée en température jusqu'à 180°C à raison de 5°C/min et un palier final à cette température pendant 6 min. L'azote est utilisé comme gaz vecteur à un débit de 20 ml/min.

Préparation des échantillons

3,3,5-Triméthylcylcohexanol-cis libre plasmatique et urinaire. 50 μl de la solution d'étalon interne (cyclohexanol à 40 mg/100 ml d'eau purifiée) sont rajoutés, dans un tube de centrifugation de 15 ml, à 1 ml de plasma ou 1 ml d'urine diluée au demi avec de l'eau purifiée. L'extraction par 10 ml de dichlorométhane s'opère par agitation pendant 2 min sur un vortex. Après séparation des deux phases par centrifugation (10 min à 40,000 *g* et 5°C), la phase aqueuse surnageante est éliminée. La phase organique est évaporée partiellement sous vide (environ 1 ml) au moyen d'un évaporateur rotatif,

puis transvasée dans un tube à fond conique. L'évaporation se poursuit à froid jusqu'à 50 μl sous un flux constant d'azote. 1 μl de cette solution est injecté sur la colonne chromatographique.

3,3,5-Triméthylcyclohexanol-cis total urinaire. Le protocole décrit précédemment pour le dosage de la forme libre a été adapté au problème de l'hydrolyse enzymatique; 2 ml de solution de β-glucuronidase à environ 250 unités Fishman/ml de tampon pH 4.7, sont rajoutés à 0.1 ml d'urine et placés à l'étuve à 37°C pendant 72 h. Au bout de ce temps, 0.5 ml de la solution de cyclohexanol à 40 mg/100 ml d'eau purifiée sont ajoutés puis la solution enzymatique transférée dans un ballon de distillation. Le tube est rincé avec 18 ml d'eau purifiée et les eaux de rinçage rajoutées dans le ballon. 10 ml de la solution aqueuse sont distillés; ils contiennent le 3,3,5-triméthylcyclohexanol-*cis* total et le cyclohexanol. 1 ml de distillat est repris dans un tube à centrifuger de 15 ml, agité pendant 2 min avec 10 ml d'hexane puis centrifugé pendant 10 min à 40,000 *g* et 5°C. La phase hexane surnageante est récupérée dans un ballon, évaporée partiellement (environ 1 ml) sous vide à l'évaporateur rotatif, transvasée dans un flacon à fond conique puis évaporée jusqu'à 50 μl sous un flux d'azote. 1 μl de cette solution est injectée dans le chromatographe en phase gazeuse.

Méthode de quantification

Les aires des pics du 3,3,5-triméthylcyclohexanol-*cis* (I) et du cyclohexanol (II) sont fournies par l'intégrateur Sigma 10B, et les rapports sont calculés par division respective des aires de (I) par celles de (II).

Une droite d'étalonnage dans le plasma et l'urine pour des concentrations croissantes de (I), en présence de (II), est effectuée selon la méthodologie décrite; après son analyse selon le principe de la régression linéaire des moindres carrés, elle fournie une équation qui servira ultérieurement lors de l'évaluation d'échantillons inconnus.

L'évaluation quantitative du 3,3,5-triméthylcyclohexanol-*cis* total s'effectue selon le même principe, mais au moyen d'une gamme étalon [entre 1 et 100 μg de (I)], réalisée pour chaque série d'extractions, afin d'éliminer toutes les erreurs introduites par une distillation peu reproductible jour après jour. Les concentrations en 3,3,5-triméthylcyclohexanol-*cis* conjugué sont déduites par simple soustraction entre les concentrations de formes totales et libres.

RÉSULTATS ET DISCUSSION

Analyse du 3,3,5-triméthylcyclohexanol-cis libre plasmatique ou urinaire

Chromatogramme. L'analyse en chromatographie en phase gazeuse d'un plasma ou urine vierge est présentée en Fig. 2A. La Fig. 2B montre le 3,3,5-triméthylcyclohexanol-*cis* (I) et le cyclohexanol (II) (étalon interne) après leur addition et extraction suivant la méthodologie d'extraction de la forme libre décrite précédemment. Les temps de rétention sont respectivement de 9.94 et 6.96 min pour I et II.

Fonction d'étalonnage. La linéarité de la méthode a été étudiée pour des quantités croissantes (0.1---40 μg) de 3,3,5-triméthylcyclohexanol-*cis* rajoutés au plasma ou à l'urine. Le cyclohexanol (étalon interne) se trouve dans tous les

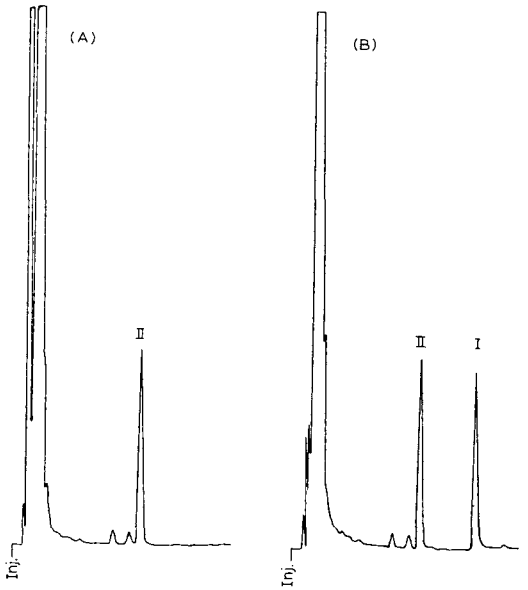

Fig. 2. Chromatogrammes d'un extrait plasmatique. (A) Plasma vierge en présence de 20 μg de cyclohexanol (II) (étalon interne). (B) Plasma + 20 μg de 3,3,5-triméthylcyclohexanol-*cis* (I) et 20 μg de cyclohexanol (II). Temps de rétention: 9.94 min pour I et 6.96 min pour II.

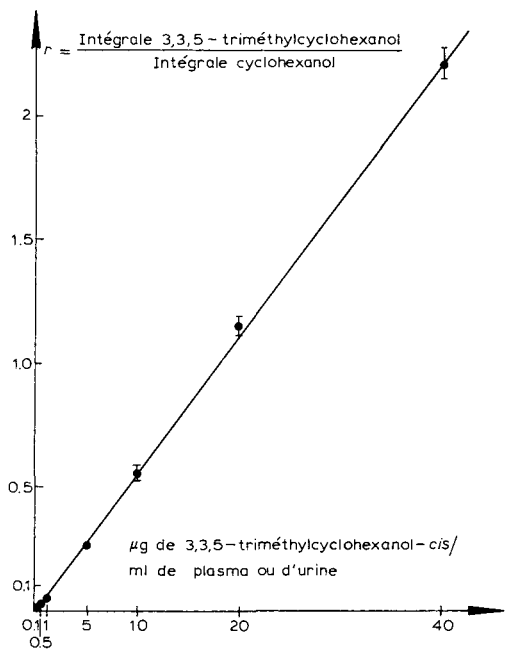

Fig. 3. Droite d'étalonnage du 3,3,5-triméthylcyclohexanol-*cis* dans le plasma ou l'urine après extraction par le dichlorométhane.

échantillons à la concentration de 20 μg/ml de plasma ou d'urine. La droite de calibration (Fig. 3) obtenue est linéaire dans la gamme de concentration choisie comme l'indique le coefficient de corrélation r entre la quantité de μg de 3,3,5-triméthylcyclohexanol-*cis* rajoutée et le rapport des intégrales 3,3,5-triméthylcyclohexanol-*cis*/cyclohexanol.

La droite de régression a pour équation:

$Y = 0.0556X + 0.00005; r = 0.9993$

où X = μg de 3,3,5-triméthylcyclohexanol-*cis* libre/ml de plasma ou d'urine, et Y = rapport des intégrales 3,3,5-triméthylcyclohexanol-*cis*/cyclohexanol.

Précision

L'extraction, pour les différentes concentrations de 3,3,5-triméthylcyclohexanol-*cis* a été réalisée pendant 6 jours consécutifs. Les résultats obtenus (Tableau I) démontrent une bonne reproductibilité interjournalière, les coefficients de variation se situant entre 2.5% (40 μg/ml) et 7.7% (0.5 μg/ml). Seule la concentration minimale (0.1 μg/ml présente un coefficient de variation important: 20%.

TABLEAU I

RAPPORT DES INTÉGRALES 3,3,5-TRIMÉTHYLCYCLOHEXANOL—*cis* LIBRE/CYCLOHEXANOL EN FONCTION DE LA QUANTITÉ DE 3,3,5-TRIMÉTHYLCYCLOHEXANOL-*cis* (EN μg/ml DE PLASMA OU URINE) APRÈS EXTRACTION DANS LE DICHLORORÉTHANE

μg/ml	Essais						$\bar{x} \pm \sigma$	C.V. (%)
	1	2	3	4	5	6		
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000 ± 0.000	0
0.1	0.005	0.006	0.004	0.005	0.003	0.006	0.005 ± 0.001	20
0.5	0.029	0.028	0.026	0.028	0.024	0.023	0.026 ± 0.002	7.7
1	0.047	0.050	0.051	0.048	0.053	0.049	0.050 ± 0.002	4.0
5	0.263	0.261	0.274	0.258	0.261	0.283	0.267 ± 0.010	3.8
10	0.587	0.591	0.567	0.523	0.524	0.552	0.557 ± 0.030	5.4
20	1.173	1.161	1.138	1.196	1.119	1.099	1.148 ± 0.036	3.1
40	2.189	2.124	2.289	2.234	2.208	2.210	2.209 ± 0.054	2.5

Rendement

La détermination du rendement de l'extraction est obtenue en comparant la droite de calibration obtenue avec des prélèvements plasmatiques ou urinaires à celle d'une gamme étalon de 3,3,5-triméthylcyclohexanol-*cis* étalon dans le dichlorométhane. La droite de régression: $Y = 0.0555X - 0.0059$ obtenue au cours de ces essais est en tout point comparable à celle obtenue après extraction. Le rendement de l'extraction peut ainsi être évalué à près de 100%.

Limite de dosage

La limite de dosage, déterminée avec une probabilité de $p = 0.05$ est de 100 ng/ml de plasma ou d'urine.

Analyse du 3,3,5-triméthylcyclohexanol-cis total

Chromatogramme. L'analyse chromatographique d'un échantillon d'urine de lapin obtenue après administration orale de 500 mg/kg de cyclandélate est présentée en Fig. 4. Les temps de rétention sont identiques à ceux obtenus lors du passage de la forme libre de l'alcool.

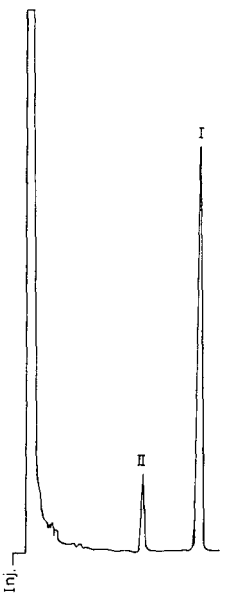

Fig. 4. Chromatogramme d'un échantillon d'urine de lapin après administration orale de 500 mg/kg de cyclandélate. Détermination du 3,3,5-triméthylcyclohexanol-*cis* total (I). Temps de rétention: 9.94 min pour I et 6.96 min pour cyclohexanol (II).

CONCLUSION

La méthode décrite est linéaire sur une gamme assez large (0.1—40 µl/ml plasma ou urine dans le cas de la forme libre, 1—100 µg/ml plasma ou urine pour la forme totale), précise, sensible et ceci malgré un protocole relativement laborieux et long d'exécution.

Elle a trouvé une application dans l'étude de pharmacocinétique et de métabolisme du cyclandélate adminstré au lapin par voie intraveineuse ou par voie orale.

Les concentrations plasmatiques et urinaires en 3,3,5-triméthylcyclohexanol-*cis* libre et conjugué ont été déterminées et la biotransformation de l'ester (cyclandélate) évaluée en fonction du temps.

Les résultats obtenus au cours de ce travail feront l'objet d'une autre publication.

RÉSUMÉ

Nous avons développé une méthode de dosage du 3,3,5-triméthylcyclohexanol-*cis* libre et conjugué dans le plasma et l'urine par chromatographie en phase gazeuse.

L'échantillon est extrait avec le dichlorométhane lors de la détermination du 3,3,5-triméthylcyclohexanol-*cis* libre ou avec l'hexane après hydrolyse enzymatique lors de l'analyse du 3,3,5-triméthylcyclohexanol-*cis* conjugué, injecté dans une colonne en acier inoxydable (Carbowax 20M à 15% sur Chromosorb W AW 100–120 mesh) et détecté au moyen d'un détecteur à ionisation de flamme. Le rendement de l'extraction à partir du plasma et de l'urine a été de près de 100%, et la limite de dosage fixée à 100 ng/ml de plasma ou d'urine.

La méthode a été évaluée dans le cadre d'une étude pharmacocinétique du cyclandélate et de son métabolite le 3,3,5-triméthylcyclohexanol-*cis*.

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CHROMBIO. 1502

GAS CHROMATOGRAPHIC ANALYSIS OF CHLOROQUINE AFTER A UNIQUE REACTION WITH CHLOROFORMATES

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SUMMARY

A specific method for the gas chromatographic determination of chloroquine (CQ) after derivatization with chloroformates, using 9-bromophenanthrene as the internal standard and a column filled with 3% OV-17 on 80—100 mesh Supelcoport is described. Derivatization with chloroformates produced a pyrrolidine derivative, 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline with CQ, and a carbamate with desethylchloroquine. The chloroformate reaction for CQ is thus selective in the presence of CQ metabolites. The method based on flame ionization detection is highly suitable for quantitation of CQ in urine.

INTRODUCTION

Chloroquine (CQ) has a worldwide use in the therapy of malaria [1], is a potent antirheumatic agent [2] and is effective in the treatment of lupus erythematosus [3]. The analysis of CQ, a secondary aromatic amine with a tertiary aliphatic amine side-chain, in biological fluids and tissues has been performed mainly by a fluorometric method [4, 5], which requires prior removal of metabolites that otherwise would be determined as CQ. Recently, a liquid chromatographic method was described for CQ and its metabolite desethylchloroquine [6]. Gas chromatographic (GC) methods previously described for CQ [7, 8] did not take into consideration the potential interference from this metabolite which, as demonstrated in the present study, elutes together with CQ. Also, these methods do not have the sensitivity

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required for analysis in blood. An improved GC method was therefore sought, based on the well known reaction of chloroformates with tertiary amines, yielding carbamates [9—11].

This paper describes the unique reaction of CQ with chloroformates to form a pyrrolidine derivative, 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline, and the carbamate of diethylamine (Fig. 1).

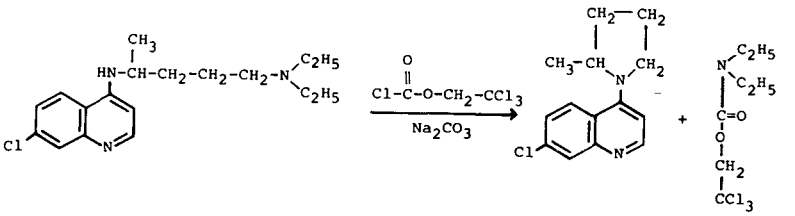


Fig. 1. The reaction of chloroquine with trichloroethyl chloroformate.

EXPERIMENTAL

Standards and reagents

Chloroquine phosphate and desethylchloroquine were kindly supplied by Sterling-Winthrop Research Labs. (Rensselaer, NY, U.S.A.). Trichloroethyl, methyl, ethyl, benzyl and isobutyl chloroformates and 9-bromophenanthrene were obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium carbonate, anhydrous, was obtained from Baker (Phillipsburg, NJ, U.S.A.). Nanograde methylene chloride was obtained from Fisher (Silver Springs, MD, U.S.A.). The alcoholic alkali solution was 0.5 *M* potassium hydroxide in methanol. Aqueous reagents were prepared from glass-distilled water and stored in glass bottles with PTFE-lined caps.

Syntheses

4-(2-Methyl-1-pyrrolidyl)-7-chloroquinoline was synthesized by a modification of a previously described method [12]. The side-chain precursor, 4-amino-1-pentanol, was prepared by reduction of 1-hydroxy-4-pentanone oxime with sodium and absolute ethanol. 4,7-Dichloroquinoline was then combined with the 4-amino-1-pentanol and heated neat, to yield 4-(4-hydroxy-1-methylbutylamino)-7-chloroquinoline, which upon action of hydrobromic acid and heat gave the pyrrolidine compound. The final product as well as the intermediates was characterized by melting point and mass spectrometry (MS).

Trichloroethyl N,N-diethylcarbamate was synthesized by dropwise addition of excess diethylamine in tetrahydrofuran to trichloroethyl chloroformate, also in tetrahydrofuran. Excess diethylamine and tetrahydrofuran were removed by evaporation. Identity was confirmed by mass spectral analysis.

Instruments

GC. A Varian Model 1440 gas chromatograph equipped with a flame ionization detector was used. The column (180 cm × 2 mm I.D.) was made of pyrex glass and packed with 3% OV-17 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min. Air and hydrogen flow-rates for the flame ionization detector

were 300 and 40 ml/min, respectively. The column temperature was 230°C (110°C for identification of carbamates of diethylamine) and the injector and detector temperatures were 240°C and 265°C, respectively.

GC—MS. Mass spectra were obtained with an LKB 9000S gas chromatograph—mass spectrometer operated at an accelerating voltage of 3.5 kV, ionization voltage of 20 eV, and a trap current of 60 μA. The column was the same as above. Fragment ions with a relative intensity of less than 5% were omitted for simplicity.

Examination of derivatization conditions

Reaction conditions were studied using the following derivatization procedure. CQ base was prepared from an alkalinized solution of CQ phosphate in water extracted with methylene chloride. The organic phase was evaporated to dryness under a gentle stream of nitrogen. To 200 μg of CQ base and 25 μg of 9-bromophenanthrene as internal standard in 200 μl of methylene chloride were added 10 μl of isobutyl chloroformate and about 10 mg of anhydrous sodium carbonate. The mixture was left at room temperature; 1—2 μl of the organic phase were injected at intervals into the gas chromatograph. The peak height ratio of the formed derivative to internal standard was measured. The percentage yield of derivative was calculated relative to maximum yield. The influence of solvent, temperature and the amounts of isobutyl chloroformate and sodium carbonate on the reaction rate was investigated, as were the comparative reactivities of methyl, ethyl, benzyl and trichloroethyl chloroformates.

Prior to GC the reaction products from CQ and isobutyl chloroformate were examined by thin-layer chromatography [silica gel GF; solvent system 25% ammonia—methanol (3:200), UV detection] and compared to CQ and synthetic 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline.

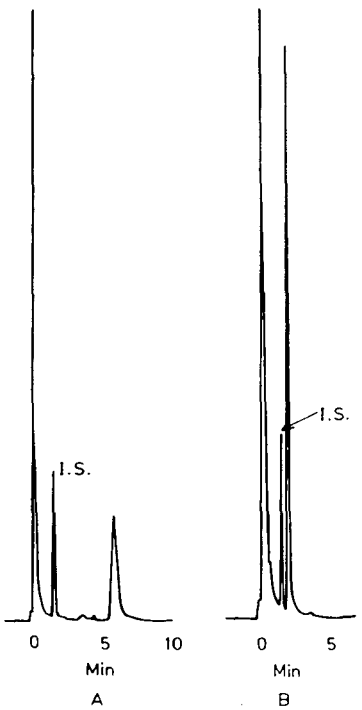
The reaction of desethylchloroquine with isobutyl chloroformate and its separation from CQ by GC were also examined.

Standard curves

Varying quantities of CQ (10—400 μg) and 10 or 50 μg of 9-bromophenanthrene as internal standard were added to 1.0-ml samples of human urine in 15-ml round-bottomed centrifuge tubes. The urine was made alkaline (pH > 13) with an equal volume of 1 *N* sodium hydroxide in 10% sodium chloride, and extracted on a reciprocating shaker for 10 min with 10 ml of methylene chloride. After centrifuging for 5 min at 2000 *g*, the organic phase was transferred to a conical centrifuge tube and evaporated to dryness in a water bath at 35°C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of methylene chloride; about 10 mg anhydrous sodium carbonate and 10 μl of isobutyl chloroformate were added and the mixture was left at room temperature for 1 h with the tubes tightly capped. Excess chloroformate was hydrolyzed by shaking the reaction mixture vigorously for 30 sec with 0.5 ml of 0.5 *M* alcoholic alkali solution. After the addition of 0.5 ml of water, shaking and centrifugation, the aqueous phase was discarded. A 1—2 μl aliquot of the organic phase was injected into the gas chromatograph.

RESULTS AND DISCUSSION

The reaction of CQ with trichloroethyl chloroformate resulted in a product with a GC retention time considerably shorter than that for the underivatized drug, relative retention 0.33 (Fig. 2B compared to Fig. 2A). The shorter retention time suggested a significant reduction of the molecular weight of CQ. The electron impact mass spectrum of this peak suggested it to be 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline (Fig. 3). The retention time and mass spectrum of the synthetic reference compound confirmed this observation. An additional GC peak associated with CQ was observed when the column temperature was decreased from 230°C to 110°C. The mass spectrum of this peak compared to that of a synthetic standard demonstrated it to be trichloroethyl N,N-diethylcarbamate (Fig. 4).



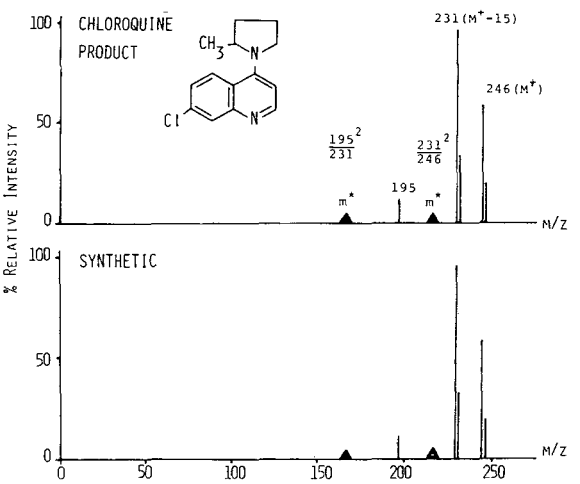


Fig. 2. Gas chromatograms of chloroquine (200 μg/ml) (A) before and (B) after chloroformate reaction with 9-bromophenanthrene (25 μg/ml) as internal standard (I.S.). Column temperature 230°C.

Fig. 3. Mass spectra of one of the derivatization products of chloroquine and synthetic 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline. An m* indicates intense metastable ions.

It is postulated that the initial reaction between CQ and trichloroethyl chloroformate yields the reactive intermediate quaternary ammonium ion generally expected from tertiary aliphatic amines and chloroformates [11]. This intermediate ion may then directly cyclize to the pyrrolidine derivative with the loss of trichloroethyl N,N-diethylcarbamate (Fig. 1). Deamination

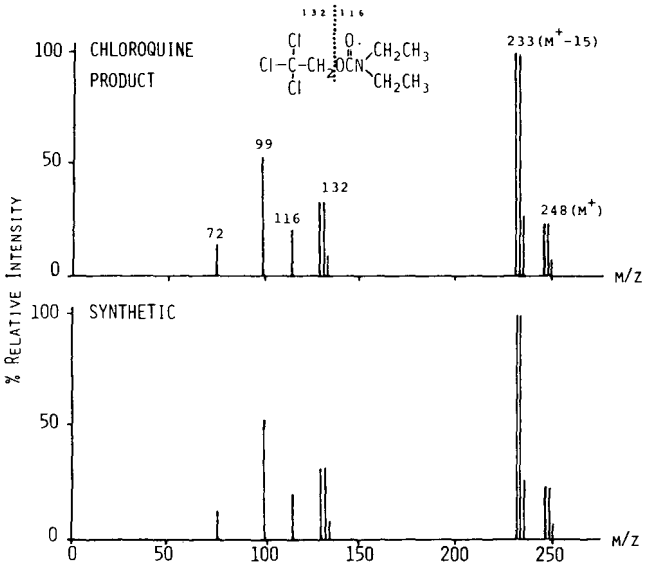


Fig. 4. Mass spectra of one of the derivatization products of chloroquine and synthetic trichloroethyl N,N-diethylcarbamate.

of the intermediate ion under formation of an organic chloride, 4-(4-chloro-1-methylbutylamino)-7-chloroquinoline in a similar fashion as shown for other tertiary amines [9, 11, 13, 14] is, however, another possibility. Such a chloride may then react with the secondary aromatic amino group, e.g. in the hot injector of the gas chromatograph, resulting in the observed cyclization. However, this chloride has been shown in the synthesis of CQ analogues [12] to be quite stable as opposed to the corresponding bromide. Moreover, it was suggested, although not unequivocally proven, by thin-layer chromatography that the pyrrolidine derivative was formed prior to the GC separation, i.e. the R_F value of the reaction product was identical to that of synthetic 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline.

When reacting CQ with several other chloroformates, e.g., methyl, ethyl, isobutyl and benzyl chloroformates, the identical cyclized pyrrolidine product was formed in addition to the corresponding carbamates of diethylamine as evidenced by GC—MS. Thus, the cyclization of CQ is a common reaction induced by all chloroformates.

The isobutyl chloroformate was the fastest reacting chloroformate, leading to complete reaction within 45 min, while the reaction with the methyl or ethyl chloroformates was complete within 2 h and after 12 h with benzyl or trichloroethyl chloroformates, when performed at room temperature using methylene chloride as the solvent and sodium carbonate as the base catalyst. In the absence of sodium carbonate the yield of the reaction with isobutyl chloroformate after 45 min was only 14%. Whereas the reaction rate in ethylene chloride and methylene chloride was about the same, it was significantly reduced in less polar solvents, e.g., hexane, heptane and benzene. Raising the reaction temperature to 50°C had no apparent effect. The pyrrolidine derivative of CQ was stable for at least 48 h.

The reaction between isobutyl chloroformate and CQ leading to the pyrrolidine derivative was highly reproducible and appeared quantitative based on comparisons with the synthetic pyrrolidine derivative. One-ml urine samples containing between 10 and 400 μg of CQ were extracted and derivatized as described in the Experimental section. There were no interferences from the biological material. The standard curve obtained was linear and passed through the origin (correlation coefficient 0.998). The coefficients of variation at the 10 and 400 μg/ml levels were 9.6% and 13%, respectively. These data indicate that this method, based on flame ionization detection, is highly suitable for quantitation of CQ in urine. It has been suggested that about one third of the administered dose of CQ is excreted unchanged in urine [15].

One of the potential problems associated with the determination of CQ in both urine and blood is the interference by CQ metabolites in the assay procedure. Without derivatization the main CQ metabolite, desethylchloroquine [15], did not separate from CQ by GC using either OV-17 or OV-1 columns. Derivatization with isobutyl chloroformate, however, produced the pyrrolidine derivative with CQ and a carbamate with desethylchloroquine, the latter derivative with a much longer retention time (t_{rel} = 3.2 compared to underivatized CQ and 9.7 compared to the pyrrolidine derivative of CQ). Structure confirmation was carried out by GC—MS. The chloroformate reaction for CQ is thus highly selective in the presence of CQ metabolites.

The chloroformate reaction with CQ appears to have several other advantages only partially explored. The cyclization of CQ markedly reduced problems of adsorption to the GC system, probably because of elimination of the highly basic aliphatic tertiary amino group. The increased volatility of the pyrrolidine derivative permitted lower column temperatures to be used for its elution. Both of these factors should promote the GC of very small sample sizes of CQ. The sensitivity of detection of the pyrrolidine derivative can be greatly enhanced, in particular by MS detection. Preliminary results also indicate that the yield of the trichloroethyl N,N-diethylcarbamate from the reaction of trichloroethyl chloroformate with CQ is also quantitative and this derivative is detectable in nanogram quantities by electron-capture detection.

To test whether the chloroformate reaction described for CQ is also applicable to other antimalarial drugs with an identical side-chain, derivatization of quinacrine was examined. Exactly the same cyclization as with CQ was found.

More generally, the results of this study add to the experience with chloroformates as derivatizing reagents for tertiary amines. Although the mechanism for the formation of the initial intermediate reaction products may be the same for all tertiary amines, the end products may differ substantially dependent on the structure of the individual molecules [9—11, 13, 14, 16] as further illustrated by our findings with CQ.

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DETERMINATION OF THE CALCIUM ANTAGONIST FLUNARIZINE IN BIOLOGICAL FLUIDS BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the quantitation of flunarizine in biological fluids including plasma, urine, milk, fecal and tissue homogenates using the analogue cinnarizine as the internal standard. As little as 1.5 ng of flunarizine per ml of plasma can be accurately quantitated, this being achieved by the combination of a selective extraction procedure and a nitrogen detector. The method has been used to determine the concentration of flunarizine in biological fluids in support of human and animal pharmacokinetic studies.

INTRODUCTION

Flunarizine, 1-cinnamyl-4[bis(*p*-fluorophenyl)methyl]piperazine (I, Fig. 1) is a new member of the calcium channel blocker family of compounds [1] synthesized and marketed by Janssen Pharmaceutica in Europe and currently

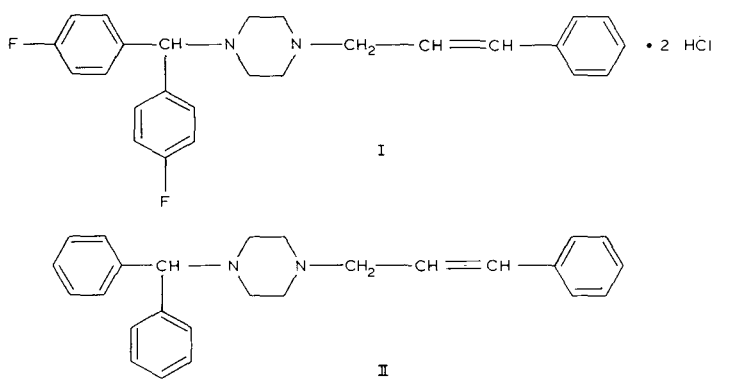

Fig. 1. Chemical structures of flunarizine (I) and cinnarizine (II).

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under clinical investigation by Ortho Pharmaceutical. In order to determine its pharmacokinetics and disposition, a sensitive and specific gas chromatographic assay was developed to quantitate flunarizine in plasma, urine, milk, fecal and tissue homogenate samples using the analogue cinnarizine (1-cinnamyl-4-diphenylmethylpiperazine) (II, Fig. 1) as the internal standard.

EXPERIMENTAL

Gas chromatography

A Varian Model 3700 gas chromatograph equipped with two thermionic specific detectors and two Varian auto samplers was used for analyses (Varian Assoc., Palo Alto, CA, U.S.A.). The 1.8 m × 2 mm I.D. glass column was packed with 3% OV-17 on 80—100 mesh Gas-Chrom Q (Supelco, Bellefonte, PA, U.S.A.). The column and injector port temperatures were maintained at 300°C and the detectors at 330°C. Flow-rates were 30—50 ml/min for the helium carrier gas, 100—170 ml/min for the air and 1—2 ml/min for the hydrogen. Bead current adjust was maintained between 550—650 and bias voltage between 5 and 6 for proper sensitivity. Chromatograms were traced on a strip-chart recorder (Houston Instuments, Austin, TX, U.S.A.). Peak heights were measured by a Model 3354 B computer interfaced with the detector by a Model 18652A A/D converter (Hewlett-Packard, Avondale, PA, U.S.A.).

Materials

Flunarizine dihydrochloride and cinnarizine were obtained from Janssen Pharmaceutica (Beerse, Belgium). PTFE-lined, screw-cap, 12-ml culture tubes were used for the extraction of the biological fluids. Kimax 3-ml conical tubes used for evaporation of the organic extract prior to injection were neutralized by immersion into a 10 *M* sodium hydroxide solution for 20 min, successive rinses with water and methanol and drying at 100°C in the oven. Hexane, methanol, ethyl acetate and toluene were glass-distilled (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and all aqueous solutions were made with glass-distilled water. The sodium carbonate—sodium bicarbonate pH 10.8 buffer [2] was stored at room temperature and replaced as needed.

Standards

A dilute (7 ng/μl) solution of flunarizine free base was prepared by weighing ten times the appropriate amount of the dihydrochloride salt and dissolving it in methanol. An aliquot (10 ml) of this solution was diluted with methanol in a 100-ml volumetric flask and used as assay standard as needed. A dilute (9 ng/μl) solution of cinnarizine was prepared and used in a similar way. Standards were kept in the freezer and checked periodically for concentration.

Analysis of biological fluids

Biological samples were stored frozen at −20°C and defrosted at the time of analysis. Milk samples were diluted 1:20 with distilled water prior to analysis. Fecal and tissue homogenates were obtained with water—methanol (50:50, v/v) in a Waring blender. Fat biopsy samples were homogenized in a mixture of hexane—ethyl acetate (3:1, v/v) in a tissue homogenizer (Akman).

Prior to analysis of samples, a drug-free biological fluid was analyzed and a calibration curve constructed for each biological fluid by adding the appropriate amounts of flunarizine (expected range) and internal standard (176 ng) to the appropriate aliquots. For analysis of unknowns, the appropiated sized aliquot (1, 4, 2, 0.5, and 20 ml, respectively, of plasma, urine, fecal and tissue homogenates and fat homogenates) was measured into a culture tube and 20 μl (176 ng) of the internal standard solution was added. All samples were analyzed in duplicate and a mini-calibration curve consisting of three concentrations (four replicates at the midpoint and two replicates each for the high and low points) covering the expected range of the unknowns, was analyzed with each set of unknowns. After addition of the internal standard, the tubes were vortexed vigorously and concentrated ammonium hydroxide (0.5 ml) and sodium carbonate—sodium bicarbonate buffer (1 *M*, pH 10.8, 1.0 ml) were added. The mixture was vortexed and hexane—ethyl acetate (3:1, v/v, 4 ml) was added. The mixture was shaken for 4 min and then centrifuged at 1500 *g* for 5 min. The organic layer was removed and the extraction repeated with another 4 ml hexane—ethyl acetate (3:1). The organic layers were combined and back extracted with hydrochloric acid (1 *N*, 6 ml). After shaking and centrifugation, the organic layer was aspirated under vacuum and discarded. The aqueous layer was then alkalinized with sodium hydroxide (10 *M*, 1 ml), vortexed and again extracted twice with the hexane—ethyl acetate mixture. After centrifugation, the organic layer was evaporated to dryness under nitrogen in a 3-ml conical tube. The residue was then reconstituted in 100 μl of toluene and 2—5 μl injected into the gas chromatograph. The concentration of flunarizine in the unknowns was determined by interpolation of the calibration curve extracted and analyzed alongside each set of samples using the peak height ratios versus concentration technique. The stability of flunarizine in the frozen plasma was established by spiking a five-point calibration curve (four replicates per point) with amounts of flunarizine (5—190 ng/ml), storing in the freezer at —20°C for three weeks, and analyzing for flunarizine. Stability in plasma was also determined by spiking 1-ml aliquots of blank plasma with 63.10 ng of flunarizine, storing and analyzing at several intervals.

RESULTS AND DISCUSSION

Flunarizine (I) and cinnarizine (II) (Fig. 1) are disubstituted piperazine derivatives, fairly volatile and very sensitive to the nitrogen detector. As little as 10 pg of these compounds injected on column can be accurately quantitated, although the practical limits of detection are determined by the background contribution of the biological matrix. The limit of 1.5 ng flunarizine per ml of plasma represents the smallest amount we could spike into blank plasma and consistently and accurately measure taking a coefficient of variation (C.V.) of 20% as the acceptance criterion. By using the 2× signal-to-noise ratio measurement a limit of detection as low as 0.5 ng/ml could be stated (Fig. 2), but day-to-day fluctuations in the background noise of the detectors makes this approach somewhat meaningless and more recently the former approach to establish the limits of detection has been recommended [3].

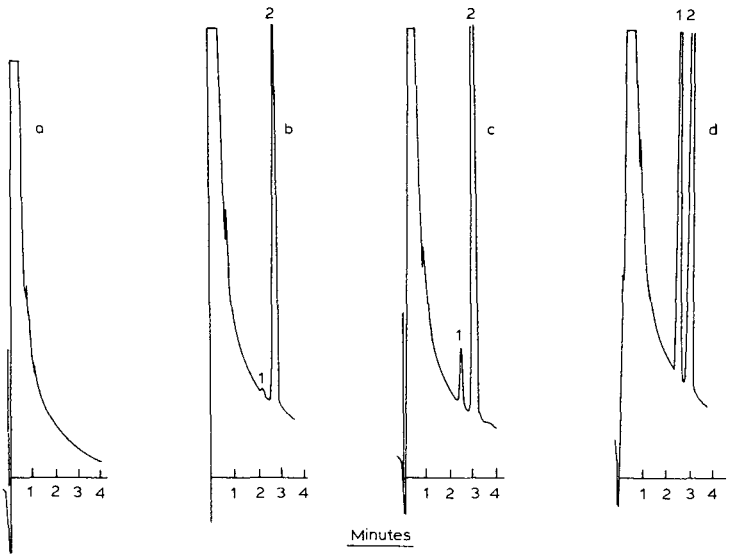

Fig. 2. Representative chromatograms of plasma samples containing: (a) no flunarizine and cinnarizine, (b) 1 ng/ml flunarizine and 176 ng/ml cinnarizine, (c) 10 ng/ml flunarizine and 176 ng/ml cinnarizine, and (d) 70 ng/ml flunarizine and 176 ng/ml cinnarizine. Peaks: 1 = flunarizine, 2 = cinnarizine.

Since flunarizine is in a very active stage of development, this methodology was required for several studies. Besides routine plasma analyses (pharmacokinetics and compliance), fecal homogenate and urinary analyses were useful to estimate absorption of the oral dose and excretion of unchanged compound. Milk analyses were used to study the extent of transport into the milk of the nursing female. Fat analyses investigated the possible accumulation into deep compartment tissues and tissue homogenate analyses were used for a variety of studies such as tissue distribution, passage through the placenta of the gestating female, etc.

The selectivity of the method was in each case demonstrated by the lack of interfering peaks in the analysis of the drug-free biological fluids. Furthermore, none of the known metabolites of flunarizine is eluted from the column under assay conditions. Linearity was demonstrated by the calibration curves constructed from each matrix (Table I). Correlation coefficients of peak height ratios versus flunarizine concentration of better than 0.990 were obtained in all cases except for the tissue homogenate matrix (r = 0.93). Accuracy and precision were established by running several replicates of each concentration. Except for the very low concentrations where the C.V. value was around 20%, most C.V. values were under 10% for the analysis of all matrices. The accuracy of the mean values was also good, with deviations from the true value generally staying under 10%, except for the lowest point in the milk curve where it was 20% (Table I).

A more extensive evaluation of the method was carried out for plasma analyses. Between-injection precision, established by repeated manual injections of the same sample extract showed a relatively high C.V. (17%) for the

TABLE I

PRECISION AND ACCURACY OF DETERMINATION OF FLUNARIZINE IN BIOLOGICAL FLUIDS

Biological matrix	Actual value (ng/ml)	Replicates (n)	$\bar{X}$	Accuracy*	C.V. (%)
Fat homogenates	28.8	6	28.3	−1.7	3.7
	46.2	6	43.4	−6.0	2.2
	124.5	6	133.7	+7.3	2.5
	249.1	6	247.6	0.6	2.9
	498.2	6	496.3	−0.4	0.9
	996.3	5	1008.7	+1.2	0.8
	1432.2	5	1472.2	+2.4	2.2
	1868.1	6	1903.4	+1.9	2.6
Urinary samples	5.8	6	5.8	0	5.1
	17.6	6	17.6	0	4.2
	29.2	6	29.2	0	2.1
	58.5	6	58.8	+0.5	1.4
	175.5	6	182.0	+3.7	2.9
Milk samples	16.6	3	12.8	−22.9	5.0
	53.2	2	53.2	0	1.5
	106.4	3	104.8	+1.5	3.8
	305.9	3	290.7	−5.0	0.6
Tissue homogenates	66.5	3	55.1	−17.1	13.0
	166.2	3	158.2	−4.8	1.8
	498.8	3	450.7	−9.6	9.9
	987.5	3	919.1	−6.9	5.1
Fecal homogenates	6.5	3	6.2	−9.4	11.6
	32.4	3	31.1	−4.0	2.9
	97.3	3	88.5	−9.0	2.3
	324.2	3	336.1	−3.7	12.1
	648.4	3	635.4	−2.0	5.9
	1296.7	3	1379.4	+7.8	5.8

*Accuracy $= \dfrac{\bar{X} - X_{\text{actual}}}{X_{\text{actual}}} \times 100.$

TABLE II

BETWEEN-INJECTION PRECISION OF DETERMINATION OF FLUNARIZINE IN PLASMA

Actual value (ng/ml)	Replicates (n)	$\bar{X}$	C.V. (%)
1.0	6	1.0	16.9
8.5	5	8.7	3.4
21.8	6	21.6	1.0
72.1	5	71.9	5.6
131.0	6	132.9	2.7
267.5	6	266.8	3.7

TABLE III

BETWEEN-REPLICATE PRECISION OF DETERMINATION OF FLUNARIZINE IN PLASMA

Replicates	$\bar{X}$	C.V. (%)
6	1.3	22.1
2	7.7	1.8
6	9.4	7.7
3	22.0	2.5
2	36.1	4.7
6	68.7	2.6

TABLE IV

BETWEEN-DAY PRECISION AND ACCURACY OF DETERMINATION OF FLUNARIZINE IN PLASMA

Day	Actual value (ng/ml)	Replicates (n)	$\bar{X}$	Accuracy*	C.V. (%)
1	1.5	4	1.4	−6.7	19.3
	7.3	4	6.8	−6.8	12.6
	22.0	4	21.5	−2.3	3.5
	73.4	4	79.9	+8.9	5.0
	146.8	4	171.1	+16.6	7.6
	293.6	4	300.2	+2.2	9.6
2	1.5	4	1.2	−20.0	13.9
	7.3	4	8.2	+11.7	12.4
	22.0	4	22.6	+2.7	5.6
	73.4	4	71.6	+2.4	2.8
	146.8	4	127.8	−12.9	3.1
	293.6	4	260.0	+11.4	0.8
3	1.5	4	1.3	−13.3	18.9
	7.3	4	6.9	−5.5	2.7
	22.0	4	22.0	0	2.5
	73.4	4	77.6	+5.7	2.3
	146.8	4	155.2	+5.7	2.4
	293.6	4	332.8	+13.3	3.0
1,2,3	1.5	3	1.3	−13.3	7.7
	7.3	3	7.3	0	10.7
	22.0	3	22.0	0	2.5
	73.4	3	76.4	+4.1	5.6
	146.8	3	151.4	+3.1	14.5
	293.6	3	297.7	−1.4	12.2

* $\text{Accuracy} = \frac{\bar{X} - X_{\text{actual}}}{X_{\text{actual}}} \times 100.$

lowest concentration, and much lower (1—6%) for the rest of the points (Table II). This indicates significant detector variability at low concentrations. Between-replicate precision, established by analyzing six replicates per concentration in our most common analytical range (0—70 ng/ml), showed very similar values to the between-injection precision indicating the reproducibility of the extraction procedure (Table III). Between-day precision and accuracy were established by the analysis of identical calibration curves on three different days and again, except for the lowest concentration, C.V. values were below 10% for most points and deviations from true values under 10% (Table IV).

The inherent variation in the absolute response of the nitrogen detector makes the use of an internal standard necessary and the evaluation of absolute recoveries somewhat difficult. Absolute recoveries, estimated by comparison of the peak heights obtained after injection of methanolic solutions of the compounds and after injection of known amounts of spiked and extracted samples were around 80% for both flunarizine and cinnarizine. Initially, losses of both compounds due to absorption to the glass during the final evaporation step were observed. This was eliminated by the strong base wash of the tubes described in the Experimental section. Siliconization, which also eliminated the problem, was found to interfere at times with the nitrogen detector. Flunarizine is also unstable in very dilute (< 5 ng/μl) solutions, therefore our lowest concentration solution was kept around 7 ng/μl and checked periodically for concentration. Flunarizine, however, was found to be very stable in frozen plasma. Analysis of a calibration curve stored for 3 weeks at —20°C and of plasma-spiked samples (63.10 ng/ml) stored for a period of over 1 year showed values for the precision and accuracy comparable to those obtained for the freshly extracted plasma samples (Table V). Only plasma samples were stored for significant amounts of time in the freezer.

TABLE V

FLUNARIZINE STABILITY IN FROZEN PLASMA

Days after preparation	Actual value (ng/ml)	$\bar{X}$	n	C.V. (%)
27	63.1	60.8	2	2.0
41	63.1	67.1	2	11.0
484	63.1	66.3	4	3.6
21	4.6	4.8	4	5.2
21	9.1	8.6	4	6.6
21	30.4	32.4	4	4.1
21	76.0	70.3	4	7.5
21	190.0	181.9	4	1.7

As previously stated, this methodology has been used among other applications, for pharmacokinetic studies. The pharmacokinetics of flunarizine after administration of a single oral dose (30 mg) to eight human volunteers were determined. Peak values at 2—3 h of around 100 ng/ml were observed and

residual levels of 2—3 ng/ml were still observed 7 days after administration (Figs. 3 and 4). To estimate the extent of absorption of the dose, fecal samples from this same study were also analyzed, as were urinary samples. The rest of the applications so far have been involved with animal studies.

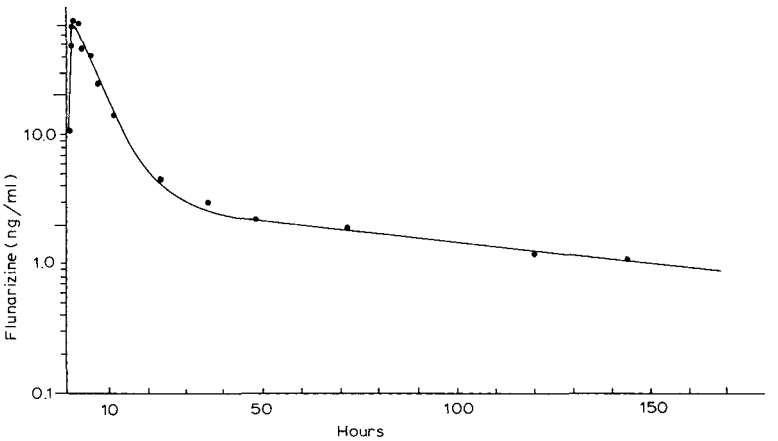

Fig. 3. Flunarizine plasma concentration versus time profile after single dose administration of 30 mg to man.

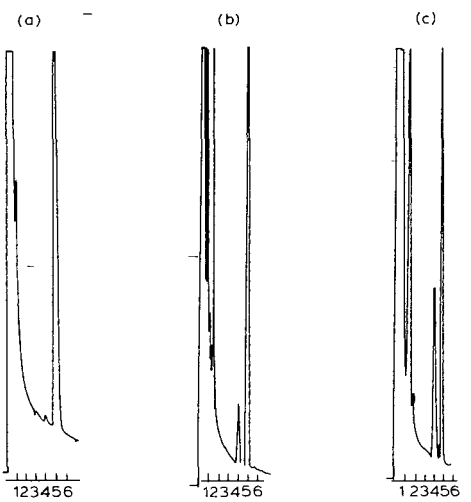

Fig. 4. Chromatograms of (a) 96-h, (b) 12-h and (c) 4-h human plasma samples obtained after administration of a single 30-mg dose of flunarizine. Concentrations found are 2, 22 and 70 ng/ml, respectively. Internal standard, cinnarizine (176 ng) was added to all the samples.

CONCLUSION

The methodology described is suitable for the determination of flunarizine in biological matrices and is sensitive enough for pharmacokinetic studies.

This sensitivity was achieved by the use of a nitrogen detector and a selective extraction procedure. The method is selective for flunarizine with no interferences from the known metabolites or endogenous materials observed.

ACKNOWLEDGEMENTS

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CHROMBIO. 1493

DETERMINATION OF ACETYLSALICYLIC ACID AND METABOLITES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new method has been developed for the determination of acetylsalicylic acid, salicylic acid, salicyluric acid and gentisic acid in plasma, urine and tissue homogenates by simple extraction with ethyl acetate, evaporation and redissolution and measuring by high-performance liquid chromatography. Linearity, reproducibility and recovery were determined. Experiments were carried out to investigate the decomposition of acetylsalicylic acid in plasma with fluoride at different temperatures. The method has been used for pharmacokinetic experiments and an example is given.

INTRODUCTION

Acetylsalicylic acid (ASA) is rapidly hydrolysed to salicylic acid (SA) and acetic acid in the body [1]. SA is further metabolized to salicyluric acid (SU) (conjugation with glycine), gentisic acid (GA) (hydroxylation), salicylacyl-glucuronide (SAG) and salicylphenolglucuronide (SPG) (conjugation with glucuronic acid) (see Fig. 1). The formation of SU and SPG is saturable at therapeutic concentrations [2, 3] and contributes to a mixed zero- and first-order kinetics for salicylic acid.

In pharmacokinetic investigations of ASA an easy and rapid method for analyses of ASA and metabolites in biological fluids is needed. Several methods for this purpose have been developed based on high-performance liquid chromatography (HPLC) [4—11]. Terweij-Groen et al. [4] determined SA in

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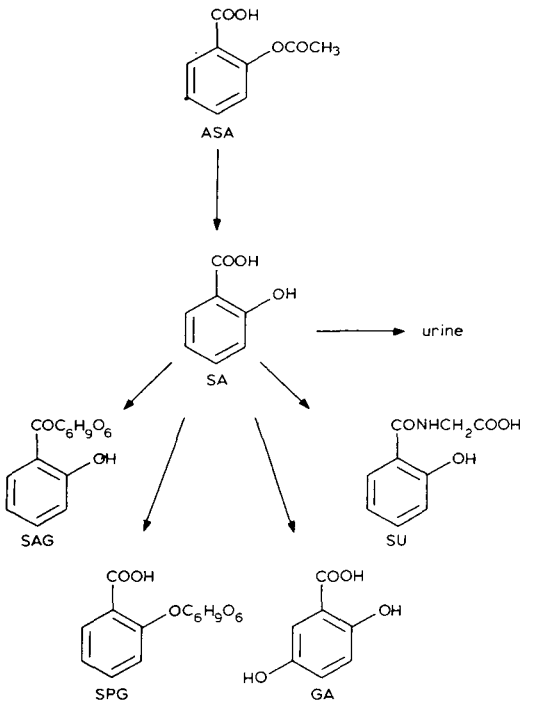

Fig. 1. Diagram showing the metabolism of acetylsalicylic acid (ASA) in humans. The formation of salicyluric acid (SU) and salicylphenolglucuronide (SPG) are saturable at therapeutic concentrations and contribute to a mixed zero- and first-order kinetics of salicylic acid (SA). SAG = salicylacylglucuronide, GA = gentisic acid.

plasma after deproteinization and injection of the supernatant, while Cham et al. [5] improved this method to include SU and GA. Maulding and Young [6] used the same method, but have improved it to include the determination in whole blood, urine and faeces. Bekersky et al. [7] determined SU and SA after deproteinization by trichloroacetic acid and extraction with diethyl ether.

None of these methods determines ASA. Harrison et al. [8] and Lo and Bye [9] determined ASA and SA after extraction with methylene chloride and chloroform, respectively, but none of the metabolites are determined by these methods. Peng et al. [10] determined ASA, SU and SA in plasma after extraction with a mixture of ethyl acetate and benzene, while Amick and Mason [11] have improved the method to include GA and determinations in urine. Rumble et al. [12] determined ASA, SU, SA and GA in plasma by deproteinization with perchloric acid and methanol. Lo and Bye [9], Amick and Mason [11] and Rumble et al. [12] used fluoride to inhibit the acetylsalicylic acid esterase, and only the two last-mentioned methods determine more than three substances.

On this basis an HPLC method was developed, by which GA, SU, ASA and SA can be determined in plasma, urine and tissue homogenates following a simple extraction by ethyl acetate, and at the same time steps have been taken to inhibit the esterase by fluoride and cooling. The method is based on the method of Amick and Mason [11], with some modifications.

EXPERIMENTAL

Reagents

Acetylsalicylic acid, salicyluric acid, gentisic acid and orthophosphoric acid are all of quality for laboratory use; salicylic acid and sodium hydroxide are Ph. Eur. grade and methanol, ethyl acetate and sodium fluoride are all of analytical quality.

Equipment

For separation and detection an HPLC pump Model M-6000 A from Waters Assoc., a Model U6K injector (Waters Assoc.), an LDC UV III Monitor (1203) detector at 280 nm and a recorder (Servo/riter II, Texas Instruments) are used. The column is 15 cm × 4 mm I.D., packed with LiChrosorb RP-18 (5 μm) and thermostatted at 45°C in a waterbath. The working pressure of the pump is 200—275 bars at a flow-rate of 1.5 ml/min.

Procedures

The blood samples are collected in vials prepared with sodium fluoride and heparin (4 mg of NaF and about 50 I.U. of heparin per 1.5 ml of blood). The vials are kept on ice for no longer than 0.5 h until further processing. The addition of sodium fluoride and keeping on ice is to minimize the hydrolysis of ASA [13]. The blood samples are centrifuged (1500 *g* for 10 min at room temperature) and the plasma is immediately extracted as follows. In a glass vial are added 50 μl of concentrated phosphoric acid, 200 μl of plasma and 600 μl of ethyl acetate. The contents are shaken for 30 sec on a whirlimixer and centrifuged at 600 *g* for 10 min at 10°C; 400 μl of the supernatant are transferred to a plastic vial and stored at −26°C until analysis. Immediately before measuring by HPLC, the 400 μl of supernatant are evaporated to dryness under a gentle stream of air (7 min) on an icebath. The residue is redissolved in 200 μl of mobile phase (methanol—water, 40:60; adjusted to pH 3.00 with 0.005 *M* phosphoric acid and sodium hydroxide), and 100 μl are injected into the liquid chromatograph. External standards are injected for every six samples and the concentration is calculated by measuring relative peak heights and correcting for dilution and recovery.

The tissue homogenates are made as follows: 500 mg of tissue and 2 ml of distilled water are homogenized on a Virtis homogenizer Model 60 K for 30 sec at 40,000 rpm. The homogenate is treated as described for plasma. For determinations in urine, the urine is diluted tenfold with water and processed as described for plasma.

RESULTS AND DISCUSSION

Chromatograms

Fig. 2a shows a chromatogram after analysing rabbit plasma without any materials added. Fig. 2b shows a chromatogram of rabbit plasma 15 min after intravenous administration of 50 mg/kg ASA. The chromatogram shows in addition to ASA, SU and SA in the rabbit plasma. No GA was measured after ASA administration, but it could be also separated. The retention times are: GA, 2 min; SU, 2.7 min; ASA, 3.7 min; and SA, 5.5 min.

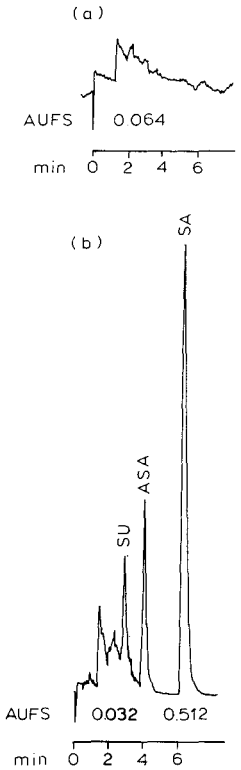

Fig. 2. Chromatograms of (a) blank samples from rabbit plasma and (b) plasma analysis 15 min after intravenous administration of 50 mg/kg ASA to a rabbit.

Linearity

Standard solutions of ASA, SA, SU and GA at different concentrations in the mobile phase (0.5, 2, 5, 10, 50, 100, 200, 300, and 500 μg/ml) were measured and the correlation between the corrected peak heights (peak height × a.u.f.s.) and the concentrations were determined. GA and SU show linearity up to 100 μg/ml (r = 0.993 and r = 0.997, respectively), while ASA and SA are linear up to 250 μg/ml (r = 0.994 and r = 0.997, respectively). Determination of higher concentrations of SA can be performed by injecting a smaller volume: 50 μl or 25 μl.

Standard solutions in plasma were measured as well, and the overall coefficient of variation for GA, SU and ASA was 4% and for SA it was 6%.

Reproducibility

To both rabbit and human plasma were added GA, SU, ASA and SA, and eight samples of each were extracted and measured. Table I shows the added and measured concentrations, demonstrating a good recovery with small variation.

Recovery

Plasma samples with different concentrations of GA, SU, ASA and SA added were extracted and measured, and the recovery was determined. Fig. 3 shows

TABLE I

REPRODUCIBILITY OF EIGHT DETERMINATIONS OF STANDARD SOLUTIONS OF GA, SU, ASA AND SA IN HUMAN AND RABBIT PLASMA

	GA	SU	ASA	SA
Human plasma				
Added (μg/ml)	24.7	45.1	63.3	80.5
Determined $\bar{x}$ (μg/ml)	24.9	42	62	72
S.D.	0.6	1	2	4
C.V. (%)	2.4	2.4	3.3	5.6
Recovery (%)	101 ± 2	94 ± 3	98 ± 3	89 ± 5
Rabbit plasma				
Added (μg/ml)	29.0	39.1	67.0	82.7
Determined $\bar{x}$ (μg/ml)	29.4	40	62	79
S.D.	0.95	1	3	3
C.V. (%)	3.2	2.9	4.7	4.3
Recovery (%)	101 ± 3	102 ± 3	93 ± 4	95 ± 4

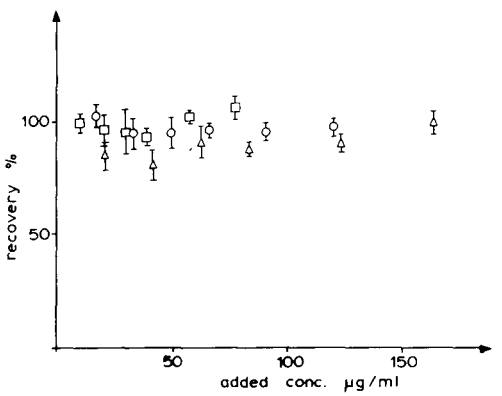

Fig. 3. Mean percentage recovery as a function of added concentration (μg/ml) to plasma of ASA (○), SA (△) and SU (□). The bars indicate the standard deviations. The percentage recovery is independent of the concentration. (SU: r = 0.646, α = 0.1204, variance ratio (VR) = 0.18. ASA: r = 0.421, α = —0.0343, VR = 0.054. SA: r = 0.883, α = 0.0832, VR = 0.88. $F_{1,4}^{0.90}$ = 4.54. F-test of dependence.)

the percentage recovery of SU, ASA and SA at different concentrations, and it is seen that the recovery does not depend on the concentration (F-test of dependence [14]).

Selectivity

In order to detect any interference, the following compounds as pure materials or tablets in solution were analysed as described for plasma: ascorbic acid, codeine, caffeine, dextropropoxyphene, inulin, paracetamol and tea. Only paracetamol interferes with SA, which should be considered when using this method.

Sensitivity

In plasma the lowest detectable concentrations were: GA, 2.5 μg/ml; SU and SA, 0.2 μg/ml; and ASA, 0.5 μg/ml. In urine: SU and SA, 10 μg/ml. In mobile phase: GA, SU and SA, 0.1 μg/ml; ASA, 0.5 μg/ml.

Storing of samples

The effect of storage of the plasma samples with added ASA and fluoride was studied after a storage time of 27 days at temperatures of 5° C, −26° C, and −80° C. Fig. 4 shows the concentration of SA formed by hydrolysis. It is seen that the samples can be stored at −80° C with only a little hydrolysis, possibly due to the thawing phase. Because of this finding the plasma samples were not stored, but were extracted within 0.5 h after blood sampling.

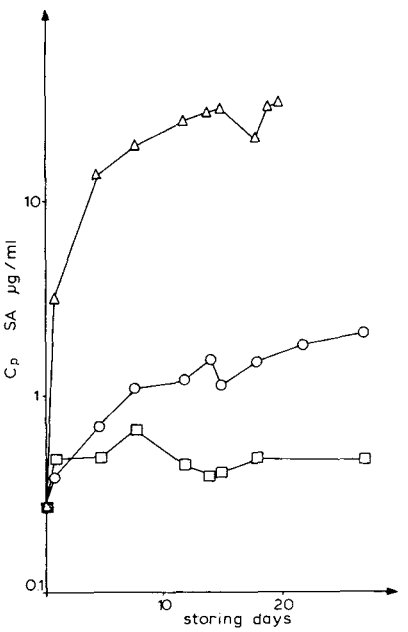

Fig. 4. SA plasma concentrations measured at different times after adding ASA to plasma with fluoride and storing at different temperatures for up to 27 days. (□), −80° C; (○), −26° C; (△), +5° C.

Use of the method

The method has been used for the determination of plasma and tissue concentration after intravenous administration of ASA or SA to rabbits and after oral administration of ASA to human volunteers. Fig. 5 shows the plasma concentration curve after administration of 650 mg of ASA per os as effervescent tablets to a human volunteer. ASA, SA and SU were measured, but no GA could be detected after ASA administration.

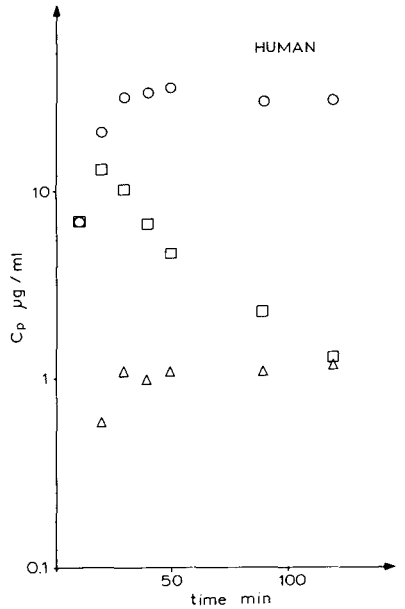

Fig. 5. Plasma concentrations of ASA (□), SA (○) and SU (△) after oral administration of 650 mg of ASA as effervescent tablets to a male human volunteer weighing 76 kg.

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CHROMBIO. 1500

QUANTITATIVE DETERMINATION OF O-(β-HYDROXYETHYL)-RUTOSIDES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure for the quantitative determination of O-hydroxyethylated rutosides by high-performance liquid chromatography is described, which can be used for the detection of these modified flavonoids in human serum. Serum samples are processed by the addition of acetone, which removes most of the proteins. After passing the supernatant through a microcolumn of Amberlite XAD-2 and washing with water, the hydroxyethylated rutosides are eluted with methanol. The eluate is concentrated in vacuo. The methanolic solution of the residue is chromatographed on RP-8 columns using UV and fluorescence detectors. The mono- to tetrahydroxyethylated constituents and their corresponding aglycones could be separated with a step gradient, starting with a solvent system of water—methanol—acetic acid (70:30:6) followed by a mixture of water—ethanol—acetic acid (70:30:6). Alternatively, the rutosides can be separated by a linear gradient of water—acetonitrile. An almost linear calibration curve and about 80% recovery are obtained. A detection limit of 1 mg/l is achieved. Pharmacokinetic studies in human volunteers are described.

INTRODUCTION

O-(β-Hydroxyethyl)-rutoside (HR) has been used successfully in the therapy of various diseases like venous insufficiency and radiation damage [1, 2]. In order to obtain reliable information about the pharmacokinetic characteristics of HR, a method had to be developed which allowed quantification of

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the drug in human serum. The development of a suitable assay for HR in blood is a particularly difficult problem because HR is a mixture of rutosides, which are hydroxyethylated to a different degree (mono- to tetrahydroxyethyl) and at different positions [1]. High-performance liquid chromatography (HPLC) in a reversed-phase system proved to be an efficient and sensitive method to solve the problem. Processing of serum samples and chromatographic separation is described including a successful application to a pharmacokinetic study after intravenous administration of HR to human volunteers.

MATERIALS

Chemicals

The commercially available mixture of the drug O-(β-hydroxyethyl)-rutoside (HR) was provided by Zyma (Munich, G.F.R.). The individual components of this flavonoid mixture were obtained by purification on Sephadex LH-20, obtained from Pharmacia (Uppsala, Sweden). Subsequent acid hydrolysis of the separated compounds yielded their aglycones [3, 4]. The individual components were identified by gas chromatography and mass spectrometry [5].

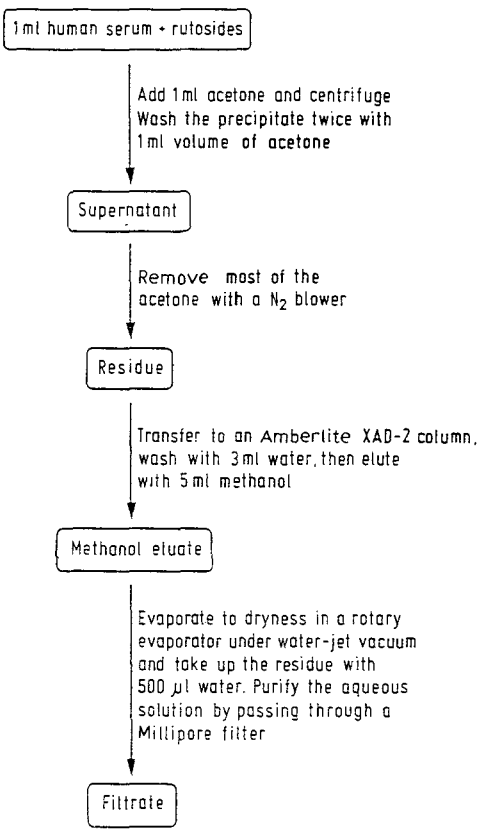

Fig. 1. Processing scheme for O-(β-hydroxyethyl)-rutosides in human serum.

Freshly distilled solvents were used, together with the resin Amberlite XAD-2, supplied by Serva (Heidelberg, G.F.R.).

Blood samples

The serum samples were obtained from hospitalized volunteers, whose identity was unknown to us. Serum samples were kept deep frozen and thawed at room temperature before use.

METHODS

Processing of serum samples

In each case 1 ml of serum was processed (Fig. 1). After acetone precipitation of most of the serum proteins, chromatography of the residue was carried out on an Amberlite XAD-2 column (50 × 6 mm). The salts, free sugars and other interfering components were first removed with water; finally the rutoside mixture was eluted with methanol and concentrated in vacuo.

High-performance liquid chromatography

HPLC measurements were made with equipment from Waters Assoc., consisting of two pumps (type M 6000A), a solvent programmer (Model 660), an integrator and a UV detector (Model 440) with a fixed wavelength of

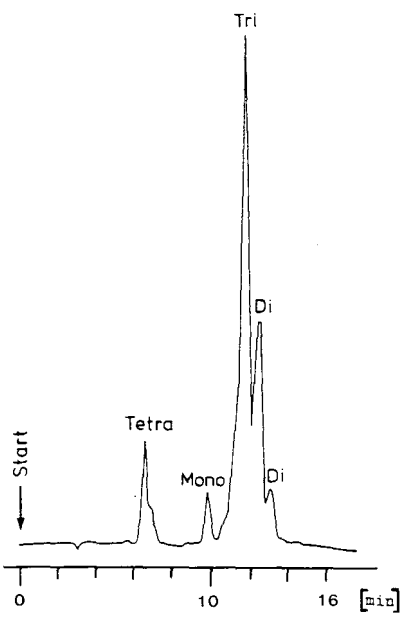

Fig. 2. HPLC chromatogram of the standard mixture of the O-(β-hydroxyethyl)-rutosides (HR). The assignment of the individual components is based on chromatographic comparison with the pure compounds of confirmed structure. Conditions: LiChrosorb RP-8 (10 μm, 250 × 4 mm, Merck); mobile phase, water—methanol—acetic acid (70:30:6); flow-rate, 0.9 ml/min; ambient temperature.

254 nm. Fluorescence detection was performed with an instrument from Schoeffel (Model FS 970) with an excitation wavelength of 355 nm, and emission at 460 nm and above. The fluorescence detector was connected to a Hewlett-Packard liquid chromatograph (type 1084B) equipped with an automatic sample applicator and integrator.

The columns used were either LiChrosorb RP-8 (10 μm, 250 × 4 mm) or LiChrosorb RP-8 (5 μm, 100 × 4 mm), both from Merck (Darmstadt, G.F.R.). The step gradient was performed with two solvent systems: water—methanol—acetic acid (70:30:6, v/v, system A) and water—ethanol—acetic acid (70:30:6, v/v, system B). For separation of rutosides and aglycones in a single run, system A was applied for 6 min, then system B for 14 min, both at a flow-rate of 0.9 ml/min. Alternatively, for the separation of the rutosides a linear gradient of water (pH 3 adjusted with sulphuric acid)—acetonitrile was used. The samples were introduced in volumes of between 10 and 100 μl via a sample loop.

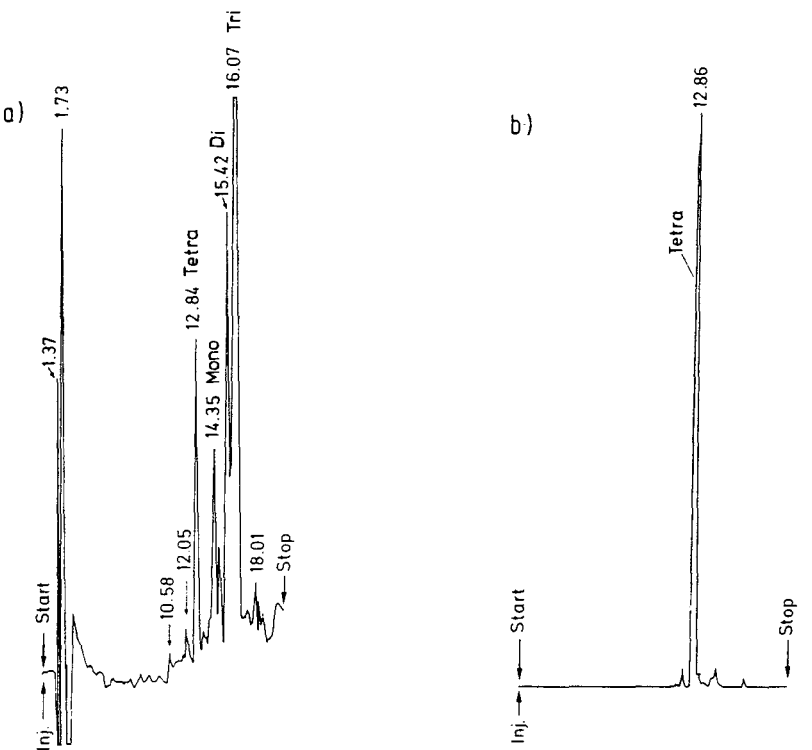

Fig. 3. (a) HPLC separation of HR. Conditions: LiChrosorb RP-8 (250 × 4 mm, 10 μm, Merck); mobile phase, gradient acetonitrile—water (pH 3); UV detection at 256 nm. (b) Conditions as in Fig. 3a but with fluorescence detection (excitation, 355 nm; emission, 460 nm).

RESULTS

Separation of aglycones and glycosides

Preliminary experiments were carried out in order to optimize the separation of HR in mixtures with their aglycones. Excellent separation of the glycosides was achieved using solvent system A (Fig. 2). The glycosides could be separated as well with the solvent mixture acetonitrile—water (pH 3) under gradient elution (Fig. 3a and b). The rutosides and their corresponding aglycones can be readily separated in a single run when elution is performed with a step gradient (Fig. 4) with solvent system A followed by solvent system B.

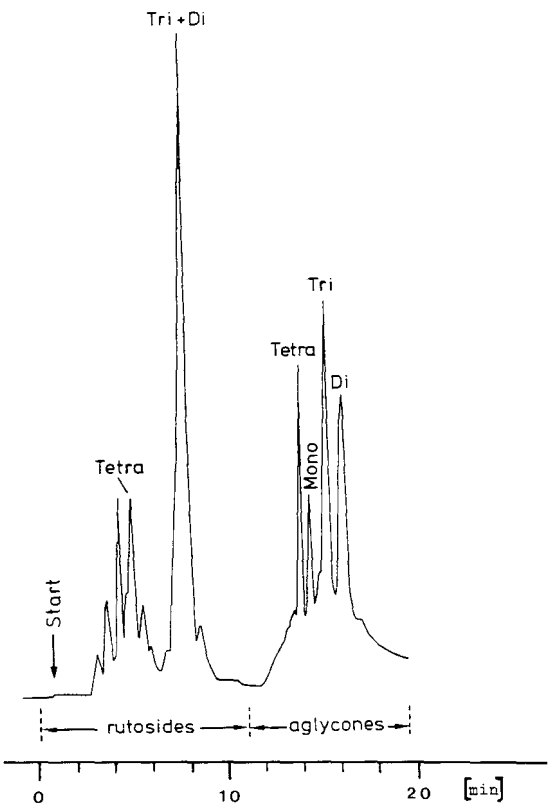

Fig. 4. HPLC separation of HR and HR aglycones in a single run. Conditions as in Fig. 2 but with a step gradient: system A (water—methanol—acetic acid, 70:30:6) for 6 min, then system B (water—ethanol—acetic acid, 70:30:6) for 14 min.

Quantitative determination of HR in human serum

In order to prepare a calibration curve quantities of 10, 20, 50, 100, 150, 200, 250 and 300 μg of HR were each added to 1 ml of human serum and the mixtures processed in accordance with the scheme shown in Fig. 1. The procedure was carried out twice for each quantity. A 10-μl volume of each filtrate was subjected to HPLC and an integrator used to measure the peak areas corresponding to the di- and tri-ethers on the one hand and the tetra-ether on the

other. Because only the tetra-ether displayed a reasonable fluorescence, the di- and tri-ethers were determined by UV absorption. In both cases we obtained calibration curves which displayed a linear correlation between the amount of HR added and the observed peak area. The recovery was determined by comparison of the areas given by pure HR and by HR determined in the serum; it was about 78% (±8). The limit of detection is at least 1 μg of HR per ml of serum, added before processing. Fluorescence detection of the tetra-ether was even more sensitive, with a detection limit of about 100 ng/ml serum.

Detection of HR in human serum after intravenous administration

In the first series of experiments we used the step gradient system and UV detection. Three male volunteers received a bolus injection of 1.5 g of HR at 8 a.m. Blood samples were then taken at 15, 30, 45, 60, 90 min and at 2, 3, 4, 6, 7, 12 and 24 hours. Each serum sample (1 ml) was processed as described in Fig. 1 and subjected to HPLC. The sums of the peak areas for the di- and tri-ethers were calculated on the basis of the calibration curve. The change in the blood HR level is shown in Fig. 5. The nature of the measured compounds was confirmed by collecting the di- and tri-ethers and identifying them by mass spectrometry [3—5]. In the second series the acetonitrile—water gradient system and fluorescence detection as well as UV absorption were used. Three different volunteers were treated as outlined above and serum concentrations of di- and tri-ether as well as those of the tetra-ether were determined. The results are outlined in Fig. 6.

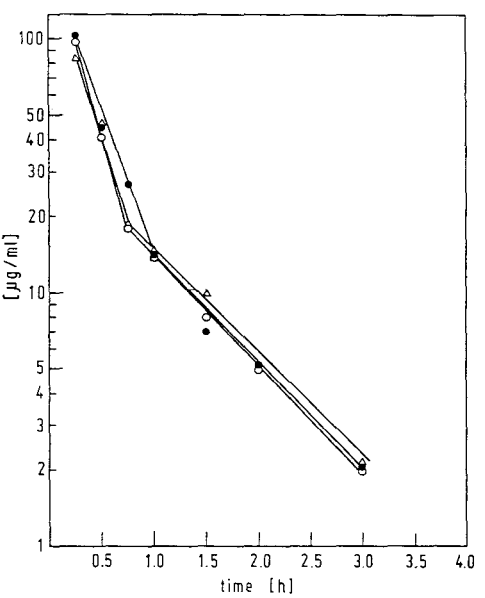

Fig. 5. Change in the blood HR level following intravenous injection of 1.5 g of HR for each of three male volunteers. The points are calculated from the peak areas of the di- and tri-ethers of HR.

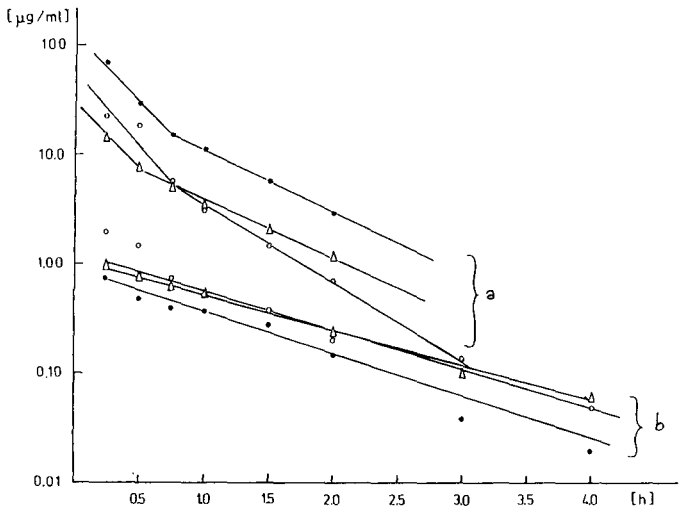

Fig. 6. Semilogarithmic concentration—time curves for each of three subjects following intravenous administration of 1.5 g of HR. (a) Di- and tri-ethers, (b) Tetra-ether.

DISCUSSION

Two test series were carried out independently in each respect leading to identical results, thus confirming the reliability of the HPLC assay of O-(β-hydroxyethyl)-rutosides. Furthermore, the results of the pharmacokinetic studies after intravenous administration are in accordance with the results obtained by Förster [6] and Griffiths and Hackett [7], who used thin-layer chromatography and ^{14}C-labeled HR. In addition, the HPLC results fully confirm the concentration—time curves of our group, obtained by circular dichroism measurements of serum samples after intravenous administration of HR [8].

The concentration—time curves of all six subjects are very similar in shape (Figs. 5 and 6). Measurable amounts of di- and tri-ethers are no longer present after 4 h. However, fluorescence detection clearly revealed traces of tetra-ether in all three subjects after 6—8 h. The biphasic elimination curve for the di- and tri-ethers could indicate a distribution of these compounds into deeper compartments. A closer examination of the serum chromatograms from the intravenous tests revealed the following differences in comparison with chromatograms of pure HR solutions in water and the serum HR standard solutions. Soon after intravenous administration of HR, a new UV-positive and fluorescent peak with a retention time of 12.1 min appears, which probably originates from a metabolite. This new peak decreases at the same rate as the HR components. Thus the possibility of accumulation of the metabolite can be excluded. The question of whether this metabolite is identical with those mentioned in the literature [7] to originate from biliary excretion will be the subject of further experiments.

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CHROMBIO. 1501

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF FLECAINIDE, A NEW ANTIARRHYTHMIC, IN HUMAN PLASMA AND URINE

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SUMMARY

A simple, selective, sensitive, and accurate high-performance liquid chromatographic method for the quantitation of flecainide in human plasma and urine is described. The method is based on initially washing the sample with hexane followed by a single extraction with hexane. The extracted drug and internal standard are chromatographed on a Zorbax TMS column with a mobile phase consisting of acetonitrile—1% acetic acid in 0.01 *M* pentanesulfonate (45:55, v/v). The eluent is monitored at 308 nm.

This method can routinely quantitate plasma or urine flecainide concentrations as low as 22 ng/ml with a 1-ml sample and 11 ng/ml with a 2-ml sample with no interference from endogenous substances and many drugs and their metabolites. The standard curve is linear over a concentration range of 22—1746 ng/ml. The precision and accuracy of the described method are suitable for monitoring flecainide levels in therapeutic, tolerance, and pharmacokinetic studies in humans.

INTRODUCTION

Flecainide acetate, 2,5-bis-(2,2,2-trifluoroethoxy)-N-(2-piperidylmethyl)benzamide acetate (R-818), is a new antiarrhythmic agent. Studies in laboratory animal models indicated the effectiveness in converting experimentally induced auricular and ventricular arrhythmias and demonstrated greater potency than reference agents such as lidocaine, procainamide and quinidine [1—4]. Studies in normal human volunteers indicated a relatively long half-life ($t_{1/2}$ = 14 h) and its potential for sustained therapeutic activity [5—9]. Flecainide acetate is undergoing extensive study in humans worldwide [5—10]. Currently, measurement of flecainide plasma levels is accomplished by first derivatizing flecainide with pentafluorobenzoyl chloride and then analyzing by gas—liquid chromato-

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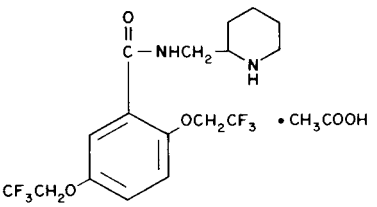
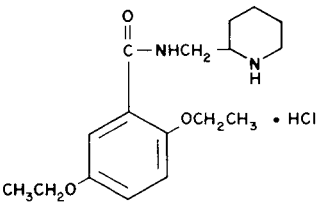


Flecainide Acetate



Internal Standard

graphy (GLC) with electron-capture detection [11]. Sensitive fluorometric methods have been developed for the monitoring of flecainide in human plasma but suffered from lack of specificity due to interference by other drugs [12, 13]. A high-performance liquid chromatographic (HPLC) procedure with the use of a fluorescence detector has recently been developed [14]. The method has a wide linear range (50—5000 ng/ml), however, it requires mechanically cutting a commercial column and the use of a sophisticated spectrofluorometer. Furthermore, it is not an internal standardization procedure. In this report, we describe a simple, rapid, selective, and accurate HPLC method which requires common LC instrumentation and is capable of routinely monitoring flecainide plasma levels in humans following either single or chronic dosing of flecainide acetate in research settings or for non-research clinical management purposes.

EXPERIMENTAL

Chemicals and reagents

The methanol and acetonitrile were Omnisolv® (MCB Reagents , Cincinnati, OH, U.S.A.) and the hexane used for extraction was Nanograde® (Mallinckrodt, St. Louis, MO, U.S.A.). The 1-pentanesulfonate was purchased from Regis (Morton Grove, IL, U.S.A.) and the trimethylamine hydrochloride from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were AR grade.

Chromatography system

The analysis was performed on a modular liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 710 B Intelligent Sample Processor (Waters Assoc., Milford, MA, U.S.A.), and a Model 1203 UV Monitor III with a 308-nm filter (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The separation was achieved on a Zorbax TMS column, 15 cm × 4.6 mm (6 μm particle size, Dupont, Wilmington, DE, U.S.A.), protected by a guard column (Waters Assoc.) packed with Permaphase ETH (35 μm particle size, DuPont). Peak heights were measured by a Model 4100 Computing Integrator (Spectra Physics, Santa Clara, CA, U.S.A.).

The mobile phase was prepared by mixing 450 ml acetonitrile, 5.5 ml glacial acetic acid, and 544.5 ml 0.01 *M* aqueous 1-pentanesulfonate. The mobile phase was filtered through a Millipore membrane (pore size 0.45 μm) and degassed prior to use. The flow-rate was 2.0 ml/min.

Preparation of standard solutions

Standard solutions of flecainide were prepared by diluting a 10 mg/l aqueous stock solution of flecainide acetate. The concentrations used were 22, 44, 87, 262, 524, 873, and 1746 ng flecainide per 0.5 ml. The internal standard solution, 2,5-diethoxy-N-(2-piperidylmethyl)benzamide hydrochloride (S-15277), was prepared by diluting a 6.3 mg per 100 ml aqueous stock solution to 250 ng per 50 μl.

Extraction procedure

Pipet 1 ml plasma or urine into a 150 × 16 mm glass culture tube with a polyethylene-lined cap, add 50 μl internal standard solution (or water for blank), 0.5 ml flecainide standard solution for calibration curve (or water for unknown samples and blank), 1 ml 0.5 *N* aqueous hydrochloric acid, and 10 ml hexane. Cap the tubes, shake on a reciprocal mechanical shaker (tubes in horizontal position) at a speed of 256 cycles/min for 10 min and centrifuge for 5 min at 840 *g*. Aspirate the hexane phase; add another 10 ml of hexane and wash a second time. Aspirate the hexane phase and add the following, in this order, to the aqueous phase: 0.2 ml 0.2 *M* aqueous trimethylamine (TMA) hydrochloride, 10 ml hexane, and 1 ml 1 *N* aqueous sodium hydroxide. Shake for 10 min, centrifuge for 5 min at 840 *g*, and transfer the hexane phase to a 15-ml conical centrifuge tube. Evaporate to approximately 1 ml at 60° C under nitrogen; rinse the tube wall with 1 ml methanol, and evaporate to dryness. Reconstitute the residue with 200 μl of the mobile phase and inject 150 μl into the liquid chromatograph.

Method of calculation

The calculation of flecainide concentration for unknown samples was performed automatically by the Model 4100 Computing Integrator. A least-squares linear regression line was calculated from the concentration of the standards and the peak height ratio of flecainide over the internal standard. For every unknown sample thereafter, the peak height ratio was compared to the linear regression line to determine the unknown concentration. The peak heights can also be measured manually when the response is presented with the use of a 10-mV recorder.

RESULTS

Chromatographic separation

Baseline separation was achieved under the experimental conditions described with retention times of 3.5 and 5.2 min for the internal standard and flecainide, respectively. There was no interference with flecainide and the internal standard by endogenous materials from human plasma. Typical plasma chromatogram tracings are shown in Fig. 1. Similar results were obtained from human urine (Fig. 2).

Extraction recovery

Because of the relatively low concentration of flecainide (a basic molecule) analyzed in this procedure, surface adsorption processes play an important role

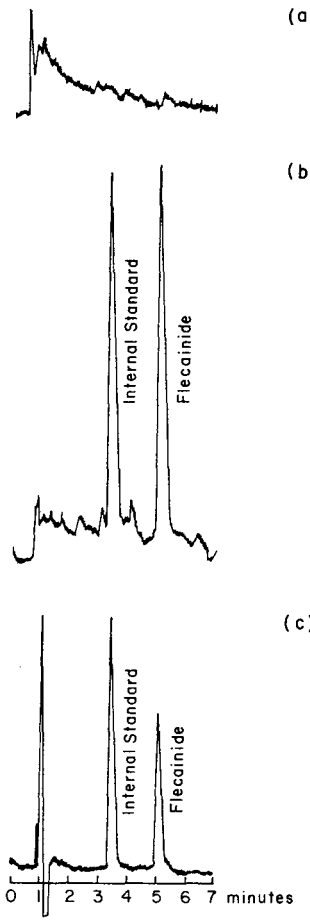


Fig. 1. Typical chromatograms of human plasma extracts for flecainide determination. (a) Blank plasma, (b) plasma of human dosed with flecainide acetate with the internal standard (250 ng) added, and (c) flecainide and internal standard.

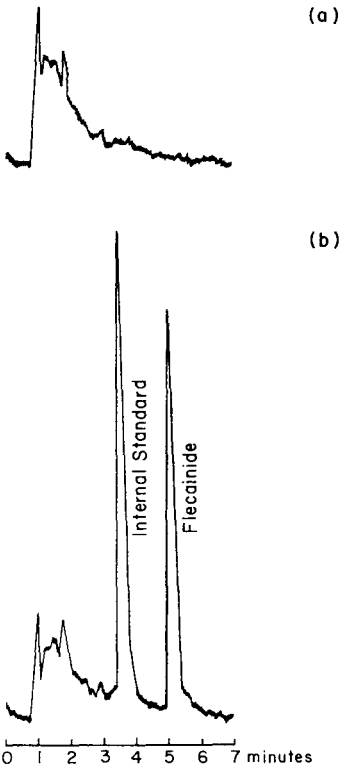


Fig. 2. Typical chromatograms of human urine extracts for flecainide determination. (a) Blank urine, (b) urine of human dosed with flecainide acetate with the internal standard (250 ng) added.

in the results of the quantitative determination. The extraction recovery from human plasma was monitored in quadruplicate (triplicate for the 698 ng/ml level) at four concentration levels: 175, 349, 698, and 1310 ng/ml. The extraction recoveries of flecainide were 61.5, 74.5, 70.7, and 73.5% at the concentration levels mentioned above. The relative standard deviations were less than 8% and indicated good reproducibility in extraction recovery between samples. The 175 ng/ml level showed a somewhat lower extraction recovery and thus the use of an internal standard with physical properties similar to flecainide becomes very important. In this assay procedure, the internal standard has been able to compensate for the potential difference in extraction recovery between concentration levels.

Calibration curve

A calibration curve was prepared daily with each batch of unknown samples. The calibration curve consisted of eight single standards: a blank, 22, 44, 87, 262, 524, 873, and 1746 ng/ml. The correlation coefficient for the peak height ratio vs. flecainide concentration (by linear regression) is usually 0.998 or better indicating a high degree of linearity in the calibration curve over the concentration range studied.

Sensitivity

Under the experimental conditions, this procedure can routinely quantitate a flecainide concentration in plasma as low as 22 ng/ml with a 1-ml sample. Concentrations lower than 22 ng/ml can be detected but quantitation was not established. At the 22 ng/ml concentration, the peak height was at least five times the level of the background noise. When a 2-ml sample is used, the minimum concentration that can be quantitated is 11 ng/ml. The urinary concentrations of flecainide are generally much higher than plasma concentrations, therefore sensitivity is not as critical. In fact, in many instances, urine samples have to be diluted before being analyzed.

Selectivity

Many drugs which may be given concomitantly with flecainide acetate were tested for interference in the assay (Table I). The types of drugs tested include β-blockers, analgesics, central nervous system stimulants, cardiac glycosides, anticoagulants, antihypertensives, bronchodilators, vasodilators, and antiarrhythmics. These drugs were tested by direct injection into the liquid chromatograph and their retention times were compared with those of flecainide and internal standard. With two exceptions (quinidine and dipyridamole), the drugs did not show any interference with the flecainide assay, either because of a different retention time or very little UV absorption at 308 nm. A secondary peak (presumably dihydroquinidine) associated with quinidine showed an incomplete separation from flecainide and affected its quantitation. Dipyridamole had a retention time identical to that of the internal standard, but when the drug was added to the plasma, it did not extract under the conditions used here, thus dipyridamole in plasma does not affect the flecainide assay. Although propranolol did not achieve baseline separation from the internal standard, there was enough separation to allow accurate quantitation of flecainide. Some of these drugs: bretylium, procainamide, digoxin, triamterene, mexiletin, thiazide, dilantin, theophylline, propranolol, and quinidine, were also tested by collecting plasma samples from patients who had received these drugs and processing these plasma samples according to the flecainide procedure. Again with one exception (bretylium), the plasma metabolites of these drugs did not interfere with the flecainide assay. In the case of quinidine, the dihydroquinidine-like peak practically disappeared, presumably metabolized in the human body. Thus, this assay procedure can be applied to a variety of clinical situations where a combination of drugs are administered.

TABLE I

DRUGS TESTED FOR INTERFERENCE

Drugs	Amount injected (ng)	Retention time (min)
Flecainide acetate	200	5.17
Internal standard	125	3.50
N-Acetylprocainamide	300	1.88
Acetylsalicylic acid	2000	1.54
Bretylium tosylate	1000	*
Caffeine	1000	*
Diazepam	50	2.97
Digitoxin	1000	*
Digoxin	1000	*
Dilantin	15,000	*
Dipyridamole	100	3.50**
Disopyramide	1000	*
Heparin sodium	1000	*
Hydralazine HCl	100	1.61
Lidocaine HCl	1000	*
Methyldopa	100	1.34
Mexiletin HCl	1000	*
Practolol	5000	1.46
Procainamide HCl	100	1.80
Propranolol HCl	100	3.04
Quinidine sulfate	2500	4.14
Quinine sulfate	2500	4.14
Sulfinpyrazone	1000	2.58
Theophylline	1000	*
Thiazide HCl	10,000	1.26
Tocainide	1000	*
Triamterene	100	1.86

*No peak after 30-min elution.
**When the drug was added to plasma, it did not extract under the flecainide acetate assay procedure.

Precision and accuracy

The intra-day precision and accuracy of the assay were determined by spiking blank human plasma with flecainide at four concentration levels: 175, 349, 698, and 1310 ng/ml, in quadruplicate. These spiked samples were carried through the entire procedure. The results are shown in Table II. The intra-day precisions, expressed as relative standard deviations, were 3.2, 0.7, 6.4, and 0.9% for 175, 349, 698, and 1310 ng/ml, respectively. The accuracies for the above mentioned concentrations, expressed as relative errors, were —0.1, 7.8, 9.8, and —2.4%, respectively. The inter-day precision was established by analyzing samples at three concentration levels: 175, 437, and 1310 ng/ml, for four consecutive days. Excellent day-to-day reproducibility was demonstrated at these concentrations with a relative standard deviation of 5% or less in each case. The precision and accuracy data indicate that this method is more than adequate for the routine measurement of plasma flecainide concentrations for clinical management as well as for research purposes where more precise data are needed.

TABLE II

INTRA-DAY PRECISION AND ACCURACY (n = 4)

Sample concentration (ng/ml)	Mean ± S.D. (ng/ml)	Relative standard deviation (%)	Relative error (%)
175	175 ± 5.5	3.2	—0.13
349	376 ± 2.5	0.7	7.8
698	766 ± 49.0	6.4	9.8
1310	1278 ± 11.8	0.9	—2.4

Correlation of HPLC and GLC methods for plasma flecainide

The GLC method [11] has been used to measure flecainide concentrations in a large number of metabolic, tolerance, and therapeutic studies in humans. To compare the GLC and HPLC methods, about 60 human plasma samples from a clinical study were analyzed by both methods. The results are shown in Fig. 3. Excellent correlation between methods was obtained (correlation coefficient = 0.99). Thus, the HPLC method appears to be capable of providing the same quality of quantitation as the GLC method, yet the HPLC procedure is

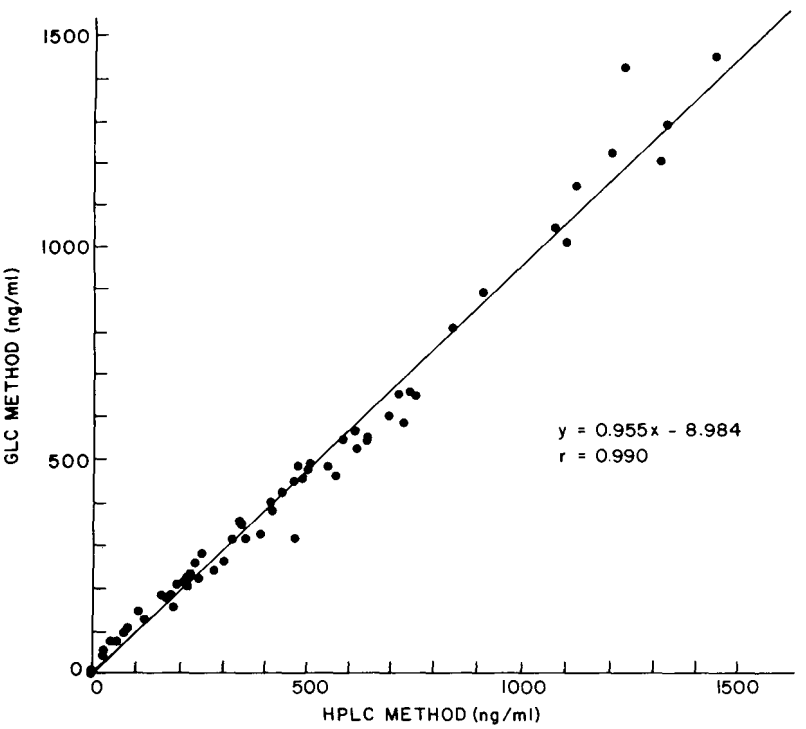


Fig. 3. Comparison of the HPLC method for the determination of flecainide in plasma with an established GLC method.

much simpler in sample preparation, less demanding in laboratory equipment, and easier to reproduce between laboratories. The method is suitable for routine clinical monitoring and pharmacokinetic studies.

DISCUSSION

Extraction

The sample preparation is accomplished by two hexane washings, one hexane extraction, and one evaporation. The entire procedure requires only one transfer; the hexane extract is transferred into a conical centrifuge tube for evaporation. Thus, the need for glassware and manual transfer is kept to a minimum. Furthermore, the initial two hexane washes at an acidic pH remove all the neutral and acidic substances soluble in hexane, thus drastically reducing the potential interference. The final hexane extraction at a strong basic pH (1 ml, 1 *N* sodium hydroxide) selectively extracts flecainide and excludes some of the known urinary metabolites of flecainide in humans [15].

The use of hexane in both the washing and extraction steps resulted from studying the effect of various solvents and solvent combinations on the extent of extraction and cleanliness of the chromatogram. The present sample preparation procedure affords about 70% extraction recovery. The use of diethyl ether in place of hexane substantially improves the extraction recovery, but at the same time, an extra peak is eluted which interferes with the internal standard. Furthermore, a memory peak is adversely affected. The residue resulting from the evaporation of the ether extract cannot be totally dissolved in the mobile phase, thus centrifugation prior to injection into the HPLC system is needed. The use of diethyl ether also requires an explosion-proof centrifuge.

The combinations of hexane and diethyl ether, hexane and chloroform, and hexane and toluene were also tried. Among the combinations, all except hexane–toluene improved the extraction recovery over the pure hexane system. The hexane–diethyl ether still suffers from the same problems as the pure ether system. The hexane–chloroform system affords a slightly better extraction recovery, but chloroform is considered to be a potential health hazard. Upon consideration of all aspects: the extent of extraction, cleanliness of the chromatogram, fewer steps in sample preparation, no need for special equipment (such as explosion-proof centrifuge) and the use of a nonhazardous solvent, hexane was chosen for both the washing and extraction steps.

In the present procedure the plasma is washed twice with hexane. A reduction in the number of hexane washes will result in the appearance of a peak interfering with the internal standard and also an increase in the size of a memory peak.

Trimethylamine (TMA) is known to minimize glass adsorption of low concentrations of amines. The addition of TMA for the extraction of nanogram levels of basic compounds has been a general practice in our laboratory. TMA solution is generally prepared by bubbling TMA gas through benzene and determining the final molarity of the benzene solution by titration. For some laboratories, this titration method might not be available. Thus, an extraction survey was made to find a suitable substitute which can be conveniently

obtained and does not significantly sacrifice the quality of the method. TMA in benzene yields the most consistent extraction recovery of flecainide and the cleanest chromatogram. Triethylamine also meets the requirements, but many extra peaks appeared on the chromatogram. TMA hydrochloride salt is commercially available and converts to the free base during the extraction step. The TMA hydrochloride salt performs well, but with slightly lower recovery than the TMA free base in benzene. For the purpose of providing an extraction procedure which is the least demanding on laboratory facilities the TMA hydrochloride was chosen and has been used for all data reported here. However, for those who have the titration capability, the TMA free base in benzene is recommended (0.2 ml of 0.2 *M* TMA in benzene).

Chromatography

The present chromatographic procedure is a result of the comparison of various chromatographic procedures. The criteria analyzed were the sharpness and symmetry of the flecainide peak with a capacity factor (k') between 3 and 6. An ion suppression technique on two C_{18} reversed-phase columns and one TMS column, an ion-pair chromatographic technique on a TMS column, and the use of a PRP-1 column (Hamilton) were tried. Among the columns tested, none were able to provide a flecainide peak as sharp and symmetrical as the ion-pair technique on the TMS column. During the submission and revision period of the manuscript, it was found that a C_1 column (IBM) also afforded excellent chromatographic characteristics.

There is more than one minute difference in retention times for the flecainide and internal standard peaks. In certain instances, when the absence of other drugs in the unknown sample is assured, the elution process can be speeded up by increasing the acetonitrile content in the mobile phase, thus the throughput of the assay is increased. When the absence of other drugs is not certain, the elution parameters should not be changed. For example, increasing the elution process will deteriorate the resolution between propranolol and the internal standard.

Detection and sensitivity

In the reported procedure, the eluted flecainide and internal standard are monitored with an LDC UV Monitor III detector equipped with a 308-nm filter. The maximum UV absorption of flecainide is at 298 nm. Monitoring the effluent at 298 nm will increase the response by 10% in comparison to that at 308 nm, while at 313 nm (such as with the Waters 440 UV detector) the response decreases by 27%. Thus, with the use of a 313-nm filter, the 22 ng/ml standard can no longer give consistent and reproducible results. However, experimental results indicate that when a 2-ml plasma or urine sample is used, the quantitation of 22 ng/ml at 313 nm can be restored. Similarly, a 11 ng/ml plasma or urine sample can be quantitated with a 2-ml sample at 308 nm. When a 2-ml sample is extracted, additional hexane washings have to be made.

The reported procedure is capable of quantitating 22—1746 ng/ml with a 1-ml sample. This range adequately covers the plasma concentrations in human subjects receiving a single or multiple dose. Although the procedure provides a wide linear range of reference standards, in practice a plasma calibration curve

of 22—873 ng/ml for a single-dose administration, and a 44—1397 ng/ml calibration curve for a multiple dose administration is recommended.

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CHROMBIO. 1509

DETERMINATION OF TAMOXIFEN AND METABOLITES IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN FLUORESCENCE ACTIVATION

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SUMMARY

Sensitive and reproducible analyses were developed for assaying tamoxifen, monohydroxytamoxifen, N-desmethyltamoxifen, metabolite E [*trans*-1(4-hydroxyphenyl)1,2-diphenylbut-1-ene] and a new metabolite, metabolite Y [*trans*-1(4-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene] in human serum using high-performance liquid chromatography (HPLC). Three different systems were developed for specific purposes. All chromatography was performed using serum extracts made with hexane—butanol. Detection was by fluorimetry of phenanthrene derivatives formed by on-stream UV irradiation with a newly described device for post-column irradiation of the HPLC stream. This device may be of use in other HPLC systems requiring post-column photochemical reactions.

INTRODUCTION

Tamoxifen is a nonsteroidal triphenylethylene anti-estrogen used routinely in the treatment of breast cancer and in basic endocrinology studies in the laboratory [1]. Consequently it is important to have convenient, sensitive and specific methods for assay of the parent drug and its metabolites in order to study pharmacokinetics, drug interactions, and the metabolism of tamoxifen in different species. Currently available methods use thin-layer (TLC) or high-performance liquid chromatographic (HPLC) methods [2—4] with detection based upon measurement of fluorescence developed by UV photochemical conversion of the triphenylethylene nucleus to fluorescent phenanthrenes (Fig. 1). The previously reported liquid chromatographic methods [3, 4] are based upon UV irradiation of extracted metabolites followed by chromatographic separation of the resulting phenanthrenes. While this method has

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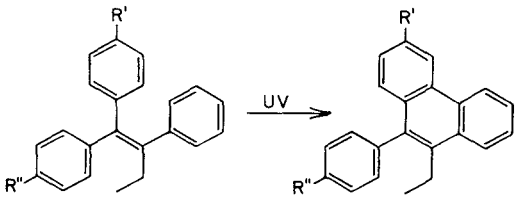

(1)	TAMOXIFEN	$R' = OCH_2CH_2N(CH_3)_2$	R'' = H
(2)	N-DESMETHYLTAMOXIFEN	$R' = OCH_2CH_2NHCH_3$	R'' = H
(3)	4-HYDROXYTAMOXIFEN	$R' = OCH_2CH_2N(CH_3)_2$	R'' = OH
(4)	METABOLITE E	R' = OH	R'' = H
(5)	METABOLITE Y	$R' = OCH_2CH_2OH$	R'' = H

Fig. 1. The chemical structure of tamoxifen and some of is metabolites, and the general reaction forming fluorescent derivatives of phenanthrene when these compounds are exposed to UV light.

generated useful information [5], the published chromatograms [3] exhibited broad and irregular peaks, probably the result of further photochemical degradation of the phenanthrenes. In an attempt to improve upon this system, we have developed an assay based upon on-stream activation of the fluorescence after chromatographic separation on HPLC columns. This paper describes a new on-stream post-column UV irradiation device and three HPLC systems for resolution of tamoxifen, several of its established metabolites, and a new metabolite, metabolite Y, from serum samples.

MATERIALS AND METHODS

Solvents and reagents

Solvents were HPLC grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Diethylamine and triethylamine were from Aldrich (Milwaukee, WI, U.S.A.).

Tamoxifen, N-desmethyltamoxifen, ICI 99,311 [1-(4-β-dimethylamino-ethoxyphenyl)-2,2-diphenylacrylonitrile], [^{3}H]monohydroxytamoxifen (42 Ci/mmole), metabolite E [*trans*-1(4-hydroxyphenyl)1,2-diphenylbut-1-ene], and metabolite Y [*trans*-1(4-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene] (Fig. 1) were gifts from ICI (Pharmaceuticals Division), Macclesfield, Great Britain. Stock standard solutions were prepared in appropriate solvents (hexane or methanol), and were kept dark and refrigerated. Working standards were prepared by dilution of the stock solutions in the chromatographic solvent.

Apparatus and chromatographic conditions

A schematic representation of the system is shown in Fig. 2. A Perkin-Elmer Model 601 pumping system (Perkin-Elmer, Norwalk, CT, U.S.A.) was used. Flow from the column passed directly into a Rheodyne injector (Model 7010, Rheodyne, Cotati, CA, U.S.A.) used as a valve to direct the stream either to the fluorescence activation unit or to by-pass this unit. Thus, examination of replicate chromatograms with and without photochemical activation permitted

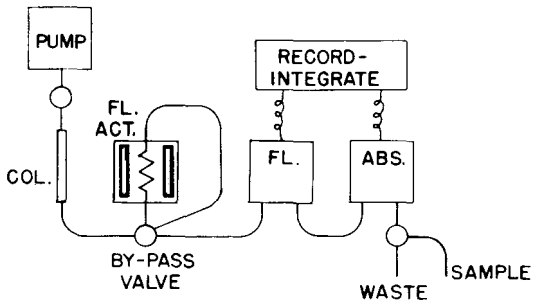

Fig. 2. Schematic representation of the HPLC system for analysis of tamoxifen metabolites using post-column activation of fluorescence by on-stream irradiation with UV light. Abbreviations: FL.ACT., fluorescence activator; COL., HPLC column; FL., fluorimeter with flow cell; ABS., spectrophotometer with flow cell. Components are described in the text.

distinction between fluorogenic components and those with native fluorescence.

The fluorescence activator consisted of a quartz capillary coil (approximately 70 cm × 0.2 mm I.D., 6 mm O.D.) interposed closely between two mercury UV lamps (Mineralite shortwave mercury lamps rated at 1200 $\mu W/cm^2$ at 15 cm) all in an air-cooled housing. Aluminum foil reflectors were placed in the housing to provide maximum luminosity on the quartz coil. Ozone generated from this unit was conducted to an exhaust hood. Each mercury lamp was separately powered so that illumination intensity could be varied. The chromatographic stream from this activator was directed to a filter fluorimeter (Fluorichrom, Varian Instruments) fitted with a 220- or 254-nm interference filter in the primary and a 360-nm filter in the secondary light path. The stream from the fluorimeter in some experiments was passed through a flow spectrophotometer (Model LC-55, Perkin-Elmer) set to measure absorption at 260 nm. A three-way valve in this exit stream allowed diversion of the stream to waste or to a fraction collector from which specific fractions could be recovered for further study such as mass spectrometry. The fluorimeter signal was recorded using an electronic recording integrator (Hewlett-Packard Model 3390A) which recorded peak areas and retention times.

Sample preparation

This procedure for the extraction of metabolites was suitable for both HPLC and TLC. All glassware used was washed with sulfuric acid—dichromate, distilled water, and rinsed with methanol prior to use. The serum sample was pipetted into conical centrifuge tubes (15 ml capacity) having PTFE-lined caps. Internal standard (ICI 99,311, [^{3}H]monohydroxytamoxifen, or metabolite E) in 10 μl of methanol, was added to serum samples, mixed and allowed to stand for at least 5 min before extraction with hexane containing 2% butanol. Serum volumes of 0.01—0.1 ml were extracted once with 1.0 ml of hexane–2% butanol; serum volumes larger than 0.1 ml were extracted once with 10 volumes of hexane—butanol. Tubes were mixed on a vortex mixer for 15 sec, centrifuged for 10 min and a portion of the supernatant fluid was transferred to 2.0-ml conical vials (Reacti-vials, Pierce Chemicals, Rockford, IL, U.S.A.) and evaporated at 55° C under nitrogen. Dried samples were redissolved

in 50 or 100 μl of HPLC solvent and aliquots were used for injection. All manipulations were carried out under subdued light or under yellow fluorescent light to minimize exposure to UV light.

System I: tamoxifen, N-desmethyltamoxifen, and monohydroxytamoxifen

Resolution of the major serum components, tamoxifen, N-desmethyltamoxifen, and monohydroxytamoxifen was achieved using a C_{18} reversed-phase column (Whatman ODS-2, 10 μm particle size, 25 × 0.46 cm) eluted isocratically with absolute methanol containing 0.04% diethylamine acetate pumped at 2.0 ml/min. Using these conditions monohydroxytamoxifen has a retention time of 3.3 min, tamoxifen 5.4 min and desmethyltamoxifen 7.1 min, with baseline resolution (Fig. 3). Since it was not possible to find a suitable internal standard for this system, [^{3}H]monohydroxytamoxifen was used in tracer amounts to determine extraction and procedural recoveries. The appropriate fraction was collected from the HPLC stream and counted by liquid scintillation techniques. The quantity added was below fluorescence detection levels. Metabolite E, reported to be a metabolite of tamoxifen in the dog [6], was eluted at the void volume.

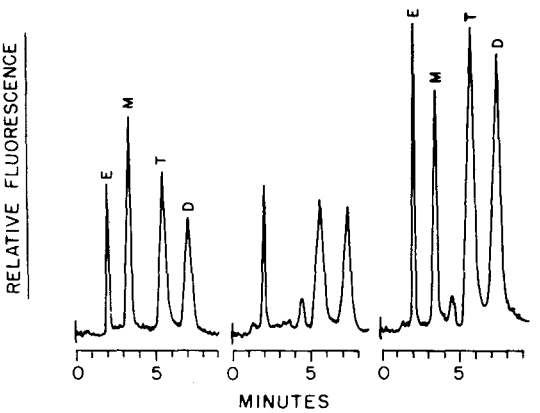

Fig. 3. Chromatography of tamoxifen metabolites on a C_{18} reversed-phase column (System I). The first panel shows the resolution of a standard mixture of 25 ng each of metabolite E, monohydroxytamoxifen (M), tamoxifen (T), and N-desmethyltamoxifen (D). The second panel is a chromatogram of an extract equivalent to 20 μl of serum from a patient (I.J.) receiving 300 mg/m^2 of tamoxifen daily. In the third panel is shown a chromatogram of the same serum fortified with the four standard compounds. Chromatographic conditions are described in the text.

System II: tamoxifen and desmethyltamoxifen

Since a suitable internal standard for the reversed-phase system (System I) could not be found, a normal-phase system was developed using a silica column (Alltech 600-Si, 10 μm particle size, 25 × 0.46 cm, Alltech, Deerfield, IL, U.S.A.) eluted isocratically with methanol–water–triethylamine–acetic acid (98:2:0.03:0.3, v/v), and pumped at 3.0 ml/min. Using these conditions, N-desmethyltamoxifen had a retention time of 2.4 min, tamoxifen 3.7 min, and compound ICI 99,311, as an internal standard, eluted at 4.5 min. In this system monohydroxytamoxifen is not resolved from tamoxifen. Since monohydroxy-

tamoxifen is a very minor serum component in patients receiving usual doses of tamoxifen, it does not interfere significantly with the tamoxifen assay.

System III: metabolites E and Y

Since fluorogenic material including metabolite E was eluted with the void volume of the above system, a third system was developed which would retain this possible metabolite. This system used a silica column (Alltech 600-Si, 10-μm particle size, 25 × 0.46 cm), eluted isocratically with hexane containing 1.3% isopropyl alcohol. At a flow-rate of 3.0 ml/min, this system resolved metabolite E (retention time 3.9 min) and a second fluorogenic metabolite, identified as metabolite Y (Fig. 1) by co-chromatography and mass spectra, eluting at 8.0 min. Structural identification and biological properties of metabolite Y are reported elsewhere [7].

RESULTS AND DISCUSSION

Fig. 1 shows the structures of the compounds studied and depicts the UV-catalyzed formation of fluorescent phenanthrenes which provides a sensitive method for detection. The fluorescence of these phenanthrenes was enhanced in acidic or neutral solvents, hence the diethylamine or triethylamine used in the solvents in Systems I and II was neutralized with acetic acid to improve sensitivity.

A schematic representation of the chromatographic system is shown in Fig. 2. The sample stream, by means of the Rheodyne valve, may be directed to the fluorescence activator, or may by-pass the activator. In this way duplicate injections can be compared with and without fluorescence activation and fluorogenic peaks thus identified. When it is desired to collect a given peak without photochemical alteration, the by-pass mode can be used and peaks collected either by elution time, or by detection of UV absorption in the flow spectrophotometer. Thus, unaltered fractions may be recovered for other studies without significant photochemical degradation.

The resolution of a mixture of standard compounds containing metabolite E, monohydroxytamoxifen, tamoxifen and desmethyltamoxifen using the C_{18} reversed-phase system (System I) is illustrated in the first panel of Fig. 3. The second panel is a chromatogram of a serum extract from a patient who had received 300 mg/m^2 of tamoxifen daily for 32 days. The third panel is a chromatogram of the same serum to which were added 25 ng of each of the four standard compounds. Extraction efficiency of internal standard ([^{3}H]-monohydroxytamoxifen) or of added tamoxifen and N-desmethyltamoxifen averaged 85% and the limit of detection of each compound was about 0.2 ng per injection.

Since monohydroxytamoxifen was virtually undetectable in serum extracts using this technique, and since compound ICI 99,311 was found to be useful as an internal standard in our silica column system (System II), it has been more convenient to use System II (Fig. 4) for routine measurements of tamoxifen and its major metabolite, N-desmethyltamoxifen, in sera. The third and fourth panels of Fig. 4 show resolution of standards and of metabolites from serum from a patient receiving the usual dosage of tamoxifen (20 mg/m^2/

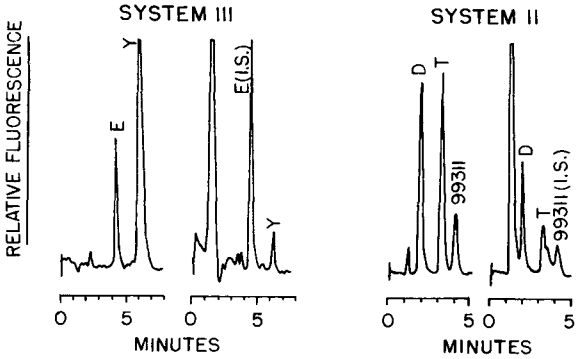

Fig. 4. Chromatograms of extracts from 0.5 ml of serum from a patient receiving normal dosages of tamoxifen (20 mg/m²/day), resolved on System III (silica column eluted with 1.3% isopropyl alcohol in hexane), and in System II (silica column eluted with methanol—water—triethylamine—acetic acid described in the text). Compound ICI 99,311 was used as an internal standard (I.S.) in System II and metabolite E was used as an internal standard in System III. Metabolite E was not present in patient sera without the internal standard spike.

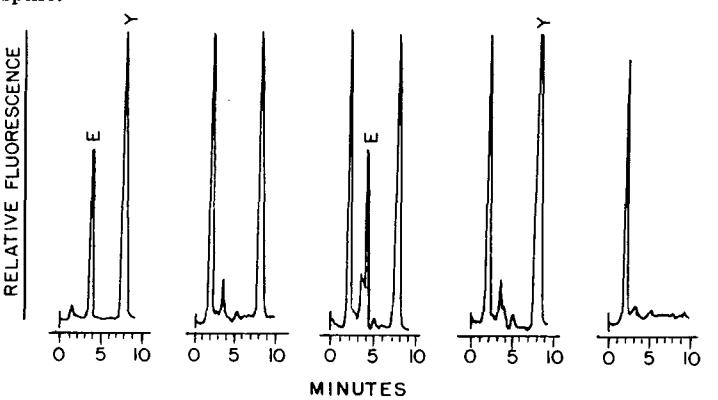

Fig. 5. Chromatography of extracts of serum from a high-dose tamoxifen patient (300 mg/m²/day) using System III and showing the presence of a peak (retention time 8.0 min, panel 2) which co-chromatographs with metabolite Y (panel 4). Panel 2 shows that metabolite E is not detectable in this sample and that E is readily resolved from a minor peak (panel 3). Note that fluorescence disappears when the fluorescence activator is off (bypassed) in the last panel.

day). The limit of detection of tamoxifen and N-desmethyltamoxifen was about 0.2 ng per injection.

Metabolite E, a metabolite in the dog and a potential metabolite in humans, was eluted along with other fluorogenic material in the void volume in Systems I and II. To resolve such possible metabolites in this void volume, a silica system (eluted with hexane–isopropyl alcohol) was developed (System III) which retained metabolite E for a reasonable time (Figs. 4 and 5). Under these conditions, tamoxifen, N-desmethyltamoxifen and monohydroxytamoxifen were not eluted. In a high-dose patient (Fig. 5), there was no convincing evidence of the occurrence of metabolite E. However, in this high-dose patient and also in a normal-dose patient (Fig. 4, panel 2) there was a second fluorogenic peak which co-chromatographed with the compound designated metab-

olite Y (Fig. 5, panel 4). The last panel of Fig. 5 indicates that, except for material with native fluorescence in the void volume, no fluorescence is detectable when the column stream by-passes the UV activator.

Identification of peak Y was by co-chromatography with authentic metabolite Y (kindly provided by Dr. Adam, ICI) and by gas chromatography and mass spectral data of appropriate derivatives [7].

The three systems described gave linear standard curves and were reproducible from day to day within limits of column and equipment stability. Standard curves of tamoxifen, N-desmethyltamoxifen and metabolite E recovered from control or patient sera assayed using System II and System III, were linear in the range from 2—200 ng/ml of serum with correlation coefficients, r, of 0.988—0.999. Triplicate analyses using System II carried out on three separate days had a coefficient of variation of 6.8%. Within-day variation was similar. Using System III with an internal standard of metabolite E, recovery of metabolite Y standards added to blank serum averaged 97.4% of the theoretical recovery with an average coefficient of variation of 5.6% for additions in the range 12.5—100 ng for triplicate analyses carried out on two different days. Observed values were highly correlated with theoretical values ($r = 0.9986$).

It would be desirable to have a single system for the analysis of all tamoxifen metabolites. However, we have not been able to develop such a system probably because of the marked differences in properties between metabolites with and without the dimethylaminoethoxy sidechain.

There are several advantages of post-column on-line irradiation for development of fluorescence over previous methods of irradiating manually before chromatography. First, the exposure to UV is dependent only on the stability of the flow-rate and the UV lamp intensity; geometry of the system is fixed. Second, most UV-absorbing materials are resolved from the compounds of interest before irradiation, hence cannot quench their fluorescence development or react photochemically with them. Third, photochemical degradation products of the pre-irradiated sample undoubtedly produce a more complex sample which leads to a more complex chromatogram and poorer resolution, whereas post-column irradiation of the already resolved peaks does not.

The HPLC methods described here for the analysis of tamoxifen, its established metabolites, and the new component, metabolite Y, will be of considerable aid in the further study of the pharmacology, metabolism and endocrinology of this anti-estrogen in patients and in animal model systems.

The device described here for on-stream post-column UV irradiation may well have applications for other HPLC systems requiring photochemical reactions.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEFLOXACIN AND ITS MAIN ACTIVE METABOLITES IN BIOLOGICAL FLUIDS

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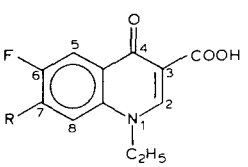
SUMMARY

We describe a high-performance liquid chromatographic (HPLC) method for the analysis of pefloxacin, a new antibacterial agent, in plasma and urine following administration of a therapeutic dose in humans. HPLC assay of pefloxacin and its two main active metabolites in urine is also described. The applicability of the methods to pharmacokinetic studies of pefloxacin in humans is demonstrated.

INTRODUCTION

Pefloxacin (Fig. 1), 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-methyl-1-piperazinyl)-quinoline-3 carboxylic acid (PF), is a new antibacterial agent, which has shown one of the highest level of in vitro activity against gram-negative bacteria among the compounds of the quinolone group; its broad and potent activity spread to gram-positive bacteria [1, 2].



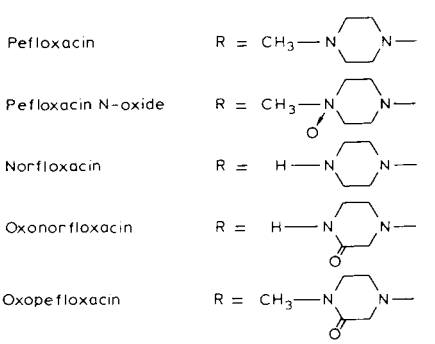


Fig. 1. Structures of pefloxacin and identified metabolites in man.

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Studies in animals and man have shown that PF is transformed into several metabolites [3]. The main metabolites (Fig. 1) identified in man are pefloxacin N-oxide, desmethylpefloxacin or norfloxacin (NF), and oxonorfloxacin (ONF), and also a minor one, oxopefloxacin. PF, NF and ONF have bacteriological activity. PF is the main active compound in human plasma (about 80% of the activity encountered), whereas NF, PF and to a lesser extent ONF account for the activity found in urine.

This paper describes a high-performance liquid chromatographic (HPLC) method for the quantitation of PF in plasma or urine. Quantitation of PF, NF and ONF in urine is also described. The technique is based on separation by reversed-phase ion-pair chromatography using an octadecylsilane stationary phase and a solution of tetrabutylammonium iodide in acetonitrile–aqueous phosphate buffer as mobile phase. An example from a pharmacokinetic study is shown.

EXPERIMENTAL

Chemicals and reagents

PF, NF and ONF, the 6-chloro analogue (I) and the 1-methyl analogue (II) of PF (internal standards) were synthesized in our laboratories. All reagents were of analytical grade.

The mobile phase consisted of a mixture of water and acetonitrile containing 0.4% of $Na_2HPO_4 \cdot 12H_2O$ and 0.2% of tetrabutylammonium iodide, adjusted to pH 9.4 with 1% triethylamine. Two solvents were prepared: solvent A was only distilled water; solvent B was a 2:3 (v/v) acetonitrile–water mixture.

Stock solutions of PF, NF and internal standards were prepared by dissolving 1 mg/ml of each compound in 0.01 *M* sodium hydroxide. A solution, prepared daily, of ONF 0.1 mg/ml in dimethyl sulphoxide (DMSO) was used.

Preparation of samples

Analysis of PF in plasma or urine. Stock solutions of PF and I were diluted in water to obtain working daily solutions which were used to prepare plasma and urine standards.

Plasma standards were between 0.125 and 1.50 μg of PF for 1 ml of plasma with 1.50 μg of I, and between 0.5 and 3 μg of PF for 0.5 ml of plasma with 3 μg of I.

Urine standards were between 1 and 10 μg/ml of PF with 5 μg of I, and between 5 and 100 μg/ml of PF with 50 μg of I.

Analysis of PF, NF and ONF in urine. Working solutions of PF, NF and II were prepared by diluting the stock solutions in water. Concentrations of PF and metabolites between 5 and 60 μg/ml in urine with 30 μg/ml of II were obtained from these solutions and the solution of ONF in DMSO.

Plasma and urine analysis of PF

To 0.5 or 1 ml of plasma in a 15-ml glass centrifuge tube were added 300 or 150 μl of the internal standard I solution (10 μg/ml), 0.5 ml of 0.5 *M* phosphate buffer (pH 7.4) and 10 ml of chloroform. The tube was manually shaken for 10 sec, centrifuged and the upper aqueous phase was discarded. The solvent

was transferred into a clean tube and evaporated to dryness in a 60°C water bath under a stream of air. The residue was dissolved in 50 μl of 1% ammonia and 10 μl of this solution were injected into the chromatograph.

To 1 ml of urine in a glass tube were added 50 μl of the internal standard I solution (1 mg/ml or 100 μg/ml), 0.5 ml of phosphate buffer and 10 ml of chloroform. The operation was then carried out as for plasma.

Urine analysis of PF, NF and ONF

To 1 ml of urine in a glass tube were added 300 μl of the internal standard II solution (100 μg/ml) and 0.5 ml of 0.5 *M* phosphate buffer (pH 7.0). The mixture was twice extracted for 20 sec with 10 ml of chloroform–isopentanol (10:1, v/v).

After combining the two extracts in a clean glass tube, the solvent was evaporated to dryness and the residue was dissolved in 100 μl of 1% ammonia; 10 μl of this solution were injected into the chromatograph.

HPLC system

A Varian Model 5020 liquid chromatograph was used, equipped with a Pye-Unicam LC-UV detector operating at 270 nm and a LiChrosorb RP-18 column, particle size 10 μm (Merck, Darmstadt, G.F.R.), 100 × 4.6 mm I.D.

The mobile phase was 52% of solvent A + 48% of solvent B for PF assay; for PF, NF and ONF assay a gradient of B was used starting from 20% of B and rising at a rate of 2.5% per min for 10 min. The flow-rate was 2 ml/min.

RESULTS AND DISCUSSION

PF assay in plasma or urine

Fig. 2 depicts representative chromatograms from blank or spiked plasma or urine and plasma or urine samples from a subject given pefloxacin. Under the described chromatographic conditions, PF and internal standard had retention times of 2 and 3.6 min, respectively, so that an injection could be made every 6 min. No interference was observed except by sulfamethoxazole and trimethoprim.

Calibration curves plotted as the peak height ratio of PF to the internal standard versus the concentration of PF were constructed and linear curves were obtained. The equations for the calibration curves were as follows:

Plasma	
0.125–1.5 μg/ml:	$Y = 1.2980X + 0.0136; r = 0.9997$
1–6 μg/ml (0.5-ml sample);	$Y = 0.571X - 0.072; r = 0.999$
Urine	
1–10 μg/ml:	$Y = 0.201X + 0.099; r = 0.990$
5–100 μg/ml:	$Y = 0.0230X + 0.0416; r = 0.9997$

The concentration ranges studied in plasma (0.1–6 μg/ml) and urine (1–100 μg/ml) covered the expected concentrations of PF in biological fluids after a single administration of the drug in humans. The detection limit was 0.05 μg/

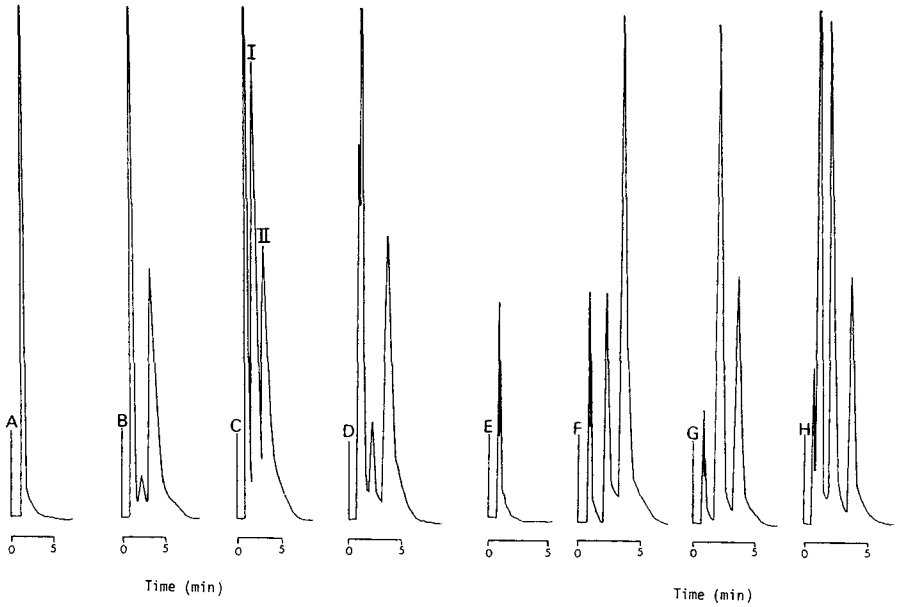

Fig. 2. Chromatograms of plasma and urine samples. (A) Blank plasma extract. (B, C) Extracts from blank plasma spiked with 0.125 μg/ml (B) or 1.5 μg/ml (C) of PF (I) with 1.5 μg of internal standard (II). (D) Plasma extract from a subject given an intravenous dose of 400 mg of pefloxacin. (E) Blank urine extract. (F, G) Extracts from blank urine spiked with 10 μg/ml (F) or 30 μg/ml (G) of PF with 30 μg of internal standard. (H) Urine extract from a subject orally given 400 mg of drug.

ml in plasma and 0.5 μg/ml in urine. The overall recovery of the method was 90% for both PF and I.

Spiked plasma and urine samples were repeatedly analysed to check the reproducibility of the technique. Table I shows the results obtained for intra-day assay and inter-day assays from samples stored at 4°C for eight weeks.

Although the pH of the mobile phase was alkaline, we were able to use every

TABLE I

REPRODUCIBILITY OF PF ASSAY IN HUMAN PLASMA AND URINE

Inter-day assays were performed over an eight-week period.

	Biological fluid (1 ml)	Amount of PF added (μg)	Coefficient of variation (%; n = 10)
Intra-day	Plasma	0.25	6.4
		2	5.2
	Urine	50	2.1
		100	2.1
Inter-day	Plasma	1	9.8
		5	4.8
	Urine	25	5.2
		100	5.2

column for about 400 assays, taking care to clean it with water after each run and periodically to change the head column frit.

PF, NF and ONF assay in urine

Representative chromatograms obtained from blank or spiked urine and from a subject given pefloxacin are shown in Fig. 3. Retention times of ONF, NF, IS and PF were, respectively, 3, 4, 6.7 and 8.8 min. The N-oxide metabolite of PF was contaminated with polar components of the urine which were first eluted from the column (see c, Fig. 3). Using the described conditions, the

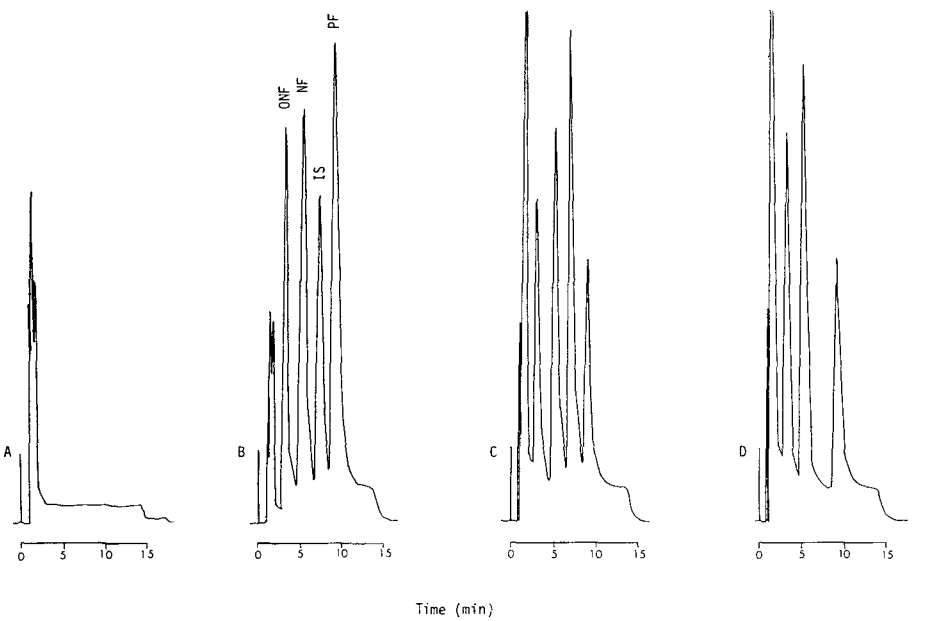

Fig. 3. Chromatograms of urine samples for PF and active metabolites assay. (A) Blank urine extract. (B) Extract from 1 ml of blank urine spiked with 25 μg of ONF, 50 μg of NF and PF and 30 μg of internal standard. (C, D) Urine extracts from a subject orally given PF with (C) and without (D) internal standard.

N-demethylated metabolite was not separated from a minor one, oxopefloxacin; we neglected this metabolite since HPLC dosages from acidified urine extracts showed that it accounted for less than 1% of the dose against 20% for NF.

Standard curves plotted as the peak height ratio of ONF, NF or PF to the internal standard versus concentrations of the metabolites are presented in Fig. 4. The equations for the calibration curves were as follows:

ONF: $Y = 0.050X - 0.015; r = 0.998$
NF: $Y = 0.028X - 0.076; r = 0.997$
PF: $Y = 0.033X - 0.012; r = 0.998$

The detection limit was 1 μg/ml for PF and ONF and 3 μg/ml for NF. Recover-

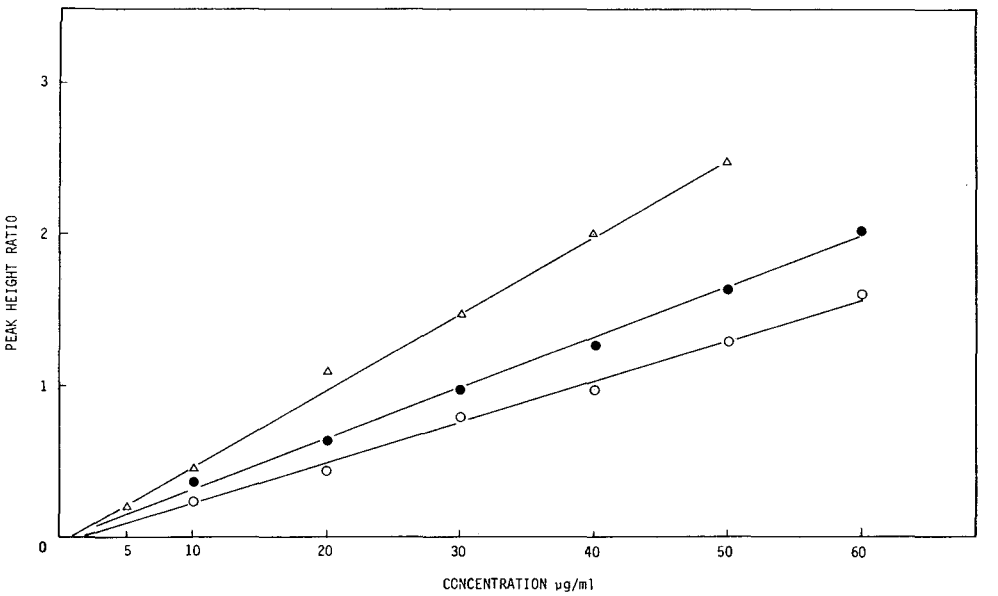

Fig. 4. Standard curves for PF (●——●), NF (○——○) and ONF (△——△) in urine (30 µg of internal standard).

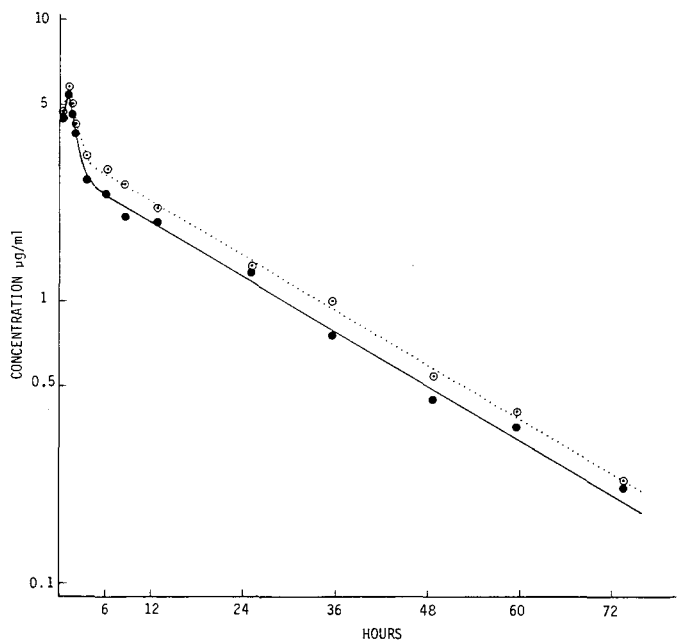

Fig. 5. Plasma level time curve obtained for PF (●——●) and with microbiological assay (○ - - - ○) following a 1-h infusion of 400 mg of pefloxacin to a patient.

ies were 94% for PF and ONF, 65% for NF and 95% for internal standard.

Coefficients of variation, determined on ten samples of 1 ml of urine overloaded with 10 μg of ONF, 40 μg of NF and 20 μg of PF (covering expected ratios of concentrations) were, respectively, 7.1, 5.5 and 4.5%.

KINETIC STUDIES

The method was applied to pharmacokinetic studies of PF in humans. Figs. 5 and 6 show the log plasma levels and cumulative urinary excretion curves versus time after administration of a single intravenous dose of 400 mg of PF to a patient suffering from renal and cardiac insufficiency.

In each figure, values found by microbiological assay are also plotted; they showed that PF was the main active compound in plasma whereas activity found in the urine was due to PF and metabolites.

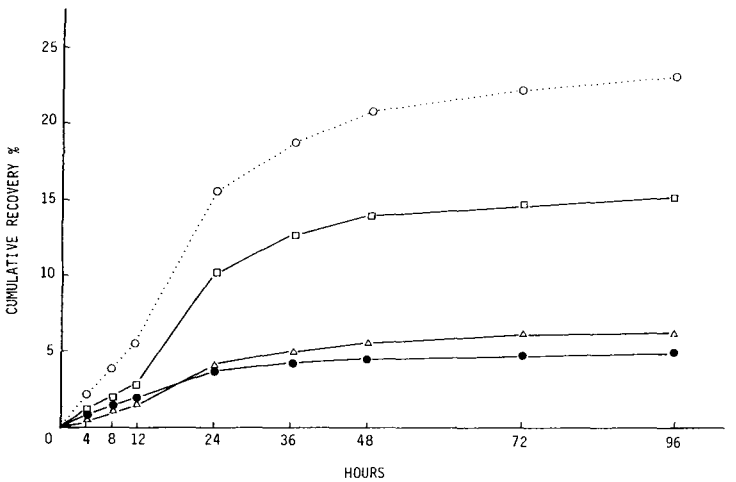

Fig. 6. Cumulative urinary excretion of PF (●——●), NF (□——□) and ONF (△——△), and microbiological assay (○ - - - ○) observed after a single infusion of 400 mg of pefloxacin to a patient.

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Note

Reversed-phase high-performance liquid chromatography method for separation of collagen tryptic peptides

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Recent methods developed for use with high-performance liquid chromatography (HPLC) for the separation of large peptides or proteins include gel chromatography with Toyo Soda TSK columns or Waters I series columns in 6 *M* guanidine HCl, 1 *M* Tris buffer or 0.1 *M* potassium phosphate, pH 7.0, and reversed-phase chromatography on octadecylsilane (C18) or cyanopropyl (CN) columns with a variety of acid aqueous—organic eluents [1—7]. In particular, collagen alpha chains (approximately 93,000 daltons) from Types I, II and III collagens have been resolved on cyanopropyl columns using a pyridine—acetate—propanol gradient [8]. Cyanogen bromide peptides of collagen have also been separated on reversed-phase columns [7]. However, separation and identification of tryptic peptides of collagen are still performed principally using gel electrophoresis or low-pressure column chromatography [9—11]. The current investigation presents a method for the isocratic separation of collagen tryptic peptides on large pore size, octadecylsilane, reversed-phase columns.

MATERIALS AND METHODS

Columns

The reversed-phase column used in these experiments was a DuPont Zorbax

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octadecylsilane (C18, ODS), 25 cm × 4.6 mm, with 5 μm particle size and 150 Å pore size (DuPont prototype column, 25-85-3).

Sample preparation

Bone diaphyses from four-week-old osteoblastoma chicks were placed on ice and stripped of periosteum and marrow, cut into 5-mm^3 pieces with bone rongeurs and pulverized to 200—400 mesh powder (at temperature of liquid nitrogen) using a Spex freezer mill (Spex, Metuchen, NJ, U.S.A.). Bone powder was demineralized in repeated changes of 0.5 *M* EDTA, 0.05 *M* Tris buffer pH 7.4 at 4°C, the residue washed exhaustively in 0.02 *M* NH_4HCO_3 and water and the residue lyophilized and weighed. A portion of the residue (largely bone collagen) was suspended in 10 m*M* calcium chloride in 50 m*M* Tris buffer pH 7.5, heated at 65°C for 20 min, cooled to 37°C, then mixed with trypsin (1% w/w, TPCK trypsin, cat. No. 30C637, Millipore, Freehold, NJ, U.S.A.) and incubated at 37°C for 2 h with stirring [10, 11]. A second trypsin portion (half of the starting amount) was added after 2 h and hydrolysis continued for two more hours [10, 11]. After hydrolysis, the residue was lyophilized, weighed, and a portion dissolved at 37°C for 20 min in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 (guanidine HCl obtained from Research Plus Laboratories, Denville, NJ, U.S.A.). Samples were sedimented at 1000 *g* for 5 min; then the supernatant fluid was removed for chromatography.

Liquid chromatography systems

Three separate chromatography systems were employed: (1) the DuPont Model 8800 controller with the Model 870 triple head pump, single sample injector, column heater, variable-wavelength detector (set at 214 nm, 8-μl flow cell) and recorder; (2) a Beckman 110 pump, single sample injector, Hitachi Model 110-10 variable-wavelength detector (set at 214 nm, 20-μl flow cell) and recorder; (3) a Waters Assoc. system controller, WISP 710 B, multisample injector, two Model 6000A pumps, a Model 441 absorbance detector (214 nm, 12-μl flow cell) and a data module.

In some experiments, sample fluorescence was monitored using a Perkin-Elmer fluorometer (Model 204-A, excitation 325 ± 5 nm, emission 395 ± 5 nm). Selected peptides were collected from five individual separations, dialyzed against water, lyophilized, and subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS) acrylamide gels.

CHROMATOGRAPHY

Mobile phase solvents were filtered through a 0.22-μm filter and degassed prior to use.

Low-pressure chromatography

For chromatography by conventional means, samples (120 mg) were applied to 300 cm × 1.5 cm columns of Sephadex G-50 superfine gel in 2 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.5 at a flow-rate of 15—20 ml/h.

Isocratic HPLC

Samples were applied to the column in mobile phase and chromatographed at 35°C in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 at a flow-rate of 1 ml/min (pressure 40 bar). In other experiments, concentrations of guanidine HCl up to 2 *M* were used, the pH was varied from 6.5 to 7.5, the temperature varied from 22°C to 60°C, and the flow-rate altered from 0.3 ml/min to 2.0 ml/min.

HPLC gradients

Linear gradients (60 ml) consisting of 1—500 m*M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 at 1 ml/min and more shallow gradients were utilized. Moreover, gradients of guanidine HCl and methanol were also utilized.

RESULTS

Molecular weight determination of the tryptic peptides of collagen in 1 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 on a TSK-2000 column and two I-60 columns indicate a range of 10,000—20,000.

Fig. 1 depicts a chromatogram of 120 mg of bone collagen on a G-50 superfine gel eluted with 2 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.4 monitored at 230 nm. A large front peak (1) containing highly crosslinked collagen

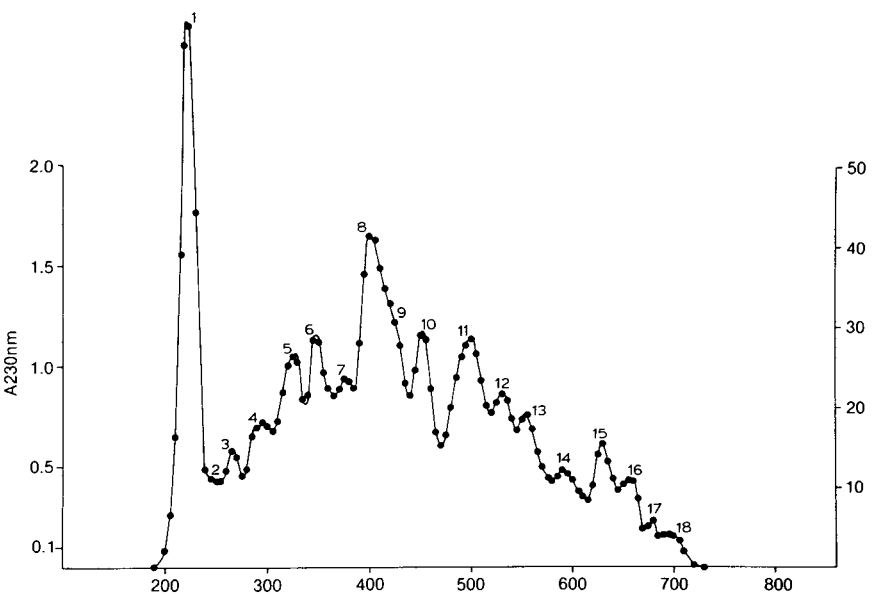

Fig. 1. Chromatogram of 120 mg of bone collagen (not reduced with $NaBH_4$) tryptic peptides from an osteoblastoma from a three-month-old chicken dissolved in 3.0 ml of 5 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.2 at 22°C. The sample was separated on a 300 × 1.5 cm column of G-50 superfine gel in 2 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.5 at a flow-rate of 15—20 ml/h. Fractions of 5 ml were collected and monitored at 230 nm. Eighteen peaks were resolved using this method; peaks 1 (void volume peak) and 3 were heterogeneous after isolation and separation on acrylamide gels.

peptides elutes in the void volume. At least 17 other peaks were detected; peaks 1 and 3 were heterogeneous by SDS gel electrophoresis (Fig. 1). In particular, peak 3, a fraction containing the fluorescent crosslink 3-hydroxypyridinium, is comprised of at least three peptides [12].

Fig. 2 is a chromatogram, typical of separations on the Zorbax C18 column at 35°C in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 with 100 μg of sample applied. However, less than 10 μg of sample can be chromatographed containing at least 30 peaks ($A_{214\ nm}$). Three of these peaks (peaks 1, 21 and 25 in Fig. 2) exhibited fluorescence (excitation = 325 nm, emission = 395 nm), indicating presence of 3-hydroxypyridinium [11]. Identical peaks (peak 21, Fig. 2) from five separations (1 mg sample size) were collected, dialyzed against water, lyophilized, and electrophoresed on 10% SDS gels. Each gave a single, faint, Coomassie-Blue reactive band in an acrylamide gel (data not shown).

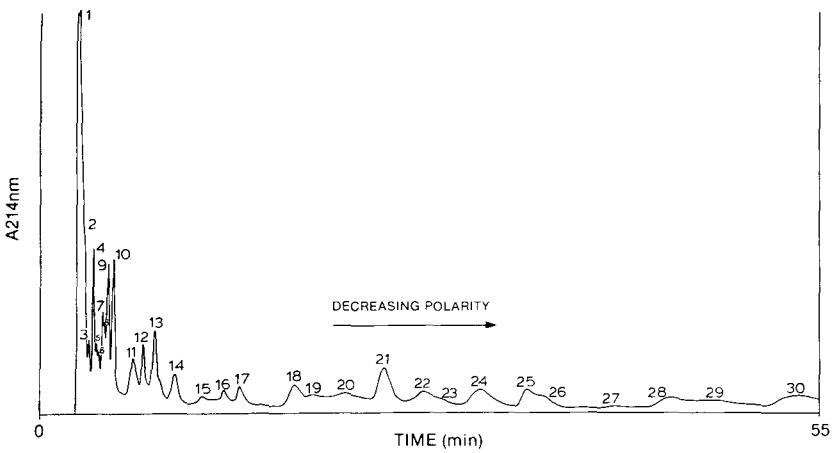

Fig. 2. Chromatogram of 100 μg of bone collagen tryptic peptides from an osteoblastoma from a three-month-old chicken dissolved in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8. The sample was injected onto a 25 cm × 4.6 mm octadecylsilane (C18) column, 5 μm particle size, 150 Å pore size (prototype DuPont column) and eluted at 1 ml/min at 35°C with mobile phase as above. Thirty distinct peaks were resolved, three of which (peaks 1, 21 and 25) fluoresced when analyzed with a Perkin-Elmer fluorometer at excitation 325 nm and emission 395 nm. In some experiments, a dye-binding assay (Bio-Rad) was used to monitor peptides in column fractions. Peak 21 was collected, concentrated and electrophoresed on a 10% SDS gel. A single Coomassie-Blue staining band was visualized.

The baseline returned to zero after 2 h for most collagen tryptic peptide samples tested and no peaks were detected during 18 h of additional run time. Series injections of the same sample yielded excellent reproducibility in peak retention times and shape.

The chromatogram in Fig. 3 depicts separation at room temperature (22°C) in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8. At least 47 peaks were detected ($A_{214\ nm}$).

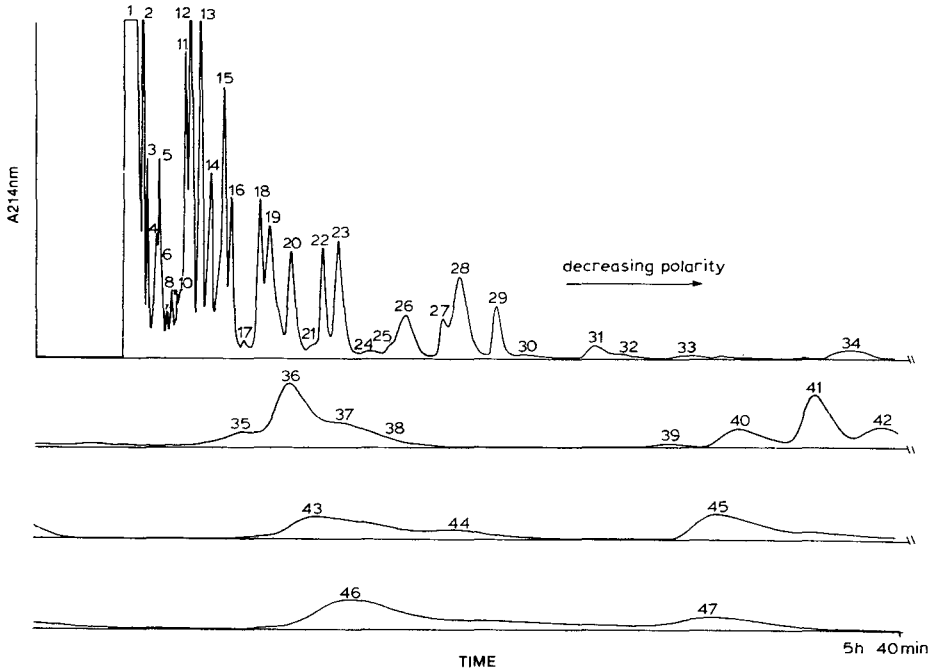

Fig. 3. Chromatogram of 50 μg of bone collagen tryptic peptides from an osteoblastoma from a three-month-old chicken dissolved in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8. The sample was injected onto a 25 cm × 4.6 mm octadecylsilane (C18) column, 5 μm particle size, 150 Å pore size (DuPont prototype column), and eluted at 1 ml/min at 22°C with mobile phase as above. Forty-seven distinct peaks were resolved. The total run time was 5 h 40 min. In some experiments a dye-binding assay (Bio-Rad) was used to monitor peptides in column fractions.

DISCUSSION

Reversed-phase chromatography is a reliable method to resolve tryptic peptides of collagen. Moreover, the latter technique permits greater resolution, reduces run time (1—2.5 h per sample) and is useful for microgram quantities of peptides. By comparison, molecular sieve-type gels offer less resolution in a single separation of a complex peptide mixture, require longer run times (days) and usually require milligram quantities of sample. The advantage in the latter case is the analysis of larger sample sizes, useful when preparing large quantities of a single peptide.

In addition, state-of-the-art protein separation columns such as the Toyo Soda (TSK 2000 or 3000, Toyo Soda Manufacturing Co., Tokyo, Japan) or Waters I-60 series columns with 2 *M* guanidine HCl, 0.05 *M* Tris buffer pH 7.4, used with HPLC, did not yield sufficient resolution of collagen tryptic peptides compared with that of reversed-phase columns (patterns were similar to that in Fig. 1 for samples separated on one TSK 2000 and two I-60 columns joined in tandem).

Isocratic separations in 0.05 *M* guanidine HCl, 0.05 *M* Tris buffer pH 6.8 at 35°C, 1 ml/min yielded the most satusfactory results (Fig. 2). These con-

ditions yielded good sample solvation, resolution and separation of collagen tryptic peptides in 1—2 h for most samples. The room temperature separation (Fig. 3) yielded more peaks (47) than did that at 35°C (30 peaks) but required 4 h and 40 min longer to effect the chromatography. Increasing the molarity of guanidine HCl up to 2 *M* decreased retention time to approximately 20 min, but yielded fewer peaks with reduced resolution, particularly in early eluting peaks. Solvent strengths above 0.5 *M* guanidine HCl were unsatisfactory, 0.1 *M* to 0.5 *M* yielded poor results, and those below 0.1 *M* yielded good to excellent results. The influence of pH in the narrow range of 6.5—7.5 had no influence on resolution (data not shown). Increased temperature decreased retention times, but also reduced the total number of peaks eluting (data not shown). The range of 35—41°C yielded similar and satisfactory results at the 0.05 *M* guanidine HCl concentration, whereas 45—60°C yielded increasingly poor results.

Programmed flow-rate increases were useful in reducing total chromatography time in the isocratic mode. For instance, a flow-rate of 0.5 ml/min was used for 10 min to increase separation of early eluting peaks, was increased to 1 ml/min for 10 min (to a point where peaks 19 and 20 elute, Fig. 2), then was increased to 2.0 ml/min for another 10 min (to the region of peak 26), then up to 3.0 ml/min for 10 min, with a return to initial conditions for 5 min before the next sample application. The latter method can be used with the Waters Assoc. LC system which is flow programmable. Flow-rate is a major consideration with any peptide separation when fraction collection is involved. Peptides separated by a matter of seconds are difficult to collect, therefore reduced chromatography time is not always beneficial. A total run time of 1 h allows for collection of 0.5-ml fractions with, in most cases, one-tube peaks. Use of a peak collector simplifies peptide collection.

Gradient elution with increasing guanidine HCl concentration from 1 m*M* to 500 m*M* was not satisfactory due to baseline shifts at 214 nm. More shallow gradients were tried (50—100 m*M* salt) but baseline shifts proved to be an overwhelming problem. Increasing the wavelength to 230 nm or 280 nm for collagen did not solve the latter problem. Methanol—guanidine HCl gradients gave the same results — unsatisfactory rises in baseline. Most recently, acetate—acetonitrile or trifluoroacetic acid—acetonitrile gradients have been tried, but some samples did not completely dissolve in initial mobile phase and some collagen peptides appeared to bind very strongly to the stationary phase in acetonitrile.

Few reports have been published on the use of halide-containing mobile phases in mildly acidic conditions for peptide elution from gel permeation or reversed-phase columns [6]. Most manufacturers do not recommend the use of halides, especially in acid conditions, in stainless-steel HPLC equipment. However, the pump heads, tubing and columns in the DuPont and Beckman systems have been used for approximately 1 year with either 0.05 *M* or 2 *M* guanidine HCl, respectively, with no demonstrable adverse effects.

The total number of collagen tryptic peptides separated by these systems was 30 at 35°C and 47 at 22°C. The theoretical number of peptides obtainable if trypsin hydrolyzed at every lysine (33 residues) and arginine (53 residues) residue in the alpha 1 chain of Type I collagen would be 86. Some of

the lysine residues are involved in crosslink formation, thus reducing the total number of possible peptides. Although every tryptic fragment may not be resolved, the isocratic method for separation of collagen tryptic peptides reported in the present investigation not only has the best resolution for collagen tryptic peptides reported to date, but also has great flexibility in sample loading. Sample amounts can be applied ranging from micrograms to several milligrams without causing major changes in the elution times. It is possible to apply a 1-mg sample, collect a particular peak, dialyze and lyophilize the sample, and prepare enough of one peptide in a day, to do amino acid and/or sequence analysis. This procedure represents a significant advance in collagen tryptic peptide resolution as well as providing the most rapid method for analysis to date.

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Note

Isocratic reversed-phase liquid chromatographic separation of 3′,5′-cyclic ribonucleotides

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The importance of the naturally occurring 3′,5′-cyclic ribonucleotides is well known [1]. Of the methods used in cyclic nucleotide research, the liquid chromatographic techniques appear to be the most advantageous [2]. However, the time required to achieve separations of the five naturally occurring cyclic nucleotides with the previously published methods is too long for routine work [2—4]. Furthermore, a simultaneous separation of the cyclic nucleotides along with corresponding nucleosides, nculeotides and bases, which is essential in studies of cAMP and cGMP metabolism, has been difficult to achieve. Therefore, we developed conditions for the individual 3′,5′-cyclic ribonucleotides from a mixture of the five naturally occurring cyclic nucleotides, and for the simultaneous separation of cAMP and cGMP in a mixture of nucleosides, nucleotides and bases. The proposed method utilizes the reversed-phase mode of high-performance liquid chromatography and isocratic elution with a totally aqueous buffer eluent which facilitates automated procedures for repetitive analyses. In addition, this system alleviates the need for gradient elution which can hamper the detection of compounds at very low levels due to baseline fluctuations.

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EXPERIMENTAL

The chromatographic system was a DuPont 8820 (DuPont Instruments, Wilmington, DE, U.S.A.) equipped with a controlled-temperature column compartment, 254-nm UV detector, HP 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) and strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). The column (250 × 4.6 mm I.D.) was packed with 6-μm spherical octadecyl silica (Zorbax-ODS, DuPont Instruments) and was heated to 35°C.

The eluent was 4.0 m*M* $(NH_4)_2HPO_4$ and 4.0 m*M* $(NH_4)H_2PO_4$, pH 3.0 (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Standards were obtained from Sigma (St. Louis, MO, U.S.A.) and were dissolved in the mobile phase at ca. 100 μM.

RESULTS AND DISCUSSION

The liquid chromatographic separation of the five naturally occurring 3′,5′-cyclic ribonucleotides has been reported using isocratic or gradient elution [2—4]. Using our conditions, excellent resolution of these compounds was obtained in 10 min (Fig. 1). The sensitivity of this system is in the 5—10-pmol range. Sample-to-sample reproducibility was excellent and there was no appreciable loss of efficiency or resolution due to the use of relatively high flow-rate. In addition, the reproducibility between different batches of the Zorbax-ODS columns was good.

Since the cyclic nucleotides are often present in extremely low concentrations compared to the levels of the structurally similar nucleosides, nucleotides and bases, a chromatographic system is needed which will separate the cyclic

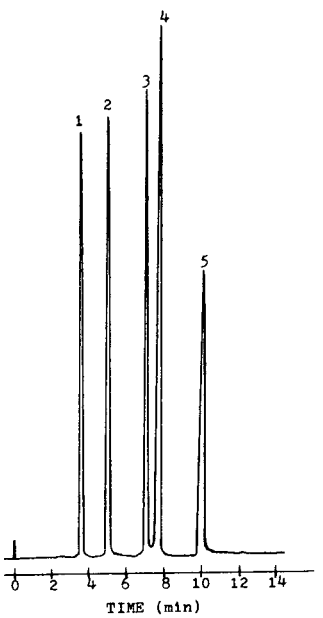

Fig. 1. Isocratic separation of the five naturally occurring 3′,5′-cyclic ribonucleotides. Flow-rate: 2.5 ml/min; other conditions as given in text. Peaks: 1 = cCMP, 2 = cUMP, 3 = cGMP, 4 = cIMP and 5 = cAMP.

nucleotides from their congeners. In addition, studies of cAMP (or cGMP) metabolism require the determination of the cyclic nucleotide with its metabolites [5]. Using a flow-rate of 1.5 ml/min (all other conditions remaining the same), a simultaneous separation of the cyclic nucleotides, cAMP and cGMP, and their nucleosides, nucleotides and bases was achieved (Fig. 2). The time required for the separation of the thirteen purine compounds is under 20 min. Resolution of the slightly retained compounds was improved by operating at ambient temperature, however analysis time is increased and retention times were not as reproducible.

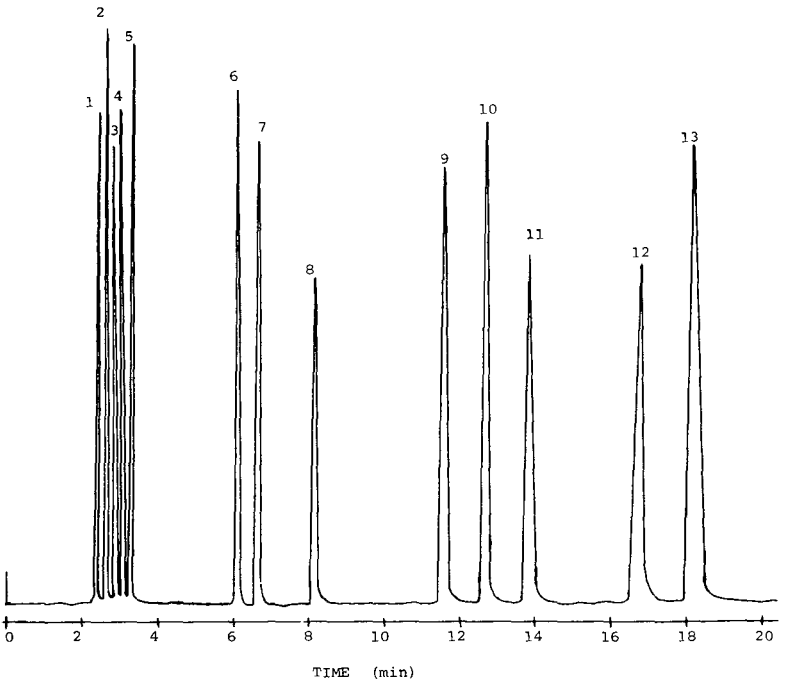

Fig. 2. Isocratic separation of cAMP and cGMP, and associated nucleosides, nucleotides and bases. Conditions are the same as in Fig. 1, except flow-rate is 1.5 ml/min. Peaks: 1 = ATP (GTP), 2 = GDP, 3 = ADP, 4 = GMP, 5 = AMP, 6 = Gua, 7 = Hyp, 8 = Ade, 9 = cGMP, 10 = Guo, 11 = Ino, 12 = cAMP and 13 = Ado.

An isocratic system employing a simple aqueous eluent is ideal for routine work in biomedical research or the clinical laboratory. The separations shown here can be used to determine the concentrations of cAMP and cGMP in cellular or tissue extracts, as well as the activities of the enzymes in the metabolic pathways [5, 6].

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Note

Simple and sensitive method for the determination of propylthiouracil in blood by high-performance liquid chromatography

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Propylthiouracil (PTU) has been used in the treatment of hyperthyroidism and liver disease. Despite widespread use of PTU, pharmacokinetic studies in man and laboratory animals have been hampered by the lack of a specific method of sufficient sensitivity to determine PTU in biological fluids. The colorimetric method [1] lacks specificity and sensitivity. The use of radioactive isotopes [2, 3] and a method of high-performance ion-exchange chromatography [4] are not feasible for extensive clinical studies. A gas chromatographic technique [5] which involves the conversion of PTU to its salt form with tetrapropylammonium hydroxide is time consuming and technically difficult. A recent method using high-performance liquid chromatography (HPLC) [6] offers specificity and high sensitivity, but did not include an internal standard.

A simple and sensitive method for the determination of PTU in blood using HPLC is described, along with a basic pharmacokinetic analysis after a single intraperitoneal administration to rats. The blood samples were deproteinized with phosphotungstic acid (PTA) reagent and the clear supernatant obtained after centrifugation was directly analysed in the HPLC system.

EXPERIMENTAL

Materials

PTU (H3 420-3, Aldrich, Milwaukee, WI, U.S.A.), 4-hydroxy-6-methyl-2-thiopyrimidine (HMTP, H-2502, Sigma, St. Louis, MO, U.S.A.), HPLC grade acetonitrile (A-998, Fisher, Fair Lawn, NJ, U.S.A.), potassium monobasic phosphate (P-284, Fisher) and PTA reagent (RO-1196, BDH, Toronto, Canada) were used. Water was deionized and then triple distilled.

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Apparatus

HPLC determinations were performed with a Beckman Model 330 isocratic liquid chromatograph, A Model 110A pump, a Model 160 ultraviolet detector operating at 280 nm with the absorbance detector sensitivity set at 0.005 a.u.f.s., and a Hewlett-Packard 3390A recording integrator. Separation of PTU was performed on a 250 mm × 4.6 mm I.D. Altex Ultrasphere ODS column (C_{18} reversed phase, particle size 5 μm) with isocratic elution. A mixture of acetonitrile and 0.025 *M* potassium monobasic phosphate in water, pH 4.6 (20:80, v/v) was used as a mobile phase. The phosphate buffer was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) prior to mixing. A flow-rate of 2.0 ml/min (17 MPa) at ambient temperature was employed in the present study.

Preparation of standard solutions

Standard solutions of PTU (0.1 mg/ml) and HMTP (0.1 mg/ml) were prepared in water in volumetric flasks by immersing them in a hot water bath and stored subsequently at 4°C. the appropriate concentrations of standard solutions were prepared by diluting the stock solutions with water.

Analytical procedure

A series of spiked rat blood samples (0.4 ml) in polypropylene tubes was prepared by mixing 0.1 ml blood with 0.3 ml water containing varying amounts of PTU, from 0.031 to 4.0 μg (corresponding to 0.31—40.0 μg/ml blood), and 1.0 μg HMTP as an internal standard. A spiked human blood sample containing 0.063 μg PTU (corresponding to 0.63 μg/ml blood) and 1.0 μg HMTP was prepared as above. The samples were deproteinized with 25 μl PTA reagent for 90 min at room temperature and then centrifuged for 15 min at 31,550 *g*. A 20-μl aliquot of the clear supernatant obtained (pH 4.4) was injected into the HPLC system with a 50-μl Hamilton syringe. The same procedure was followed using varying amounts of PTU in 0.425 ml water with no blood and PTA reagent.

Standard curve

Known amounts of PTU (0.031—4.0 μg) in 0.4-ml aliquots of the spiked blood samples were taken through the entire procedure, HMTP (1.0 μg) being added to each sample as an internal standard. An identical set of PTU samples was made up in 0.425 ml water with no blood or PTA reagent. To construct the standard curve, the PTU/HMTP response ratios were plotted against the concentrations of PTU in μg/ml. Actual amounts of PTU injected into the HPLC system were 1.5—188.2 ng, while the amount of HMTP was 47.1 ng.

Animal study

To study the suitability and applicability of the present method to animal studies, male rats weighing 430—470 g (CBL, Montreal, Canada) were administered intraperitoneally (i.p.) with 20 mg/kg PTU (in 2.0 ml saline per 100 g body weight, pH 6.7). Rats were housed under constant environmental conditions and food was removed 24 h prior to the administration of PTU. Blood samples (0.1 ml) from the tail vein were collected into polypropylene

tubes containing 0.3 ml water at various time intervals after administration. The samples were analysed as described above for the spiked samples.

RESULTS AND DISCUSSION

Clearly separated peaks representing PTU and its internal standard, HMTP, were seen in water and rat blood (Fig. 1). The chromatogram obtained with a human blood sample showed the same result. No interfering endogenous compounds were found on the chromatograms obtained with the blank rat or human blood. Under the present experimental conditions, the elution volumes for PTU (retention time, 3.6 min) and the internal standard HMTP (retention time, 1.9 min) were 7.2 and 3.8 ml, respectively. Maximum absorbance of both PTU and HMTP were found to occur at 275 nm, and in all subsequent studies these were analysed at 280 nm.

The linearity of the concentration and response relation was established over the range of 0.31—40.0 μg/ml blood (r = 0.9998; slope, 0.050; y-intercept, 0.0028). Analytical recovery of PTU (0.031—4.0 μg) added to the spiked blood samples calculated for peak areas by comparison with results obtained with water samples was 74 ± 3% ($\bar{x}$ ± S.D.). The mean between-run coefficient of variation recovered from blood samples was 3.5% over the range of 0.31—40.0 μg/ml (in each concentration, n = 6), whereas the within-run coefficient of variation at 0.31 μg/ml (n = 6) was 5.6%.

The in vivo study illustrates that the method is suitable for the pharmacokinetic study of PTU following administration of a single dose (Fig. 2). The blood level of PTU was measurable (0.2 ± 0.04 μg/ml, $\bar{x}$ ± S.E.M., n = 5) within 1 min after i.p. administration. The peak level of 27.8 ± 0.3 μg/ml occurred at 90 min. The blood PTU appeared to have a $t_{1/2}$ of about 6 h.

The presence of PTA reagent in samples of blood and water was found to interfere with the maximum absorption of PTU and HMTP at 280 nm. Under the present conditions, the values obtained for peak areas of PTU and HMTP

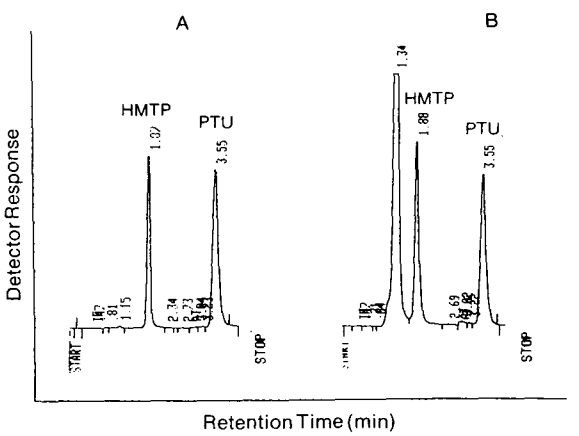

Fig. 1. Isocratic separation of PTU and its internal standard, HMTP. (A) Chromatogram of PTU and HMTP standards in water. (B) Chromatogram from rat blood sample showing peaks of PTU (11.8 ng) and of internal standard, HMTP (47.1 ng).

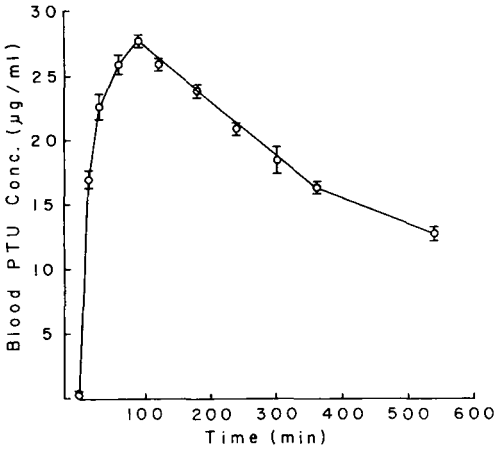

Fig. 2. The concentration of PTU in blood of rats after i.p. administration of 20 mg/kg. Results are the means ± S.E.M. for five rats at each time interval.

from blood samples with PTA reagent were 26% and 8% lower than those obtained with water samples with no PTA reagent. However, the ratios of peak area (PTU/HMTP) remained constant, although 25% lower than the absolute values. Concentrated PTA reagent (25 μl) added to water samples containing PTU and HMTP obscured the PTU and HMTP peaks, and addition of PTA reagent diluted 1:10 to water samples resulted in a significant decrease but detectable response for PTU (60% or higher). The amount of PTA reagent used in the present study was sufficient to deproteinize blood samples, but less than 25 μl (10—20 μl) did not produce complete deproteinization. Addition of PTU before or after the deproteinization of blood samples with PTA reagent showed the same quantitative results which indicated that there appeared to be no protein-binding interactions. Storage of blood samples at 4°C for up to two months did not alter the quantitation of PTU.

The present method is simple and sensitive, and comparable to the method of Giles et al. [6]. However, inclusion of HMTP as an internal standard for the determination of PTU improved the quantitation, i.e., an average drop of the between-run coefficient of variation from 4.2 to 3.5%. Use of PTA reagent for the deproteinization of blood further simplified the sample preparation. In the present study, no extraction, purification and concentration procedures of samples before the HPLC system were found to be necessary.

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Note

Determination of trimethadione and its metabolite by gas chromatography with flame-thermionic detection and its application to pharmacokinetic studies of the drug in carbon tetrachloride-treated rats

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We have already reported a rapid and sensitive gas chromatographic method for the determination of plasma trimethadione (TMO) and its metabolite, 5,5-dimethyl-2,4-oxazolidinedione (DMO) [1]. We have shown that pharmacokinetic of TMO when administered in vivo is a good indicator for estimating drug-metabolizing enzyme activities in control, phenobarbital and hepatotoxins-treated rat liver [1—4]. Thus, it is expected that TMO could be suitable as a model drug for estimating drug-metabolizing enzyme activities in human. However, the detection limit of TMO and DMO of the previous gas chromatographic method seems to be not sufficient if this method is applied to humans, because the administered dose of TMO should be as low as possible because of its antiepileptic pharmacological effect. In this respect, we developed a more sensitive method for the determination of low concentrations of TMO and DMO by using gas chromatography equipped with a flame-thermionic detector (FTD). In addition, a pharmacokinetic study by using this new sensitive method was carried out in carbon tetrachloride-treated rats.

EXPERIMENTAL

Materials

TMO was purified from commercial 66.7% powder (Mino-Aleviatin®; Dainippon, Osaka, Japan). Paramethadione (PMO, internal standard) was synthesized from DMO by the method of Spielman [5]. DMO was purchased from Tokyo Kasei (Tokyo, Japan), olive oil from Sanko (Tokyo, Japan) and carbon tetrachloride from Wako (Osaka, Japan). All other chemicals were of reagent grade.

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Extraction procedure

To 50 μl plasma in a 2.5-ml tube were added 100 μl of 5 *M* monobasic sodium phosphate, a minimum quantity (about 50 mg) of sodium sulphate and magnesium sulphate, and 100 μl of ethyl acetate containing 5 μg of PMO as internal standard. The tube was shaken for 2 min and centrifuged at 1500 *g* for 8 min. A 2-μl volume of the organic phase was then analyzed by gas chromatography.

Gas chromatography

Analysis of plasma TMO and DMO was carried out with a Shimadzu GC-7A instrument equipped with an FTD. The glass column (50 cm × 2.6 mm I.D.) was packed with 5% PEG 6000 on 80—100 mesh Chromosorb W HP (Chromato Supply, Tokyo, Japan). The column oven temperature was raised from 100°C to 190°C at a rate of 16°C/min and held at 190°C for 5 min. The injection port and detector were at 210°C. Helium, hydrogen and air flow-rates were 50, 90 and 230 ml/min, respectively.

Animal studies

After overnight fasting, male Wistar rats (Japan Laboratory Animal, Tokyo, Japan), weighing 196—230 g, received intravenous administration of 10 mg/kg of TMO. Carbon tetrachloride was dissolved in olive oil and administered orally (0.5 ml/kg) 24 h prior to the intravenous administration of TMO. Blood samples (0.1—0.15 ml) were obtained from the jugular vein at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h after the intravenous administration of TMO.

Pharmacokinetic studies

Concentration—time curves of TMO and DMO were drawn on semilogarithmic scales. The half-life ($T_{½}$), metabolic rate constant (K_m) and elimination rate constant (K_{el}) were calculated from linear regression analysis. The apparent volume of distribution (V_d) was calculated from the ratio of the given dose to the plasma concentration extrapolated to the time zero. The area under the curve (AUC) was calculated by the trapezoidal rule, and area to infinite time was added by integration (C_t/K_{el}), where C_t is the last value of TMO concentration; K_{el} was calculated from the equation:

$$K_{el} = 0.693/T_{½}$$

Metabolic clearance (*Cl*) was calculated according to the equation

$$Cl = 0.693V_d/T_{½}$$

For statistical analysis a paired Student's *t*-test was used.

RESULTS AND DISCUSSION

Fig. 1 shows the gas chromatogram of TMO, DMO and the internal standard PMO. The results indicate that there was a good separation between TMO, PMO and DMO. The retention times for TMO, PMO and DMO were 1.7, 4.1 and 6.3 min, respectively. The calibration graphs showed the linear relationship between the peak height ratios of TMO or DMO to the internal

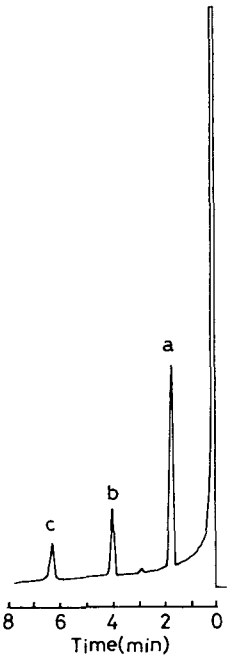

Fig. 1. Gas chromatogram of TMO (a) and DMO (c) in rat plasma. PMO (b) was used as internal standard. Retention times for a, b and c were 1.7, 4.1 and 6.3 min, respectively.

standard (PMO) in the concentration ranging from 0.1 μg/ml to 15 μg/ml (r = 0.956). This method was capable of measuring at least 0.05 μg/ml of TMO and DMO, and was ca. 100-fold more sensitive than the method reported previously [1]. Extraction recovery of plasma TMO and DMO (0.1, 0.2, 0.5, 1, 5, 10 and 15 μg/ml) were 98.65 and 97.24 with standard deviations of ± 4.3 and ± 5.4% (n = 4), respectively. No interfering peaks appeared when phenobarbital, phenytoin, pentobarbital, acetazolamide, carbamazepine and primidone, which are usually administered to the patients in combination with TMO, were added to plasma. From these results, it is reasonable to note that the method presented for the determination of TMO and DMO could be fully applicable to cases even if a lower dose of the drug was administered to animals or humans. Next, we carried out the pharmacokinetic study of TMO following the administration of a lower dose of the drug as compared to those reported previously [1—4]. The results of these experiments is shown in Table I. The plasma concentration of TMO in olive oil-treated rats reached its peak at 0.5—1 h, and plasma DMO reached its peak at around 9 h and then gradually decreased. The changed patterns of plasma TMO and DMO levels were almost similar to those reported previously [2]. In contrast, the peak levels of plasma concentration of TMO in carbon tetrachloride-treated rats were delayed to 5.7 h. Additionally, DMO production of the carbon tetrachloride-treated group occurred to a lesser extent than in the olive oil-group. Since K_{el} values thus appeared to reflect K_m values properly, estimation and analysis of K_{el} values may enable us to predict the drug-metabolizing capacity in the given cases (r = 0.988, Fig. 2). For the pur-

TABLE I

PHARMACOKINETIC PARAMETERS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF TMO TO RATS PRETREATED WITH CARBON TETRACHLORIDE

Values are means ± S.E. (n = 5). Rats were pretreated with carbon tetrachloride (0.5 ml/kg) 24 h prior to TMO (10 mg/kg, i.v.) administration.

Treatment	$T_{½}$ (h)	V_d (l)	Cl (l/h)	K_m (1/h)	AUC (μg/ml·h)
Olive oil	1.61±0.04	0.220±0.007	0.095±0.003	0.463±0.027	18.03±0.92
Carbon tetrachloride	5.73±0.60*	0.260±0.002**	0.033±0.003*	0.063±0.018*	61.39±0.02*

*$P < 0.01$.
**$P < 0.02$.

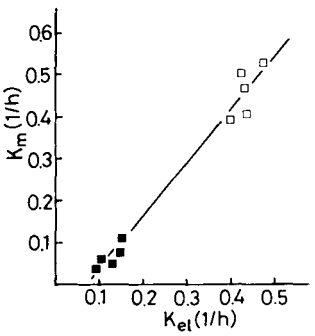

Fig. 2. Correlation between K_m and K_{el} of TMO in rat plasma pretreated with carbon tetrachloride. □, Olive oil; ■, carbon tetrachloride. Rats were pretreated with carbon tetrachloride (0.5 ml/kg, p.o.) 24 h prior to TMO (10 mg/kg, i.v.) administration. Each group consisted of 5 male rats. r = 0.988, y = 1.3x — 0.1, n = 10.

pose of shortening the time required for estimation of the drug-metabolizing capacity, we studied the possible correlation between K_{el} values and ratios of plasma DMO to TMO at 1 and 2 h after intravenous administration of TMO. As shown in Fig. 3, there was a good correlation between the ratio of DMO to

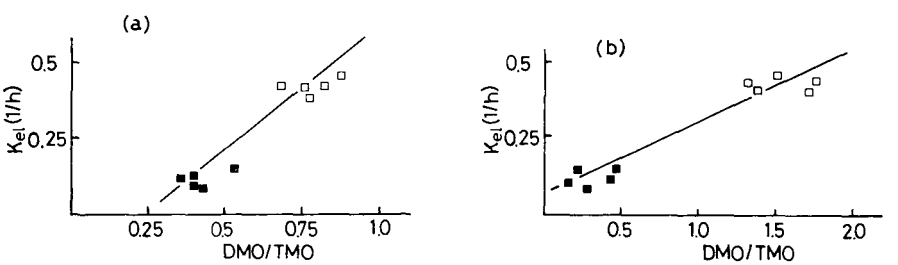

Fig. 3. Correlation between DMO/TMO ratio and K_{el} in rat plasma 1 (a) and 2 h (b) after intravenous administration of TMO in rats pretreated with carbon tetrachloride. □, Olive oil; ■, carbon tetrachloride. Rats were pretreated with carbon tetrachloride (0.5 ml/kg, p.o.) 24 h prior to TMO (10 mg/kg, i.v.) administration. Each group consisted of 5 male rats. (a) r = 0.955, y = 0.8x — 0.19, n = 10; (b) r = 0.958, y = 0.24x + 0.07, n = 10.

TMO in plasma and K_{el} values at 1 and 2 h after intravenous administration of TMO, respectively (r = 0.955 at 1 h and r = 0.958 at 2 h). The present investigation has revealed that the modified gas chromatographic method is sufficiently sensitive to determine plasma TMO and DMO in animals even when a lower dose of the drug was administered. Additionally, the results of the pharmacokinetics of TMO are well compatible with those reported previously [2].

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Note

Dosage du diltiazem plasmatique par chromatographie en phase gazeuse

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(Reçu le 13 juillet 1982; manuscrit modifié reçu le 22 septembre 1982)

Le diltiazem est utilisé dans le traitement de l'angor pour ses propriétés anticalciques [1]. La technique de chromatographie en phase gazeuse décrite par Rovei et al. [2] permet de le doser avec une sensibilité de 10 ng/ml dans le plasma des patients traités.

Nous avons tenté d'améliorer cette technique en la rendant plus rapide et plus sensible; les modalités d'extraction ont été simplifiées et un étalon interne de structure très voisine de celle du diltiazem a remplacé le cyclopam.

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MATÉRIALS ET MÉTHODES

Standard et réactifs

Diltiazem (Tildiem®) (DTZ) et désacétyldiltiazem (DAD) ont été fournis par les Laboratoires Dausse, Paris, France; le prazépam (PZP) par le Laboratoire Substantia, Courbevoie, France; le propionyldésacétyldiltiazem (PDAD) et le butyryldésacétyldiltiazem (butyryl-DAD) ont été préparés par hémisynthèse selon les modalités évoquées plus loin.

Les réactifs utilisés sont: la pyridine, le chlorure de butyryle, le chlorure de propionyle, le bicarbonate de sodium, le *n*-hexane, l'acétate d'éthyle (tous de Merck, Darmstadt, R.F.A.), le N,O-bis(triméthylsilyl)acétamide (BSA) (de Pierce, Rockford, IL, E.U.) et une solution tampon NaH_2PO_4, K_2HPO_4 0.1 *M*, pH 7.

Appareillage

L'appareil de chromatographie en phase gazeuse Perkin-Elmer F-30 équipé d'un détecteur azote—phosphore (NPD) a été utilisé avec une colonne de verre de 2 m × 4 mm (I.D.) contenant du Chromosorb W HP (100—120 mesh) imprégné de OV-17 à 1% (Alltech Assoc.). La colonne a été préalablement conditionnée durant 36 h à 300°C sous un débit d'azote de 40 ml/min.

Conditions opératoires

Les dosages ont été effectués en utilisant l'hélium comme gaz vecteur avec un débit de 40 ml/min à une température d'injection et de détection commune de 350°C. La température du four a été fixée à 280°C et la polarité du détecteur à —180 V avec des débits de 2 ml/min pour l'hydrogène et de 100 ml/min pour l'air.

Étalon interne

Le butyryldésacétyldiltiazem (butyryl-DAD) a été choisi comme étalon interne. Il a été préparé au laboratoire par hémisynthèse selon Kugita et al. [3] à partir du désacétyldiltiazem (DAD). Après avoir mélangé pendant 1 h à 4°C 800 mg de DAD et 3 ml de pyridine, on met en contact ce mélange avec 0.28 ml de chlorure de butyryle pendant 20 h à la même température avant d'ajouter 10 ml d'eau glacée. Après 10 min, on extrait le butyryl-DAD formé en agitant le tout en présence de 15 ml de chloroforme dans une ampoule à décanter. Cette opération d'extraction est effectuée à quatre reprises avec la même quantité de chloroforme en laissant décanter chaque fois durant 10 min. Après trois lavages successifs de l'extrait chloroformique par un égal volume de bicarbonate à 5% et deux lavages par un égal volume d'eau, le pH doit être voisin de 7. On évapore alors sous vide la phase chloroformique. Le butyryl-DAD ainsi purifié se présente sous la forme d'une huile jaune; il est solubilisé dans la pyridine et conservé à 4°C. Le titre final de la solution est égal à 160 mg/l.

L'identification du butyryl-DAD a été effectué par spectrophotométrie IR (Perkin-Elmer, 157 G), par spectrométrie RMN (Cameca, 250 MHz) et spectrométrie de masse (SM) (Ribermag). Les données expérimentales sont les suivantes:

IR: (pastille KBr) 1672 cm^{-1} (>=O, hétérocyclique; 1750 cm^{-1} (>=O, ester);
NMR: ($CDCl_3$ — étalon interne TMS). Protons aromatiques: (7.60, doublet, H), (7.38, multiplet, 2H), (7.20, multiplet, 3H), (6.85, doublet, 2H). Protons hétérocycliques: (5.10, doublet dédoublé, 2H), CH_3—O— (3.80, singulet, 3H) — $(CH_3)_2N$— (2.24, singulet, 6H) — CH_3(c)—CH_2(b)—CH_2(a)—: CH_3(c) — (0.94, triplet, 3H) — CH_2(b) (1.48, quadruplet, 2H) — CH_2(a): (2.12, quadruplet, 2H);
SM: [ionisation électronique et ionisation chimique (NH_3)] : M^+ : 442.

Étalons et échantillons plasmatiques

Un pool de plasmas a été constitué pour le contrôle des critères de qualité de la méthode. A partir de ce pool, une gamme de plasmas surchargés en DTZ a été préparée avec les titres suivants: 0 (blanc plasmatique), 25, 50, 100, 200

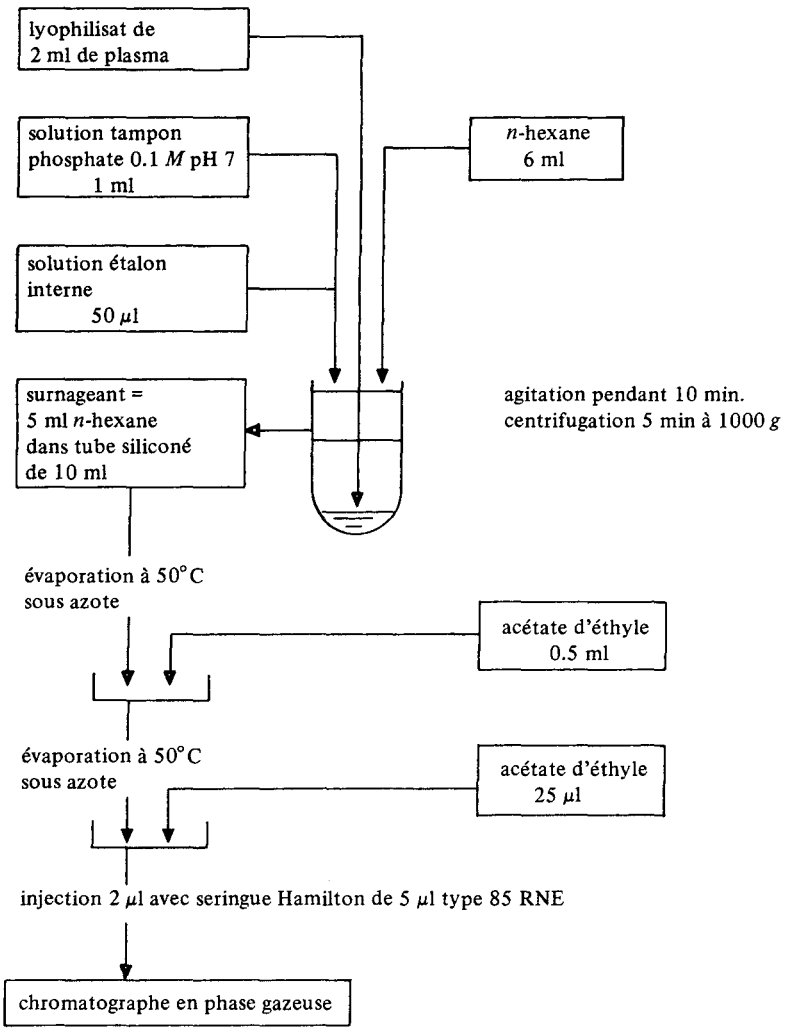

Fig. 1. Procédé d'extraction du diltiazem (DTZ) et butyryldésacétyldiltiazem (butyryl-DAD).

et 400 ng DTZ/ml. Chaque élément de cette gamme a fait l'objet de trois dosages selon le même protocole que celui auquel sont soumis les essais.

Deux ml de chaque étalon et échantillon plasmatiques sont lyophilisés dans un tube Sovirel de 10 ml (réf.: 732-01, diam. 15 mm).

Extraction et expression des résultats

Les lyophilisats sont traités d'après les indications mentionnées dans la Fig. 1.

Les concentrations sont calculées à partir de la droite d'étalonnage déterminée par les rapports des hauteurs de pics d'enregistrement DTZ/butyryl-DAD des étalons plasmatiques mesurés en fonction de la concentration en DTZ ou encore à partir du rapport des pics d'enregistrement DTZ/butyryl-DAD pour la valeur de 100 ng/ml (Fig. 2).

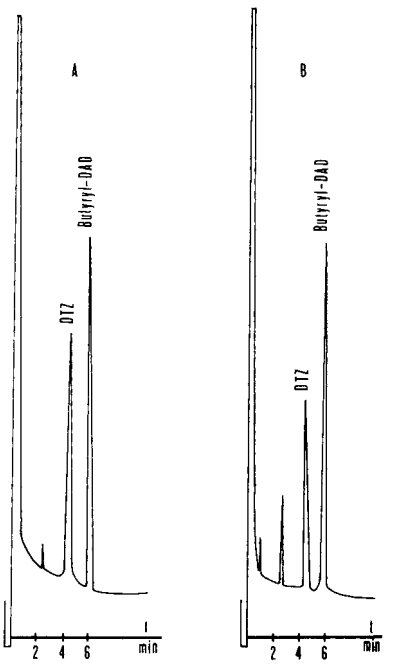

Fig. 2 (A) Chromatogramme d'un extrait plasmatique surchargé en diltiazem (DTZ) (100 ng/ml) et de son étalon interne butyryldésacétyldiltiazem (butyryl-DAD). (B) Chromatogramme de l'extrait plasmatique d'un patient traité au DTZ et de l'étalon interne butyryl-DAD; la concentration plasmatique est de 76 ng/ml 3 h après la dernière dose de DTZ administrée (3 doses de 60 mg/jour).

RÉSULTATS ET DISCUSSION

Afin de tester la fiabilité de notre technique (M_2) et ne disposant pas de l'étalon interne préconisé par Rovei et al. [2] (cyclopam), nous avons effectué une étude comparée en utilisant la technique de cet auteur (M_1) avec le butyryl-DAD comme étalon interne. De ce fait, notre discussion portera essentiellement sur les procédés de traitement du plasma et la technique d'extraction comparés.

Traitement des plasmas et procédé d'extraction

La méthode M_1 implique la congélation des échantillons de plasma si le laboratoire n'a pas mission d'effecteur une série de dosages chaque jour. Ce procédé de conservation a l'inconvenient de faire apparaître un insoluble après décongélation. Cet inconvénient se surajoute à celui d'une gélification qui se développe fréquemment en raison du rapport de phases important (phase aqueuse—phase organique, 3:6, v/v) lors de la première étape d'extraction.

La méthode M_2 évite ces deux inconvénients en préconisant la lyophilisation des plasmas. Les plasmas sont congelés au fur et à mesure de leur arrivée puis lyophilisés en série. La redissolution des constituants plasmatiques dans 1 ml de tampon est complète et le rapport de phases (1:6, v/v) est suffisamment faible pour éviter une gélification parasite.

En outre, l'efficacité de l'extraction qui résulte de ce rapport et de l'absence de cette gélification présente un gain de temps non négligeable en évitant de procéder à une deuxième extraction.

Étalon interne

Le butyryl-DAD est certainement préférable au cyclopam préconisé par la méthode M_1 en raison de sa similitude structurale avec le DTZ qui lui confert un comportement lors de l'extraction et un temps de rétention chromatographique (6 min) les plus proches de ce dernier (4 min).

Le butyryl-DAD a fait l'objet d'une étude comparée avec d'autres étalons internes tels que le prazépam (PZP) et le propionyldésacétyldiltiazem (propionyl-DAD). Le PZP a été abondonné en raison de son temps de rétention inférieur à 2 min et de son éventuelle interférence lorsqu'il est utilisé en thérapeutique.

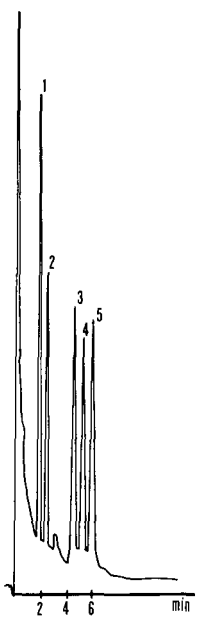

Fig. 3. Chromatogramme d'un mélange des cinq substances étalons utilisées: prazépam (1), désacétyldiltiazem (2), diltiazem (3), proprionyldésacétyldiltiazem (4) et butyryldésacétyldiltiazem (5).

Bien que comparable au butyryl-DAD par son inertie métabolique et son rendement d'extraction, le propionyl-DAD a été également abondonné pour son temps de rétention trop proche (5 min) de celui du DTZ le rendant ainsi presque confluent avec ce dernier lors de l'enregistrement (Fig. 3).

Le comportement du butyryl-DAD se révèle le même avec les deux méthodes M_1 et M_2: les hauteurs des pics d'enregistrement qui lui correspondent suivent une loi normale (test de Kolmogorof), leurs variances sont comparables (test de Student, M_1: 130 ± 44 mm, M_2: 140 ± 26 mm).

Répétabilité

Le coefficient de variation de 9 dosages d'un même pool de plasmas renfermant 100 ng DTZ/ml est de 1.5%.

Reproductibilité

Le coefficient de variation de 13 dosages de ce même pool de plasmas effectués chacun dans une série différente est de 5.5%.

Précision

L'analyse de variance entre la variable (hauteur du pic DTZ/hauteur du pic butyryl-DAD × conc. théorique) et ses facteurs méthode, concentration et interaction méthode—concentration, ne montre aucune différence significative entre elles dans la marge de concentration de 25—400 ng DTZ/ml de plasma.

Exactitude

Les concentrations déterminées avec les deux méthodes M_1 et M_2 sont corrélées avec un coefficient de régression $r = 0.999$.

Linéarité

Les courbes d'étalonnage couvrant les concentrations C thérapeutiques habituelles de 25—400 ng DTZ/ml sont linéaires pour les deux méthodes. Avec la méthode M_1, le coefficient de régression r est de 0.999 avec une équation de droite: $Y = 0.552\ C - 0.9$; avec la méthode M_2, $r = 0.998$, $Y = 0.558\ C + 0.36$, en posant Y = hauteur du pic DTZ/hauteur du pic butyryl-DAD.

Sensibilité

La sensibilité "pratique", correspondant à la concentration minimum décelable (3 fois le bruit de fond de l'appareil dans nos conditions opératoires) est de 1 ng/ml.

Spécificité

En montrant la bonne individualisation des produits étudiés, la Fig. 3 reflète la bonne spécificité de la méthode M_2.

Interférences

Nous avons procédé à deux types d'essais qui s'accordent pour confirmer la spécificité de la méthode M_2 par l'absence d'interférences avec d'autres substances:

(1) L'étude comparée du comportement chromatographique de diverses

substances susceptibles d'interférer avec le DTZ sur la colonne OV-17, 1% démontre que les températures utilisées pour les éluer en moins de 10 min sont toutes inférieures à celle de 280°C préconisée dans notre méthode M_2 pour l'élution du DTZ et du butyryl-DAD:

200°C: théophylline, phénobarbital, heptabarbital,

220°C: diphénylhydantoïnes,

250°C: imipramine, amitriptyline,

250—270°C: diazépam, clobazam, clonazépam, prazépam.

(2) D'après l'étude faite sur 54 patients hospitalisés, les médicaments associés au DTZ n'introduisent aucune interférence.

CONCLUSION

Cette méthode, utilisée depuis deux ans dans notre laboratoire, se révèle être bien adaptée aux examens de routine hospitaliers et permet, une fois la lyophilisation effectuée (de nuit), de traiter 30 plasmas en une demi-journée.

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CHROMBIO. 1513

Note

Detection of picogram concentrations of fentanyl in plasma by gas—liquid chromatography

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The popularity of fentanyl and related compounds as narcotic analgesics in clinical anaesthesia has resulted in a number of studies requiring measurement of plasma concentration. Normally, only relatively low doses of these drugs are administered and plasma concentration tends to fall rapidly to low levels — often in the pg/ml range. The detection of such low levels is important to a full understanding of the pharmacokinetics of these drugs. Radioimmunoassay [1], with a lower detection limit of 30 pg, has been the method of choice for measuring such small quantities as the gas chromatographic methods at present described are not sufficiently sensitive. Using gas—liquid chromatography (GLC) with a flame ionization detector, Van Rooy et al. [2] described a detection limit of fentanyl in plasma of 3.3 ng/ml. Gillespie et al. [3] using a more sensitive nitrogen—phosphorus detector (NPD) measured plasma fentanyl concentrations of 0.25 ng/ml. Using a gas chromatograph equipped with an NPD, a modification of the extraction method described by Van Rooy et al. [2] is presented which allows detection of picogram concentrations of fentanyl and similar compounds in plasma.

EXPERIMENTAL

Equipment

A Perkin-Elmer Sigma 1B gas chromatographic system [Perkin-Elmer (Canada) Ltd.] equipped with an NPD is used. The conditions for the analyzer in which the detector is housed are shown in Table I and are adjusted to give maximum component separation in minimum time. The voltage to the rubidium bead of the detector is adjusted for maximum sensitivity as described in the manufacturer's manual. Recorder range and attenuation are set for maxi-

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TABLE I

ANALYZER CONDITIONS FOR MEASUREMENT OF PLASMA FENTANYL CONCENTRATION BY GLC USING A NITROGEN—PHOSPHORUS DETECTOR

Column	3.05 m × 3.2 mm silanized glass
Packing	3% OV-17 on Gas-Chrom Q (80—100 mesh)
Injector temperature	310°C
Oven temperature	290°C
Detector temperature	310°C
Carrier gas flow-rate (helium)	35 ml/min
Detector gas flow-rates:	
hydrogen	3 ml/min
air	108 ml/min

mum sensitivity. The area and base sensitivities used in the analysis method are calculated using a computer programme supplied by Perkin-Elmer.

Materials and chemicals

For the NPD flame ultra high pure hydrogen and air, ultra zero gas (Matheson, Edmonton, Canada) are used. The carrier gas is ultra high pure helium (Matheson, Edmonton, Canada). Kimax 10-ml culture tubes with PTFE-lined caps are used. To prevent adsorption of drugs to the glass, the tubes are initially silanized with a 5% solution of dimethyldichlorosilane in toluene (Eastman Kodak, Rochester, NY, U.S.A.). The extraction solvent is 99% molecular pure benzene (Fisher Scientific, Fairlawn, NJ, U.S.A.).

Alfentanil is used as the internal standard. A solution of a known concentration of alfentanil in benzene is prepared using pure alfentanil hydrochloride powder.

Method

All glassware is rinsed with benzene prior to use to remove interfering substances. 1.0 ml of plasma sample, 0.1 ml of 4 *M* sodium hydroxide, 1 ml of internal standard solution and 5 ml of benzene are pipetted into clean culture tubes. These are sealed, mixed for 5 min and centrifuged for 10 min at 8000 *g*, following which the supernatants are transferred to clean tubes and evaporated to dryness at 40°C. The residues are reconstituted with 10 μl of distilled, deionized water of which 1 μl is injected onto the GLC column. Following extraction, a number of samples of known concentration of fentanyl in human plasma protein fraction are injected onto the column. Relative response factors for fentanyl as compared to the internal standard are then calculated by the Sigma 1B computer and an average factor is inserted into the analysis method to allow unknown fentanyl concentrations to be determined.

RESULTS

Using this method we have been able to measure plasma fentanyl concentration as low as 20 pg/ml. A chromatogram from a 20 pg/ml sample is shown in Fig. 1. The initial large deflection is caused by air and distilled water. The

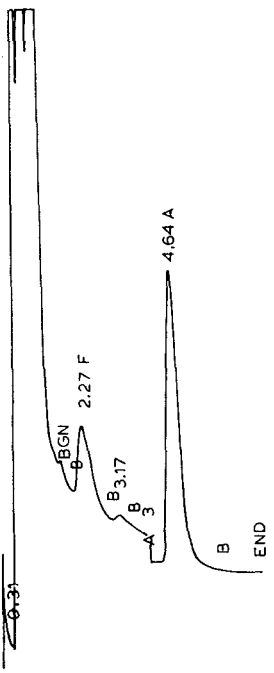

Fig. 1. Chromatogram from a 20 pg/ml sample of fentanyl. Peaks: F = fentanyl, A = alfentanil, BGN = data start time, B = computer recognition of baseline and A 3 = increase of signal attenuation. 0.31, 2.27, 3.17, 4.64 = elapsed time in min.

first peak shown (F) is from fentanyl and the second (A) is from alfentanil. As a relatively large concentration of alfentanil (2 μg/ml) is used, the signal attenuation from the detector to the computer is increased before the alfentanil is eluted otherwise maximum deflection of the pen would occur and the peak would not be displayed properly. Recovery of 20 pg/ml fentanyl was 69.4 ± 5.3%, determined by injecting known concentrations of fentanyl onto the column and comparing with extracted samples.

DISCUSSION

The major modification in the current method from that of Van Rooy et al. [2] was to use distilled water as the final solvent prior to injection of the sample onto the column. The use of an organic solvent such as benzene produces a very broad deflection over the first few minutes of the chromatogram. This completely masks the fentanyl peak from low plasma concentrations and from larger concentrations gives at best a peak superimposed on the trailing side of the solvent peak. In this situation the fentanyl peak is produced on an unstable baseline and corrections for this have to be inserted into the analysis method to enable the computer to distinguish the peak from the baseline. The current method eliminates the need for such correction factors as a stable baseline is produced.

The problem of an initial broad deflection was encountered with a wide

range of organic solvents but was eliminated with the use of distilled water. This produces a narrow initial peak, allowing the pen to return to baseline before fentanyl is eluted from the column. It seems that distilled water, being relatively inert as compared to the organic solvents, is retarded less by partitioning as it passes through the column. Also, as water is an inorganic substance, the NPD is insensitive to it. Baseline is recognised by the computer, which prints a letter B (see Fig. 1). The stable baseline enables the computer to distinguish very small peaks and thus measure accurately low concentrations of fentanyl. Furthermore, the Sigma 1B system calculates concentrations by measuring peak areas which is more accurate, particularly at low concentrations, than the measurement of peak heights described by earlier workers using less sophisticated equipment.

The internal standard is added prior to the extraction process to allow for variability in drug recovery from different samples. The computer is programmed to recognize the concentration of alfentanil as being the same in every sample and thus variability in recovery is taken into account.

ACKNOWLEDGEMENTS

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CHROMBIO. 1495

Note

Quantitative determination of cardiotonic agent MDL 17,043 in plasma by reversed-phase high-performance liquid chromatography

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MDL 17,043, 1,3-dihydro-4-methyl-5-[4-(methylthio)benzoyl]-2H-imidazol-2-one, is a new noncatechol, nonglycoside cardiotonic agent. Pharmacological studies in laboratory animals suggest that the drug will be useful in the treatment of congestive heart failure since both oral and intravenous (i.v.) administration results in dose-dependent increase in cardiac contractile force, slight increases in heart rate and small decreases in systemic blood pressure [1—3]. In order to facilitate the studies of bioavailability and pharmacokinetics of this compound, a sensitive and reliable analytical procedure was needed to measure plasma concentrations of MDL 17,043. This communication describes a reversed-phase high-performance liquid chromatographic (HPLC) method developed for this purpose and the application of the assay to a feasibility study using two male beagle dogs given single oral and i.v. doses of MDL 17,043.

EXPERIMENTAL

Materials

Ethyl acetate, acetonitrile and methanol were glass-distilled grade obtained from commercial suppliers and used as received. Sodium phosphates (monobasic and dibasic) were standard reagent grade. Glass-distilled water was used in the preparation of aqueous solutions.

MDL 17,043 and the internal standard [MDL 18,763, 1,3-diethyl-1,3-dihydro-4-(4-methoxy-benzoyl)-5-methyl-2H-imidazol-2-one] were supplied by the Merrell Dow Research Center.

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Standards

Standard solutions of MDL 17,043 were prepared in methanol. For use, 0.1 ml of the appropriate concentration of standard solution was mixed with 1.9 ml human plasma (heparin anticoagulant) to give the plasma concentrations shown in Table I. Internal standard solution was 4.16 μg/ml in methanol. To test the precision and accuracy of the assay a six-day validation study was carried out. A six-point standard curve in duplicate together with six duplicate plasma samples with concentrations unknown to the analyst were analyzed each day.

Feasibility experiment

To investigate the sensitivity and specificity of the assay, it was applied to a limited dog bioavailability study. In a crossover experiment, two male beagle dogs (A & B) were given single oral and i.v. doses of MDL 17,043 at 3 mg/kg. Periodic plasma samples were taken from each dog and the plasma concentrations of MDL 17,043 were determined by this analytical method. Two weeks separated the two legs of the experiment.

Extraction procedure

To each 2-ml standard or unknown plasma sample, 100 μl internal standard solution were added. Then 3 ml of acetonitrile were added to each tube and vortexed to precipitate plasma protein. Two ml of 0.1 *M* sodium phosphate buffer, pH 7.5, were mixed with each sample which was then centrifuged (900 *g*, 20 min). A 6-ml aliquot of the supernatant was transferred to a 25-ml screw-cap extraction tube which contained 9 ml of ethyl acetate. The compounds were extracted into the organic phase by mixing in a horizontal Eberbach reciprocating shaker for 20 min. After centrifugation, 10 ml of the supernatant were transferred to a 15-ml conical tube and evaporated (50—55°C) to dryness under a slow stream of nitrogen gas. For HPLC analysis, the residue was redissolved in 200 μl of methanol, and a 25-μl aliquot was injected into the column.

Apparatus

HPLC analyses were performed on equipment manufactured by Waters Assoc. (Milford, MA, U.S.A.); solvent delivery systems: Model 6000A; UV detection system: Model 440 (fixed wavelength at 313 nm). Samples were processed with a Waters Autoinjector (WISP Model 710A). The HPLC column used for this analysis was a DuPont Zorbax C-8, 25 cm × 4.6 mm I.D., particle size 6 μm; mobile phase: methanol—water (60:40); flow-rate: 1.0 ml/min. HPLC peaks were integrated by an Automated Laboratory Data System (Computer Inquiry Systems, Englewood Cliffs, NJ, U.S.A.).

Calibration and calculation

On each day, calibration equations were determined for the standardization samples, using linear regression analysis. These equations were used to calculate the MDL 17,043 concentrations in the coded unknown samples.

RESULTS AND DISCUSSION

Using the extraction procedures described previously, there was no endogenous material found in dog or human plasma that would interfere with the assay. Retention times for MDL 17,043 and internal standard were 6.0 and 11.5 min, respectively. A typical chromatogram is shown in Fig. 1.

Composite results for the six validation days are tabulated in Table I. The assay was linear over the range of 25—1000 ng/ml and had acceptable precision. The day-to-day variation as judged by the slope had a coefficient of variation of 1.0%.

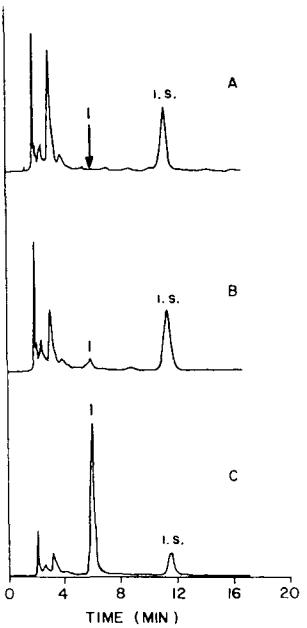

Fig. 1. Chromatograms of extracted plasma standards: (A) blank, (B) 25 ng/ml, (C) 1000 ng/ml. Peaks: 1 = MDL 17,043; I.S. = internal standard.

TABLE I

MDL 17,043 STANDARDIZATION RESULTS FOR SIX DAYS

Standard concentration of MDL 17,043 (ng/ml)	*n*	Mean peak height ratio* (%)	S.D.
0	12	0	—
25	11	26.0	2.3
100	12	102.8	3.0
300	11	308.2	6.7
600	12	627.3	10.4
1000	11	1031.5	24.8
Slope	6	1.0333	0.0102

*(Peak height of MDL 17,043/peak height of internal standard) × 100.

The accuracy of the assay was assessed by preparing and analyzing 36 unknowns in a randomly coded fashion. The mean results are shown in Table II.

Dog plasma concentrations found after i.v. and oral dosing are given in Fig. 2. MDL 17,043 was found to be readily absorbed and could be followed up to 8 h post dose. At 24 h post dose no drug was detected. Based upon area under the plasma concentration versus time curve, the absorption of MDL 17,043 was about 50% and 85% for dogs A and B, respectively. From the program CSTRIP [4], the $t_{1/2}$ values for the two dogs were 1.75 h and 2.25 h in the β-phase after i.v. dosing.

In conclusion, the validity of the method to measure plasma level was demonstrated; the method is sensitive enough to apply to pharmacokinetic and bioavailability studies of the drug in animals and humans.

TABLE II

ANALYSIS OF PLASMA CONTAINING UNKNOWN ADDED CONCENTRATIONS OF MDL 17,043

Concentration added (ng/ml)	n	Mean concentration found (ng/ml)	S.D.
0	4	0	—
25	3	24.8	2.4
50	2	47.8	—
100	6	96.7	1.9
200	2	194.6	—
300	6	304.9	3.8
500	2	505.0	—
600	6	605.2	7.8
800	2	806.2	—
1000	3	1014.9	13.6

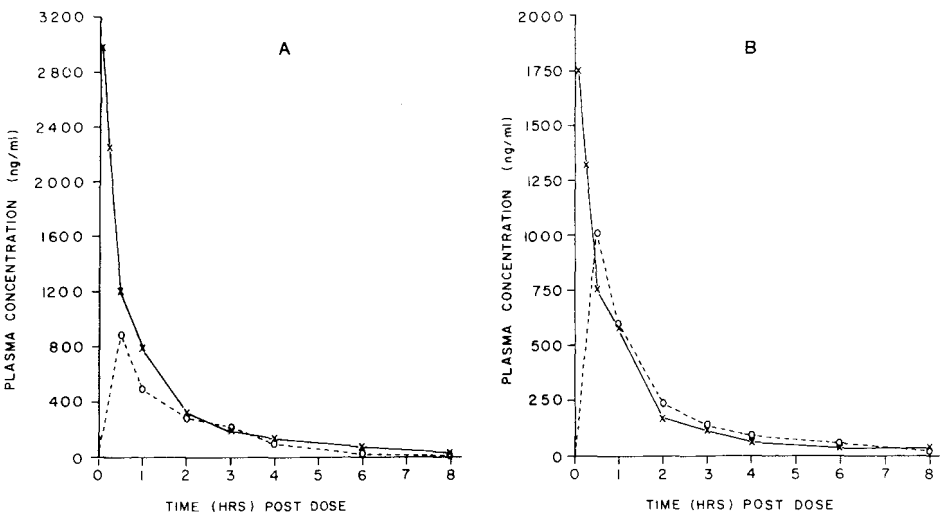

Fig. 2. Plasma concentrations of MDL 17,043 in (A) dog A and (B) dog B after oral (o— — —o) and i.v. (x——x) administration of MDL at 3 mg/kg.

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CHROMBIO. 1507

Note

Determination of dihydroergocristine and dihydroergotamine in plasma by high-performance liquid chromatography with fluorescence detection

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(First received July 2nd, 1982; revised manuscript received September 23rd, 1982)

Dihydroergocristine (DHEC) is used in the treatment of cerebral and peripheral vascular diseases, and dihydroergotamine (DHET) in the treatment of migraine. The analytical procedures commonly used for the determination of these ergot derivatives in plasma are not selective or sensitive enough for pharmacokinetic studies.

The determination of total radioactivity after the administration of labelled compound is highly sensitive but lacks selectivity since ergot alkaloids are extensively metabolized [1—4].

Several radioimmunoassay (RIA) methods have also been described recently [5—9] but although these methods are generally rather sensitive they do not always distinguish between the parent compound and its metabolites. Thin-layer chromatographic and fluorimetric methods [10, 11] are not sensitive enough for pharmacokinetic studies.

Although several high-performance liquid chromatographic (HPLC) methods for the separation of ergot alkaloids in pharmaceutical preparations have been described [12—18], only a few studies report the application of HPLC to pharmacokinetic studies of ergot derivatives [19—21].

The present report describes a rapid, sensitive and selective HPLC method for the determination of DHEC and DHET in plasma and its application to pharmacokinetic studies in rats.

EXPERIMENTAL

Reagents and standard solutions

DHEC methanesulphonate was supplied by Roussel-Maestretti (Milan, Italy) and DHET methanesulphonate was obtained from Sandoz (Basle, Switzerland).

Stock solutions were prepared in methanol at a concentration of 1 mg/ml.

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Working standard solutions with a concentration of 1 μg/ml were prepared daily by dilution in water.

Acetonitrile and chloroform for liquid chromatography were obtained from Merck (Darmstadt, G.F.R.). All other chemicals and reagents were of analytical grade (Merck, and Carlo Erba, Milan, Italy).

Chromatographic apparatus and conditions

Perkin-Elmer (Norwalk, CT, U.S.A.) series 2/2 high-performance liquid chromatograph equipped with either a Perkin-Elmer LC-75 variable-wavelength UV detector and autocontrol system or with a Perkin-Elmer 650-10S spectrophotofluorimeter was used. Samples were introduced by means of a syringe into a Rheodyne 7105 (Berkeley, CA, U.S.A.) injection valve with a 150-μl loop. An RP-8 Hibar column (10 μm particle size; 25 cm × 4.0 mm I.D.) from Merck was operated at room temperature. The mobile phase was acetonitrile—pH 7.2 phosphate buffer (9 m*M* NaH_2PO_4 and 9 m*M* Na_2HPO_4) (60:40) and the flow-rate was 1.0 ml/min. The mixture was degassed at room pressure in an ultrasonic bath for a few minutes. The column effluent was monitored at 223 nm with the UV detector. The spectrophotofluorimeter was employed with an excitation wavelength of 295 nm, an emission wavelength of 350 nm and band widths for both excitation and emission of 10 nm.

Procedure

A 1-ml volume of plasma, 50 μl of internal standard solution and 30 μl of 5 *M* sodium hydroxide solution were placed into a 12-ml tapered glass tube. After adding 7 ml of chloroform, the tubes were shaken on a reciprocal shaker for 10 min. After centrifugation at 2000 *g* for 15 min, the aqueous phase and the emulsion layer were totally aspirated off, and the organic phase transferred into fresh tubes and evaporated to dryness under a flow of nitrogen at 40°C. The residues were reconstituted in 100 μl of the mobile phase and 10—30 μl injected into the chromatograph.

In addition to the unknown samples, plasma calibration standards containing 5, 10, 25 and 50 ng of either DHET or DHEC and 50 ng of internal standard (DHEC or DHET, respectively) were run.

Calibration curves were constructed by plotting the DHEC or DHET concentrations versus the ratio of the peak heights of compounds to those of their respective internal standards.

Recovery

The percentage recovery was calculated by comparing the peak height ratios for DHEC and DHET standards prepared in mobile phase, with those obtained after plasma extracts at the same concentrations were injected.

Animals

Charles-River rats weighing 150—180 g, kept in makrolon cages at constant room temperature (21—22°C) and humidity (60%) were used. At various times after drug administration, the rats were killed by decapitation and the blood collected in plastic tubes containing 0.1 ml of sodium heparin. After centrifugation at 2200 *g* for 15 min the plasma was frozen and kept at −30°C until analysis.

Drugs were given orally in solution by gavage (5 mg/kg, 1.0 ml/kg) or by intraperitoneal injection (1 mg/kg, 1.0 ml/kg).

RESULTS

Detection

With UV detection, the absorbance spectra for DHEC and DHET showed two peaks at 282 and 223 nm. As the highest sensitivity and specificity were achieved at 223 nm, this wavelength was chosen for plasma monitoring. The minimal detectable amount of pure compounds was about 1 ng. The lower limit of detection in plasma extracts was 5—10 ng/ml.

Using fluorescence detection, the excitation and emission peak readings, recorded in spectra by stopping the column outflow into the cell were 295 and 350 nm, respectively. The minimal detectable amount of pure compounds was 0.1 ng. The selectivity and high sensitivity of this detection method increased the sensitivity for measurement of the compounds in plasma extracts to 0.5—0.7 ng/ml.

Extraction and chromatography

Both DHEC and DHET are relatively polar and quantitative extraction can be obtained only with a rather polar solvent. On the other hand, it is advisable to use a solvent with as low a polarity as possible to avoid extraction of interfering plasma substances. In our hands chloroform was a good compromise and gave extracts that were sufficiently clean, particularly for fluorescence detection; the recovery was high even after a single extraction (91 ± 4%, n =

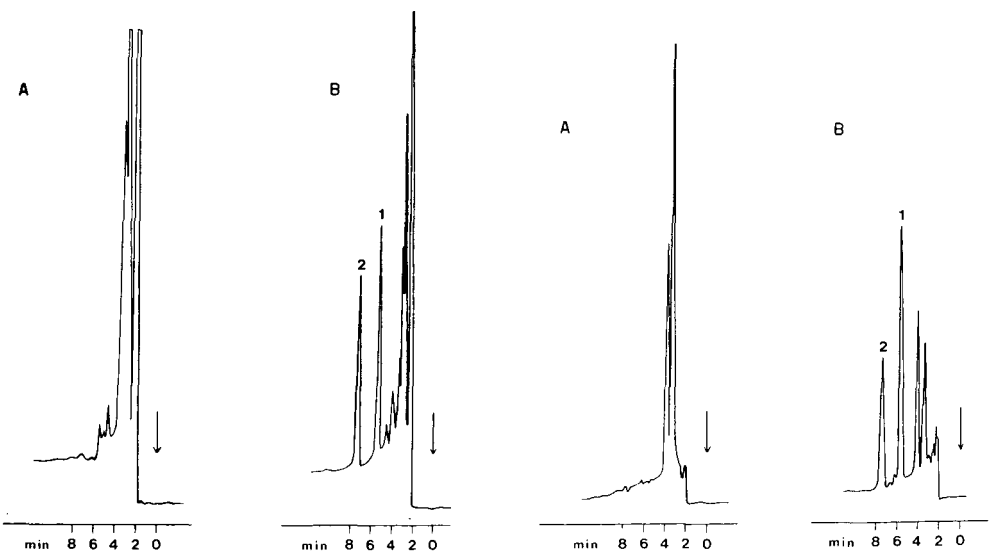

Fig. 1. Chromatograms of HPLC analysis with UV detection of plasma extract. A, Control plasma; B, control plasma containing 100 ng/ml of both DHEC (2) and DHET (1).

Fig. 2. Chromatograms of HPLC analysis with fluorescence detection of plasma extracts. A, Control plasma; B, control plasma containing 25 ng/ml of DHET (1) and 25 ng/ml of DHEC (2).

6 with an intra-assay coefficient of variation, C.V., of 3.4% for DHEC and 93 ± 6%, n = 6, with a C.V. of 2.8% for DHET). the inter-assay C.V. was found to be of 4.8% for DHEC and 5.2% for DHET. Separation of DHEC and DHET with chromatographic system described above was good (retention times: 7.9 and 5.2 min, respectively) and no interfering peaks were present after either UV or fluorescence detection (Figs. 1 and 2).

Plasma levels

The method described above has been used in our laboratory for the determination of the pharmacokinetics of DHEC and DHET. Peak concentrations after 1 mg/kg i.p. and 5 mg/kg oral administration of the drugs are shown in Table I and typical chromatograms of plasma extracts in Figs. 2 and 3. Peak concentrations were reached 10 min after i.p. and 1 h after oral administration.

TABLE I

PEAK PLASMA LEVELS (ng/ml) OF DHEC AND DHET AFTER ORAL (5 mg/kg) AND INTRAPERITONEAL (1 mg/kg) ADMINISTRATION

Values are mean ± S.E. of five determinations.

Drug	Oral	i.p.
DHEC	3.8 ± 0.9	59.9 ± 6.3
DHET	3.2 ± 0.8	53.0 ± 5.8

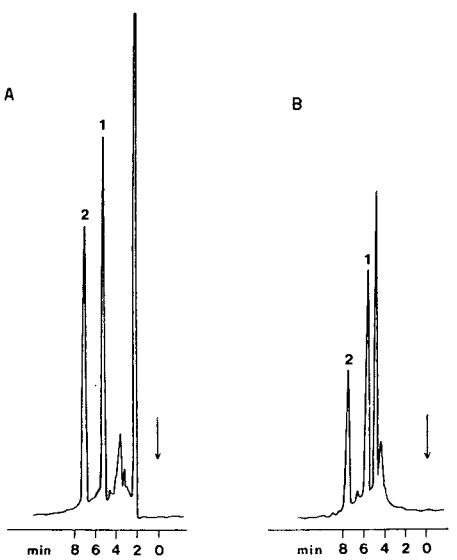

Fig. 3. Chromatograms of HPLC analysis with fluorescence detection of plasma extracts. A, Plasma extract from a rat administered 1 mg/kg i.p. of DHEC (actual concentration 52 ng/ml) (2) and containing 50 ng/ml of DHET (1) as internal standard. B, Plasma extract from a rat administered 1 mg/kg i.p. of DHET (actual concentration 33 ng/ml) (1) and containing 25 ng/ml of DHEC (2) as internal standard.

DISCUSSION

Fluorescence detection proved to be clearly more selective and sensitive

than UV detection. In the present work the sensitivity for DHET was higher than that found by Edlund [21]. This may possibly be due to the greater efficiency of the fluorimeter we used, which is equipped with two monochromators instead of filters. In fact, in our experience filters absorb as much as 70% of the light energy, while monochromators absorb less than 10%. The present chromatographic method can be used to separate the dihydro derivatives of ergotamine, ergocornine, ergocristine and ergocryptine. However, to obtain a complete separation of these compounds a chromatographic column with higher resolution efficiency such as 5 μm RP-8, has to be used. Our HPLC—fluorescence method allows us to measure plasma concentrations of DHEC and DHET after parenteral or oral administration to rats. We do not consider, however, that the sensitivity obtained with this method is great enough for kinetic studies of ergot alkaloids after oral administration of therapeutic doses to humans. Our data confirm the low oral bioavailability of ergot derivatives, the plasma concentrations after oral administration being only about 6% of those after parenteral administration. In fact, using the HPLC—fluorescence method, Ibraheem et al. [20] could measure the plasma levels of ergotamine after parenteral but not after oral administration, indicating a very low oral bioavailability of the drug.

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Note

High-performance liquid chromatographic determination of ethyl biscoumacetate in human plasma

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Ethyl biscoumacetate (EBA) is a coumarinic oral anticoagulant which differs from other drugs of its group by having a faster onset of action [1, 2]. For determination of EBA in biological fluids, only a spectrophotometric method has been reported [3]. In pharmaceutical samples, however, a high-performance liquid chromatographic (HPLC) method has been used to measure this drug [4]. In the process of studying the interaction of dipyrone with EBA [5] a rapid, sensitive and specific HPLC method suitable for measuring micro-quantities of EBA in human plasma was developed, which is reported below.

EXPERIMENTAL

Apparatus and conditions

The HPLC system consisted of a Model 224 U instrument (Waters Assoc., Milford, MA, U.S.A.) equipped with a dual-channel fixed-wavelength (254 and 280 nm) UV detector and a 30 cm × 3.9 mm I.D. column with a 10-μm (spherical) particle size (μBondapak C_{18}, Waters Assoc.). The mobile phase was methanol—water—acetic acid (56:40:4) which was pumped at a flow-rate of 1 ml/min with an initial pressure of 7 MPa.

Chemicals

All organic solvents were of analytical grade and the distilled water was deionized. Carbamazepine was used as internal standard (IS) which was extracted from tablets (Tegretol, Ciba Geigy, Iran) with benzene and recrystallized in absolute ethanol—benzene (70:30). The purity of IS and EBA (Ciba Geigy) was confirmed by the official methods [6, 7].

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Standard solutions

A stock solution of 40 mg EBA in 50 ml methanol was prepared and further diluted with water to provide four series of solutions containing 2, 4, 20, 40, 80, 120, 200, 400 and 800 μg in 3 ml of water. In 25-ml PTFE-insert screw-cap glass tubes, 1 ml blank plasma, 1 ml 0.1 *M* hydrochloric acid, 1 ml IS (40 μg/ml in methanol) and 10 ml benzene were added to the standard solutions. Tubes were shaken mechanically for 30 min. A blank solution without EBA and IS was also prepared. The benzene layer was removed and transferred into 15-ml centrifuge tubes, evaporated under vacuum and the dried residue was dissolved in 0.25 ml methanol. Aliquots of 25 μl of the latter solutions were injected into the HPLC system. The final amounts of EBA in 25 μl were 0.05, 0.10, 0.50, 1.00, 2.00, 3.00, 5.00, 10.00 and 20.00 μg, respectively, with a constant amount of 4.00 μg IS in each injected sample. Four series of standard solutions were prepared. The 280-nm peak height ratio (EBA/IS) was calculated and a standard curve was prepared by plotting the ratios versus amount of EBA. Statistical parameters were computed using a programmable calculator (Model 97, Hewlett-Packard, Corvallis, OR, U.S.A.).

Recovery

To determine the efficiency of the extraction, two series of solutions similar to those of the standard solutions were prepared but EBA (in methanol) was added to the separated benzene layer after the extraction.

Subjects

Volunteers were four healthy male students with average age and body weight of 26.5 years and 66.0 kg, respectively. They took single tablets of 300 mg EBA with 250 ml water after an overnight fast and at least 1.5 h before breakfast. Venous blood samples were taken from forearms by heparinized disposable syringes at 0, 0.5, 1, 1.5, 2.5, 3.5, 5, 7 and 9 h post-dosing. Samples were centrifuged and plasma portions were kept frozen until the time of analysis. The contents of 0.1—2.0 ml plasma were determined using the same procedure described in the *Standard solutions* section. The biological half-lives of EBA were estimated from slopes of plasma drug concentration–time curves using the last four points of the terminal phase.

RESULTS AND DISCUSSION

Fig. 1 depicts chromatograms from plasma before and 1 h after administration of a single oral dose of 300 mg EBA to a subject. No interfering peaks were noted from blank plasma. Peaks representing EBA and carbamazepine appeared 14.0 and 16.5 min, respectively, after injection into the chromatograph. The selectivity of the assay was assured by routine examination of UV absorbance ratios at 280/254 nm. Linearity and extraction recovery specifications are shown in Table I. For the pooled data the best-fit line through the points was described by $y = 0.1827x - 0.0013$ with a correlation coefficient of 0.999, indicating an excellent linear relation between the peak height ratio and the amount of injected EBA. Coefficients of variation (C.V.) varied from 2 to

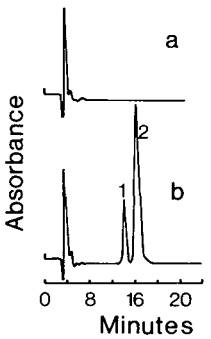

Fig. 1. Chromatograms of (a) blank plasma and (b) plasma of a subject 1 h after administration of 300 mg EBA. Peaks: 1 = EBA, 2 = carbamazepine (IS).

TABLE I

PEAK HEIGHT RATIOS (EBA/INTERNAL STANDARD) OBSERVED AT 280 nm FOR STANDARD SOLUTIONS (EXTRACTED) AND THEIR RECOVERY COMPARED WITH UNEXTRACTED SOLUTIONS

Amount added (μg)	Extracted*		Unextracted**	Recovered (%)
	Mean	C.V. (%)		
0.05	0.0077	24	0.0081	
			0.0080	95.6
0.10	0.0176	8	0.0187	
			0.0193	92.7
0.50	0.0879	5	0.0920	
			0.0904	96.4
1.00	0.1816	4	0.1882	
			0.1956	94.6
2.00	0.3609	4	0.3701	
			0.3668	97.9
3.00	0.5489	5	0.5681	
			0.5712	96.4
5.00	0.9187	4	0.9308	
			0.9453	97.9
10.00	1.8210	4	1.9250	
			1.9830	94.0
20.00	3.6535	2	3.8150	
			3.7500	96.6
Mean				95.7

*The best-fit line through the data is described by $y = 0.1827x - 0.0013$ which was used as standard curve (regression coefficient = 0.999). Mean value is the mean of four determinations.

**The best-fit line through the data is described by $y = 0.1902x - 0.0002$ (regression coefficient = 0.999).

8% within the examined range except for the solutions containing 0.05 μg per injection which was 24%. Therefore, the acceptable range was set as 0.10—20.00 μg. To measure plasma EBA concentrations below 0.1 μg/ml, therefore, larger plasma volume samples were required.

An average of 95.7% (standard deviation 1.7) was found to be extractable using this method (Table I).

Plasma EBA concentrations after administration of single oral doses of 300 mg are shown in Table II. Maximum plasma EBA concentrations (C_{max}) ranged from 10.21 to 14.62 μg/ml and were attained (T_{max}) 1.0—1.5 h post-dosing. In two subjects, 9 h after ingestion, plasma drug concentrations declined below the acceptable range (0.03 and 0.08 μg/ml in R.M. and M.J., respectively). Utilization of larger volumes of plasma and/or injection of larger volumes of extracts into the chromatograph could bring the levels within the desirable range. However, no attempts were made to increase the precision of these two samples.

TABLE II

PLASMA EBA CONCENTRATIONS (μg/ml) AND HALF-LIVES OF TERMINAL PHASES ($t_{½}$) AFTER ADMINISTRATION OF SINGLE ORAL DOSES OF 300 mg TO HEALTHY SUBJECTS

Subject	Hours								$t_{½}$* (h)
	0.5	1.0	1.5	2.5	3.5	5	7	9	
M.J.	—	2.29	10.21	10.01	7.91	2.66	0.44	0.08	0.82
R.M.	1.24	10.56	8.04	2.88	1.72	0.57	0.28	0.03	1.02
G.A.	3.32	4.11	11.62	10.10	7.24	5.62	2.08	0.79	1.66
R.A.	5.83	10.58	12.44	11.84	6.04	3.75	1.41	0.77	1.79

*Calculated from the slope of the best-fit line through the last four points.

Following oral administration of bolus 1500 mg and then daily maintenance doses of 300 mg EBA to five subjects, using a spectrophotometric method, Brodie et al. [3] were unable to quantitate the drug beyond 8 h post-bolus dose and 6 h post-maintenance dose. Therefore, they concluded that EBA does not cumulate in plasma during chronic therapy which agrees with our observation. The observed half-lives of the post-absorption phase of the plasma EBA concentration versus time curves ($t_{½}$) calculated from lines best-fitted through the terminal four points of each curve varied from 0.82 to 1.79 h with a mean of 1.36 h and standard deviation of 0.421 (Table II). This drug is usually administered in one or two divided daily doses [5, 8]. Dosing intervals of 8 and 12 h correspond to 6.7-h and 13.4-h half-lives observed after 300-mg doses, respectively. Therefore, no appreciable accumulation of EBA is expected during chronic therapy.

The average C_{max} reported by Brodie et al. [3] was 25 μg/ml after ingestion of 300-mg daily doses. No measure of deviation from this average was reported. Nevertheless, this value is substantially higher than the range observed by us. Since no significant accumulation is expected after repeated doses of EBA,

this discrepancy may be attributed to the nonspecificity of the spectrophotometric method [9].

It is worthy of mentioning that this method was also applied to measure urinary excretion of EBA. Only traces of the unchanged drug were found in urine. However, three other peaks with retention times of 5.8, 10.6 and 15.4 min and with respective UV absorbance ratios (280/254 nm) of 4.57, 0.30 and 1.70 were consistently detectable in urine of EBA-administered subjects (Fig. 2). These peaks, presumably, represent metabolites or degradation products [4] of EBA (absorbance ratio 2.5).

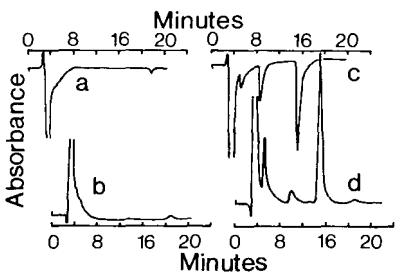

Fig. 2. Chromatograms of blank urine (a and b) and urine of a subject 2 h after administration of 300 mg EBA (c and d). Upper chromatograms represent absorbance at 254 nm while lower ones are observed at 280 nm.

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Note

High-performance liquid chromatographic assay with fluorometric detection of ketanserin, a new antihypertensive agent and serotonin S_2 antagonist in human plasma, blood and urine

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Ketanserin (R 41468) or 3-{2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl}-2,4-(1H, 3H) quinazolinedione (see Fig. 1) is an antagonist of 5-hydroxytryptamine S_2 receptors (5-HT_2) [1, 2] and a novel antihypertensive agent undergoing current widespread clinical investigations [3–7]. To date, only limited information is available concerning the concentration range of this drug in plasma, blood or urine. A radioimmunoassay method has been developed [8], but it is not generally available and its specificity has not yet been confirmed. Evaluation of pharmacokinetic data and plasma level monitoring might contribute to a better understanding of the therapeutic efficacy and mechanisms of action of ketanserin.

We report here a sensitive and selective high-performance liquid chromatographic (HPLC) assay with fluorometric detection for ketanserin. Following its extraction from plasma, whole blood or urine, ketanserin can be measured in

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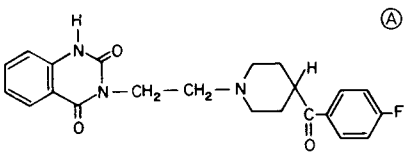
humans after therapeutic doses. Pharmacokinetic data derived from this plasma level monitoring will provide the rational basis for establishing proper dosage regimens of this new drug.

EXPERIMENTAL

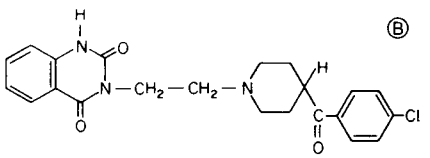
Chemicals and reagents

Ketanserin and the internal standard (R 46594) were obtained as reference compounds from Janssen Pharmaceutica, Life Sciences Products Divison, Beerse, Belgium. Chemical structures are shown in Fig. 1.

Acetonitrile, methanol, pentane and isoamyl alcohol were of nanomolar grade. Diethylamine was purified before use by elution through basic aluminium oxide, activity 60. All other reagents were of analytical grade and were purchased from Merck, Darmstadt, G.F.R. All inorganic reagents were prepared in double-distilled water.



R 41 468 (Ketanserin)



R 46594 (internal standard)

Fig. 1. Structures of ketanserin (A) and internal standard (B).

Apparatus

A Spectra-Physics solvent delivery system Model 140B was used for analysis. The pump was equipped with a Rheodyne 100-μl injection loop. For detection a Jobin Yvon Spectrofluo JY3D LC fluorimeter with excitation and emission wavelengths set at 270 nm and 410 nm, respectively, was used. The fluorometric detector was coupled to a Servocord recorder with chart speed set at either 300 mm/h or 120 mm/h. A 25 cm × 2 mm I.D. reversed-phase column equipped with a 5-cm precolumn, both packed with Nucleosil 10 μm (Macherey and Nagel, Düren, G.F.R.), were used for separation. The mobile phase was made up of water—acetonitrile (70:30) with 0.2% (w/v) diethylamine. The flow-rate was 1.00 ml/min at a pressure of 200 atm.

Sample preparation

Stock solutions of ketanserin and internal standards were stored in amber-coloured bottles at 4°C. One millilitre of quality control plasma, patient or standard plasma containing between 2 and 20 ng/ml was mixed with 0.200 ml of internal standard solution (100 ng/ml). Plasma containing high concen-

trations of ketanserin was diluted with blank plasma. Plasma or blood was alkalinized with 1 ml of 0.01 *M* sodium hydroxide in stoppered tubes; 2 ml of pentane—isoamyl alcohol (95:5) solution were added and the mixture was rotated for 10 min. Samples were centrifuged and the organic upper layer was transferred to 15-ml conical tubes. The alkalinized plasma was re-extracted with 2 ml of pentane—isomayl alcohol and the combined organic layers were evaporated under nitrogen at about 60°C. The residue was redissolved in 0.1 ml of mobile phase and injected into the HPLC column.

For urine samples buffering was needed. Accordingly, standards were prepared in 0.1 ml of blank urine and 0.01 *M* phosphate buffer (0.9 ml) pH 7.4. Aliquots (0.05—0.1 ml) of refrigerated urine were then extracted as described above for plasma.

Assignment of peaks

Qualitative designation of peaks was done by comparing their retention times to those of known reference compounds. Under our experimental conditions the retention volume of ketanserin was 4.8 ml while that of internal standard was 7.8 ml (retention times were 4.8 and 7.8 min, respectively). Ketanserin in samples was determined by calculating the ratios of peak heights of the drug to that of internal standard and relating this to concomitantly constructed calibration curves over the concentration range 2—20 ng/ml. Recovery was calculated from comparison between directly injected standards and standards subjected to the extraction procedure.

RESULTS AND DISCUSSION

Blanks from different biological samples did not show any interfering peaks even at maximum instrument sensitivity settings (Figs. 2A, 3A and 4A). A plot

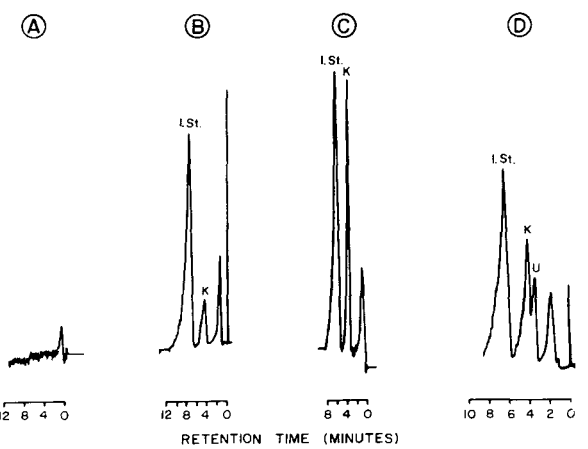


Fig. 2. Representative chromatograms of ketanserin in plasma. (A) Blank Plasma. (B) Quality control plasma: 6 ng of ketanserin (K) and 20 ng of internal standard (I. St.). (C) Standard: 20 ng of ketanserin and 20 ng of I.St. with mobile phase buffered with 0.01 *M* phosphate buffer pH 7.4. (D) A ten-fold dilution of plasma from a healthy volunteer 120 min after 40 mg oral dosing; U is a proposed metabolite. Chart speed 300 mm/h; chart speeds A, B, C were run at 120 mm/h.

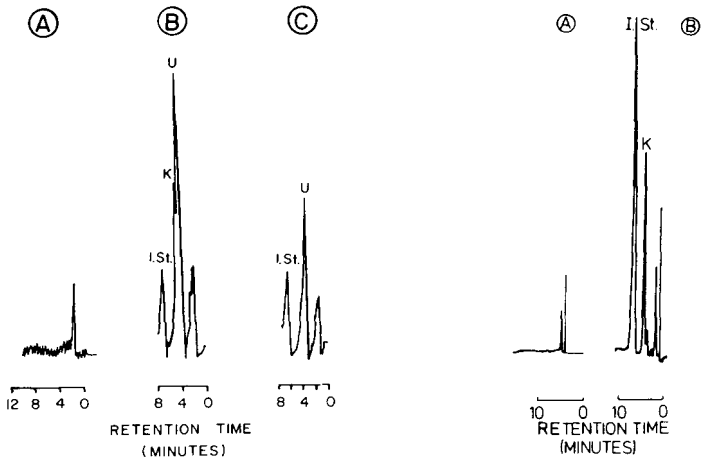


Fig. 3. Chromatogram of ketanserin in urine. (A) Blank urine. (B) A ten-fold dilution of first 24-h urine collection. (C) A ten-fold dilution of second day urine collection. I.St. = internal standard, K = ketanserin, U = unidentified metabolite.

Fig. 4. Chromatogram of ketanserin in whole blood showing (A) blank and (B) patient 1 h after an intravenous dose.

of peak height ratio versus concentration of ketanserin is linear (range 0.1—30 ng/ml) and is described by the equation $Y = 0.033X - 0.0014$ ($r = 0.9998$).

A concentration of 500 pg of ketanserin per ml plasma can be measured with reasonable accuracy. The precision of the assay was established by multiple measurements of quality control samples (12 ng/ml) stored at —20°C. The intra-assay mean ± S.D. was 12.05 ± 0.38 ng/ml and the inter-assay mean was 11.68 ± 0.36 ng/ml. The intra-and inter-assay coefficients of variation were 3.1% and 3.2%, respectively ($n = 18$). Ketanserin seemed to be stable if stored in plasma for over a three-month interval at —20°C. Frequent freezing and thawing did not reduce the reliability of ketanserin determination. Recovery was highest for plasma and lowest for urine (see Table I). At pH 7.4, fluorescence of ketanserin is enhanced over internal standard but the two compounds tended to elute too closely together (Fig. 2C). Therefore a more alkaline pH has been chosen for better separation but with some loss of sensitivity. At pH > 10.5 or < 1.5 the stability of the column was rather poor.

TABLE I

RECOVERY OF KETANSERIN IN BIOLOGICAL SAMPLES

	Recovery (% ± S.D.)	n
Plasma	94.8 ± 5.32	10
Whole blood	92.1 ± 6.81	8
Urine	78.5 ± 4.54	15

The assay seems to distinguish between ketanserin and a possible metabolite with a retention time of about 4 min. This peak is absent in ketanserin plasma solutions stored at −20° C, in ketanserin standards and blanks, but promptly appears 0.5 h after intravenous administration and 1 h after oral administration (designated "U" in Figs. 2D, 3B and 3C). This substance is also present in 24-h urine and is probably the main substance in 24-h urine of the second day of collection (see Fig. 3).

The metabolism of ketanserin by humans is at the present time poorly understood. We cannot therefore predict the rate of metabolite formation by our assay method. With the availability of purified reference standards of metabolites of ketanserin in the future, our method may be adapted for their easy estimation. In its present state the method is not suitable for the quantitation of ketanserin in urine when the concentration of the compound U is higher than that of ketanserin. We have also compared our HPLC assay with the radioimmunoassay method on spiked plasma samples and there appears to be a good correlation (see Fig. 5). The radioimmunoassay method was not suitable for determination of whole blood, and urine had to be fortified with blank plasma before being subjected to radioimmunoasay.

Fig. 6 depicts a representative ketanserin plasma concentration—time profile in a healthy volunteer following single dosing. This indicates that the assay can be used in pharmacokinetic and clinical studies.

In conclusion, the fluorometric detection of our HPLC method enables selective and very sensitive ketanserin measurements in biological samples. It is suitable for detection of very low ketanserin levels in biological fluids which will be an important prerequisite for determination of reliable pharmacokinetic data. Following the elucidation of the ketanserin metabolism in man and the availability of pure ketanserin metabolites as reference substances the method will have to be optimized for measuring ketanserin and its major metabolites.

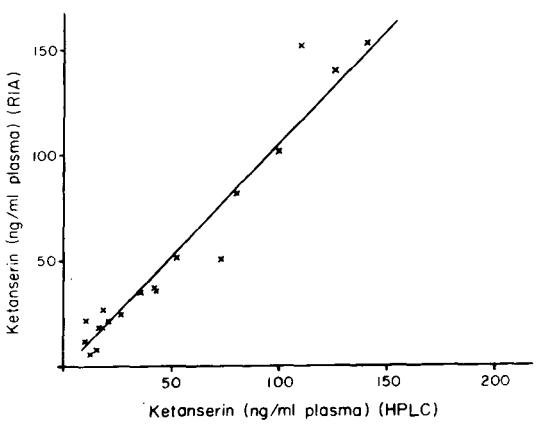


Fig. 5. Comparison of HPLC method for ketanserin with a radioimmunoassay (RIA) method. $Y = 0.998X + 0.026$, $r = 0.99$, $p < 0.001$.

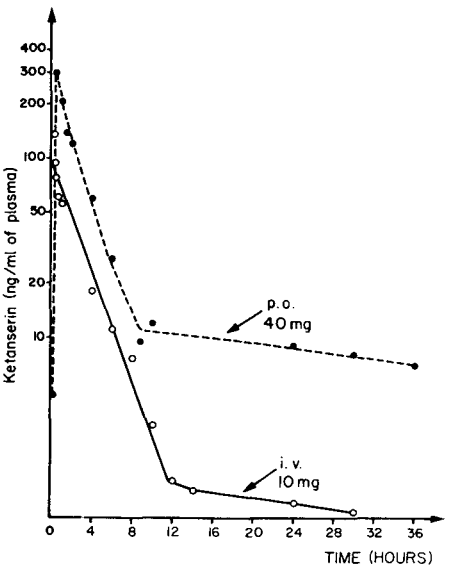


Fig. 6. Plasma concentration—time profiles of ketanserin in one subject following a single oral (p.o.) dose of 40 mg of ketanserin and a single intravenous (i.v.) dose of 10 mg of ketanserin. The pharmacokinetic parameters in this particular healthy volunteer were: oral bioavailability = 76%; elimination half-life time ($t_{½\beta}$) = 14.8 h and 11.4 h after intravenous and oral application, respectively; total body plasma clearance = 535 ml/min and 624 ml/min after intravenous and oral application, respectively; apparent volume of distribution ($V_{d\beta}$) = 10.4 l/kg and 9.3 l/kg after intravenous and oral application, respectively. Calculations were made on the basis of a two-compartment open model using a SAAM 25 computer program.

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Note

Determination of ketanserin in human plasma by high-performance liquid chromatography

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Ketanserin, 3{2-[4-(fluorobenzoyl)-1-piperidinyl]ethyl}-2,4-(1H,2H)quinazolinedione (Fig. 1), is a new serotonin antagonist which is thought to selectively block serotonin-2 receptors [1—3]. There is evidence that this agent may be useful in the treatment of hypertension, congestive cardiac failure and other clinical conditions in which platelet activation may be occurring [4—6].

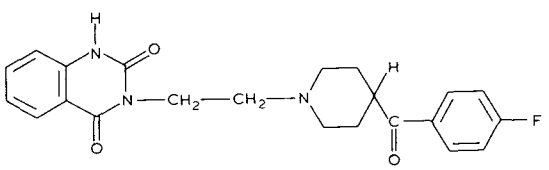

Fig. 1. Structural formula of ketanserin.

Methods for estimation of ketanserin in plasma using radioimmunoassay [7] and high-performance liquid chromatography (HPLC) [8] have been reported briefly in publications by Janssen Pharmaceutica, Beerse, Belgium, but the suitability of these for clinical studies has not been established. The present paper describes an improved alternative assay for ketanserin in human plasma using reversed-phase HPLC and the verification of its use in measuring plasma levels in man.

MATERIALS AND METHODS

Sample preparation

Plasma specimens (1 ml) were pipetted into conical glass tubes containing either 195 nmol/l or 585 nmol/l of the chloro derivative of ketanserin, the in-

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ternal standard (vide infra). After addition of 200 μl of 2 mol/l carbonate buffer (pH 12.0), the mixture was extracted with 3 ml 8% *tert.*-amyl alcohol in heptane for 3 min using a vortex mixer. The two phases were separated by centrifugation at about 1500 *g* for 5 min. The organic layer was then transferred to another conical glass tube, acidified with 500 μl of 1 mol/l hydrochloric acid and mixed thoroughly for 3 min using a vortex mixer. After centrifugation, the organic layer was discarded. The aqueous phase was rendered alkaline with 500 μl of saturated carbonate buffer and about 50 mg solid sodium chloride was added. This mixture was extracted with 8% *tert.*-amyl alcohol in heptane, as described above, and subjected to centrifugation. The organic phase was transferred to another conical tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The extract was reconstituted with 50 μl methanol and 20-μl aliquots were injected into the chromatograph.

Chromatographic analysis

A reciprocating dual-piston high-pressure pump (Constametric IIG; Laboratory Data Control, Riviera Beach, FL, U.S.A.), a sample injection valve containing a 20-μl loop (Rheodyne Model 7120; Rheodyne, Berkeley, CA, U.S.A.) and a variable-wavelength UV detector (Spectromonitor II; Laboratory Data Control) formed the basis of the chromatographic system. Analyses were performed on an octadecylsilane (ODS) reversed-phase column (Hibar LiChrosorb, 5 μm, RP-18, 125 mm × 4 mm, E. Merck, Darmstadt, G.F.R.), at ambient temperature, using methanol—acetonitrile—0.02 mol/l sodium acetate, pH 4.5 (1:4:6) as the mobile phase. The flow-rate was constant at 1.0 ml/min. Absorbance of the effluent from the column at 245 nm was recorded at a sensitivity of 0.005 or 0.01 a.u.f.s. using a strip chart recorder (Omniscribe, Houston Instruments, Austin, TX, U.S.A.).

To prolong the life of the analytical column, a pre-column (30 mm × 4 mm) containing 5-μm ODS reversed-phase packing was incorporated into the system. Reduction in chromatographic efficiency necessitated a change of pre-column after approximately 200 samples were assayed.

Chemicals and solutions

With the exception of methanol and acetonitrile which were HPLC grade (Ajax Chemicals, Melbourne, Australia), all chemicals used were of analytical quality. Water was distilled. Ketanserin was used as the tartrate monohydrate salt (R 49945; MW 563.5 daltons). The chloro derivative of ketanserin (R 46594) was supplied as the base (MW 411.9 daltons) and was used as the internal standard for the assay. These compounds were donated by Janssen Pharmaceutica, Belgium.

Stock solutions of ketanserin tartrate in methanol were prepared in concentrations ranging from 0.25 μg/ml (0.44 μmol/l) to 8.0 μg/ml (14.2 μmol/l). Two solutions of the internal standard in methanol were prepared in concentrations of 1.6 μg/ml (3.9 μmol/l) and 4.8 μg/ml (11.7 μmol/l). The solutions were stable for at least 6 months when stored at 4°C.

The following aqueous solutions were used: acetate buffer: 0.02 mol/l sodium acetate, pH adjusted to 4.5 with glacial acetic acid. Carbonate buffers: (a) 2 mol/l sodium carbonate, pH adjusted to 12.0 with saturated sodium bicar-

bonate solution, (b) saturated sodium carbonate, pH adjusted to 12.0 with saturated sodium bicarbonate solution.

Assay calibration

The assay was calibrated by addition of ketanserin and internal standard to 1-ml drug-free human plasma specimens. Calibration curves were established over a wide concentration range, and to ensure accuracy at the extremes, two concentrations of internal standard were used. The lower end of the range was calibrated with samples containing 0, 22, 44, 89 and 177 nmol/l ketanserin and 195 nmol/l internal standard. Samples containing higher concentrations, 355 and 710 nmol/l ketanserin, contained 585 nmol/l internal standard and to enable continuity of calibration, additional samples containing 0, 89 and 177 nmol/l ketanserin and the higher concentration of internal standard, were assayed.

Peak height ratios of ketanserin to the internal standard were used in quantitation of the assay.

RESULTS

Typical chromatograms of extracted plasma specimens (Fig. 2) show that control samples are free from interfering peaks. Retention times for ketanserin and the internal standard were 5.2 min and 8.1 min, respectively. Any endogenous contaminants remaining in the extracts were eluted before ketanserin and injection of specimens could be repeated immediately after elution of the internal standard.

Calibration curves for ketanserin passed through the origin and were linear up to 710 nmol/l, the maximum concentration used. When the lower quantity of internal standard was used and absorbance was monitored at 0.005 a.u.f.s.,

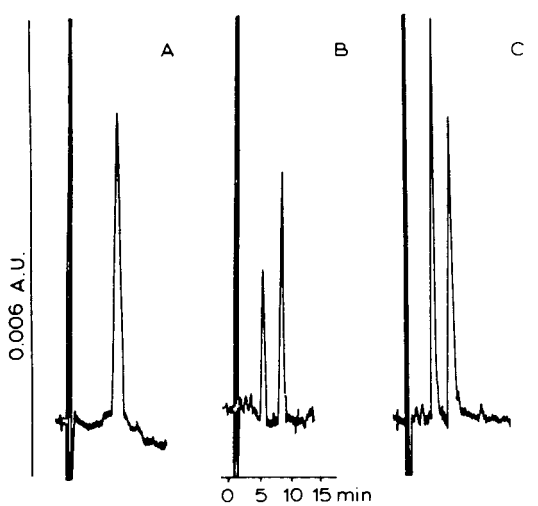

Fig. 2. Chromatograms of human plasma extracts: (A) blank plasma; (B) plasma containing 217 nmol/l ketanserin; (C) plasma containing 625 nmol/l ketanserin. All contain 585 nmol/l internal standard. The ordinate represents absorbance units (A.U.). Retention times were 5.2 min and 8.1 min for ketanserin and internal standard, respectively.

less than 20 nmol/l ketanserin was easily detectable. UV absorbance of ketanserin was monitored at 245 nm, the secondary absorbance peak. Maximum absorbance of ketanserin in the mobile phase occurred at 220 nm, but at this wavelength, the signal-to-noise ratio was lower than at 245 nm and background interference from contaminants remaining in the plasma extracts appeared in several samples.

Recovery of ketanserin from plasma following extraction was assessed by comparison of the peak heights from the plasma extracts with those from standard solutions of the drug in methanol. For ketanserin at concentrations of 44, 177 and 710 nmol/l, recovery ranged from 76 to 86%. At a concentration of 177 nmol/l, the intra-assay coefficient of variation was 4.2% (n = 7). The day-to-day precision of the assay was determined over a period of eight weeks and the interassay coefficient of variation was 3.0% (n = 7) at a concentration of 177 nmol/l, and was 8.5% (n = 7) at a concentration of 22 nmol/l.

To demonstrate the effectiveness of the assay in a clinical situation, blood samples from a hypertensive patient who had received 30 mg ketanserin by intravenous infusion, were assayed. Plasma ketanserin concentrations at 10 min, 2 h and 8 h after cessation of the infusion were 708 nmol/l, 217 nmol/l and 58 nmol/l, respectively. In the same patient, after repeated oral therapy with ketanserin (40 mg twice daily) over a priod of four weeks, plasma levels of the drug in specimens taken 2—4 h after a dose, ranged from 144 to 265 nmol/l. No interference with the chromatographic measurement of ketanserin was observed despite concurrent medication with hydralazine, methyldopa, cyclopenthiazide, prazosin, metoprolol and labetalol.

DISCUSSION

The HPLC assay method presented here is sufficiently sensitive and precise to determine plasma ketanserin concentrations in the clinically relevant concentration range. In addition, the procedure can be performed rapidly and over 30 plasma specimens per day can be assayed. This method should, therefore, be suitable for pharmacokinetic studies of ketanserin in man.

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Note

Analysis of adriamycin and adriamycinol in micro volumes of rat plasma

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Despite ten years of intensive investigation into the therapeutic effects and side-effects of the anthracycline derivative adriamycin, cardiac toxicity is still one of its most severe toxic effects. This limits the dose and consequently its application in cancer chemotherapy. As part of an investigation of the cardiac toxicity caused by adriamycin in tumour-bearing rats, pharmacokinetic data were needed to find out whether there is a correlation between histological changes of heart tissue, the dose of adriamycin and the method of administration. Hence, an assay for adriamycin and its active metabolite adriamycinol was needed to establish pharmacokinetic parameters, such as peak levels, rate of distribution and elimination. If several rats are used for one test, the results due to interindividual differences will not be reliable. Therefore, we decided to establish pharmacokinetic parameters for one single rat.

From the literature [1, 2] one can conclude that after the initial distribution phase, a biphasic disappearance pattern is to be expected. Therefore, 10—15 plasma samples are needed to obtain a complete plasma concentration disappearance curve. This limits the volume of the plasma samples to be analyzed to a maximum of 100 μl (the total blood volume of a rat is 4—6 ml).

Recently described high-performance liquid chromatographic methods that involve fluorescence detection for selective determination of adriamycin

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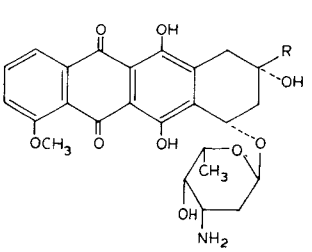

adriamycin : R = $-\overset{O}{\overset{\|}{C}}-CH_2OH$

adriamycinol : R = $-\overset{OH}{\overset{|}{C}}-CH_2OH$

daunorubicin : R = $-\overset{O}{\overset{\|}{C}}-CH_3$

and adriamycinol, are preferred to non-selective methods like total fluorescence analysis [3, 4] and radio-immunoassays [4, 5].

Reversed-phase [6—11] as well as straight-phase [12—14] methods can be used. With straight-phase liquid chromatography and gradient elution, one can analyse nearly all the metabolites, including the inactive aglycones, glucuronated and sulphonated metabolites. Reversed-phase liquid chromatography can easily be applied for the selective assay of adriamycin and its active metabolite adriamycinol. If these methods are to be applied successfully in pharmacological practice, further requirements are easy sample treatment and simple apparatus; gradient elution techniques, for instance, should be omitted if possible.

In this paper, a simple and selective high-performance liquid chromatographic method involving fluorescence detection is described. Rat plasma samples each with a volume of 100 μl were used, plasma levels being within the therapeutic range.

To test the applicability of this method in pharmacological practice, plasma disappearance curves and preliminary pharmacokinetic data for several rats have been determined. A typical plasma concentration—time curve and related pharmacokinetic data are presented.

EXPERIMENTAL

Chemicals and apparatus

Adriamycin·HCl was obtained from Farmitalia in vials of 10 mg with 50 mg lactose. Farmitalia kindly gave us samples of adriamycinol. Daunorubicin·HCl was obtained from Specia in vials of 20 mg with 100 mg D-mannitol. Chromatographic solvents and other chemicals were of analytical grade and were used without further purification. The chromatographic system consisted of a Model 6000A solvent delivery system and a U6K septumless injection system (both from Waters Assoc., Milford, MA, U.S.A.), and a Perkin-Elmer Model 204 fluorescence detector supplied with a Hellma 25-μl flow cell (Hellma, type 176.70). A Merck LiChrosorb RP-8 (50432 C8) reversed-phase column (12.5 × 4 mm I.D., particle size 5 μm) was used. The mobile phase consisted of acetonitrile—distilled water—0.1 *M* H_3PO_4 (31:61:8) containing 10 μg desipramine · HCl per ml (pH 2.3), and was filtered through a 0.2-μm filter and deaerated ultrasonically before use. The flow-rate was 1.5—2 ml min^{-1}. Fluorimetric detection was performed at excitation wavelength 470 nm and emission wavelength 565 nm. Quantitation was based on peak height ratios using the structural analogue daunorubicin as an internal

standard. The chromatographic analyses were performed at ambient temperature.

Sample pre-treatment of micro-volumes of rat plasma

The rats received i.v. injection of adriamycin. By cannulating the vena jugularis, heparinized blood samples of about 200 μl were collected in polypropylene tubes over a period of 3 days. The samples were centrifuged immediately and stored at −20°C prior to analysis. For the extraction of adriamycin and adriamycinol, 100 μl plasma was mixed with 100 μl borate buffer pH 9.0 in a conical polypropylene tube of 1.5 ml. The buffer was composed of 24.7 g boric acid, 6.8 g sodium hydroxide, 29.7 g potassium chloride and 10 mg desipramine·HCl per litre [7, 15]. Appropriate amounts of the internal standard daunorubicin were added in 10-μl volumes of an aqueous solution. A 0.50-ml volume of a chloroform—1-heptanol mixture (1:1) was added. The mixture was vortexed for 45 sec and centrifuged for 5 min at 2500 *g*. The aqueous upper layer was removed and the organic layer was transferred to a new conical polypropylene tube of 1.5 ml containing 100 μl 0.2 *M* phosphoric acid. After 1 min vortexing and 5 min centrifugation at 2500 *g*, 10 to 90 μl of the aqueous phase, depending on the expected concentration level, was injected into the chromatographic system. All the glassware used was silanized before use by treating it with a solution of 2% trimethylchlorosilane in toluene, followed by a washing procedure with methanol.

Blood sampling

Male Wistar rats all weighing about 250 to 300 g were cannulated in the left vena jugularis. The cannule was flushed with a heparin solution twice a day and after each blood sampling. Adriamycin (2 mg kg^{-1}) was administered by i.v. bolus injection. The valve of the cannule was opened and about 200 μl blood was collected in heparinized polypropylene tubes. If the rats damaged the cannule, blood was collected by orbital puncture.

Calculation

The plasma levels of adriamycin were analysed using the HP 9810, programmed with the Wagner stripping method [16].

RESULTS

The straight-phase liquid chromatographic method for the analysis of adriamycin and adriamycinol, described by Baurain et al. [14], is the only one that uses 100 μl of plasma. However, the authors showed its applicability in the μg range only. All other methods mentioned earlier in this paper — reversed-phase as well as straight phase — need 1—4 ml of plasma.

To determine adriamycin and adriamycinol in the ng range, with acceptable accuracy, using 100 μl of plasma, special techniques have to be developed to prevent loss of adriamycin and adriamycinol, because of their strong adsorptive properties. The flexibility of the sample pre-treatment is limited by the adsorptive properties. Polypropylene tubes should be used if possible; if glass-

ware is used it should all be silanized [17]. However, even with silanized or siliconized glassware, adriamycin may still be adsorbed from an aqueous solution. Desipramine·HCL, with comparable adsorptive properties, was added to the buffer in the extraction procedure and to the mobile phase in the chromatographic procedure, in order to decrease the number of active, adsorptive sites. The syringe was pre-treated with trimethylchlorosilane for the same reason and had to be washed with a 4 *M* hydrochloric acid—methanol mixture (1:9). The syringe was washed many times with water between each injection, to prevent memory effects appearing on this trace level analysis. The optimum pH value of the buffer mixed with plasma was found to be pH 9.0. Eksborg [18] mentioned pH 8.6 as an optimum, but he used an organic extraction solvent with a different composition.

As in several other investigations [9, 11, 13] the structural analogue daunorubicin was used as internal standard for comparable lipophilicity, and for comparable chemical and fluorescence properties. We found chloroform—1-heptanol (1:1) to be the best possible composition for the extraction of adriamycin, adriamycinol and daunorubicin [15]. Using this organic solvent in a phase-volume ratio of 1:5, we achieved a 95% recovery of adriamycin for the whole clean-up procedure.

Eksborg [6, 17] determined the influence of the pH and the composition of the extraction mixture. His results indicate that an adriamycinol recovery of about 90% can be expected under these circumstances; this was confirmed in practice.

It is important to vortex the mixture of plasma and buffer for 45 sec since longer vibration yields in a smaller usable organic layer, because of a kind of emulsion of precipitated protein which is formed in the organic solvent. In order to achieve an almost quantitative transfer of adriamycin and adriamycinol to a small volume of the aqueous phase, the aqueous layer must be kept at an acidic pH.

By using 0.2 *M* H_3PO_4 as the aqueous phase, we obtained quantitative recovery and negligible aglycone formation. When aqueous, acidic solutions are stored for longer than about 3 h before analysis, it is preferable to use 0.1 *M* H_3PO_4, despite its lower yield. Also the life-time of the column will increase if the pH is increased.

Eksborg compared several chemically bonded phases for optimum separation of the drug, metabolite and plasma peaks and recommended the use of RP-8 bonded phases.

We investigated several commercially available RP-8 columns and selected the RP-8 column from Merck because of its very small plate height. The composition of the mobile phase (acetonitrile—water—0.1 *M* H_3PO_4, 37:60:3) was optimized for this column. For other types of columns the composition had to be changed for maximum separation. To prevent aglycone formation during the chromatographic process, the concentration of phosphoric acid should never exceed 0.1 *M*. The capacity factors (k') of adriamycin, adriamycinol and daunorubicin proved to be 1.7, 0.7 and 5.7, respectively, under these circumstances.

As in the sample treatment, the strong adsorptive properties of adriamycin interfere with the injection system and disturb the chromatographic pro-

cesses and detection. Addition of desipramine·HCl diminishes this interference to a large extent.

In this procedure no gradient elution is needed; for routine analysis the flow-rate was kept at 2 ml min^{-1} to obtain short analysis times. Fig. 1 shows a chromatogram of the analysis of a rat plasma sample. Calibration curves for standard solutions of adriamycin in aqueous solution were linear over the studied concentration range from 0.1 ng/100 μl to 10 μg/100 μl and passed through the origin (r^2 = 0.9999). Standard deviations in replication measurements were 1.5% in the higher range, and 3% in the lower range.

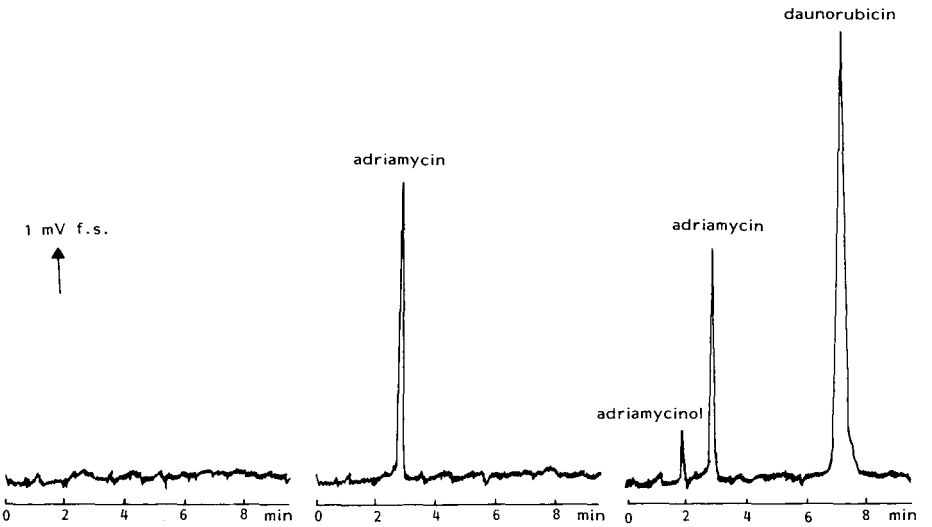

Fig. 1. A chromatogram of the analysis of rat plasma. Left, blank rat plasma; middle, blank rat plasma, spiked with 40 ng adriamycin/100 μl; right, plasma of treated rat, spiked with 60 ng daunorubicin/100 μl.

Calibration curves for the analysis of spiked plasma samples were linear over the studied concentration range of 1 ng/100 μl plasma up to 100 μg/100 μl plasma and passed through the origin (r^2 = 0.9999). Standard deviations in the analysis of plasma samples were 3% in the higher range, and 8% in the lower range.

PRELIMINARY PHARMACOKINETIC RESULTS IN RATS

We have developed the described assay of adriamycin in rat plasma in order to investigate the relation between the dosage, the method of administration of adriamycin and the toxic effects on heart tissue. To investigate the reliability of the method in pharmacological practice, we established the plasma concentration—time curves for several rats, on the basis of very small volumes of rat plasma. The blood samples were collected according to the procedure described in the experimental chapter, namely by cannulating the vena jugularis. If the rats "disconnected" the cannule, blood samples were collected by orbital puncture.

A typical plasma concentration—time curve for adriamycin and adriamycinol is shown in Fig. 2. The line, shown in this figure, is the best fit, as calculated by the computer program. The pharmacokinetic data, calculated using this computer program, are collected in Table I. A three-compartment model gave the best fit. This highly sensitive method makes it possible to measure 6—8 half-lives. The pharmacokinetic data found in this way give new insight into the distribution and elimination of the drug. The distribution volume (V_γ), the half-life time of the elimination phase ($t_{½\gamma}$) and the total body clearance (Cl_{tot}) are remarkably large, compared to earlier published data [19, 20]. Further pharmacological experiments are in progress.

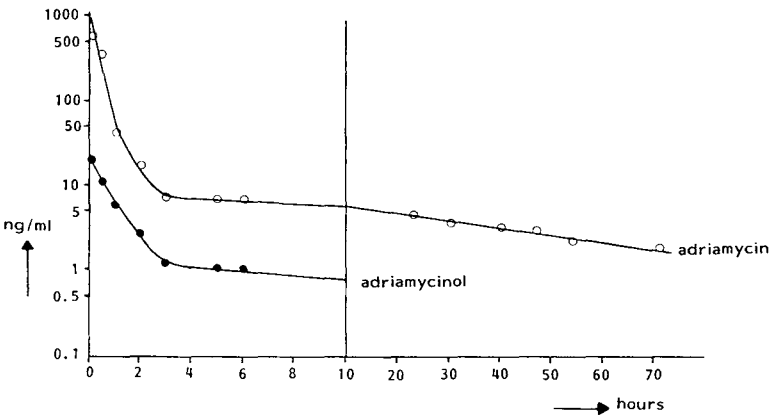

Fig. 2. A typical log plasma concentration—time curve for one rat (adriamycinol could only be analysed for up to 10 h).

TABLE I

TYPICAL PHARMACOKINETIC DATA FOR THE ANALYSIS OF ADRIAMYCIN OF THE log PLASMA CONCENTRATION—TIME CURVE FROM FIG. 2

Wistar rat; 267 g, 2 mg adriamycin/kg by i.v. bolus injection

$C = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + P \cdot e^{-\gamma t}$			
A:	996.6 ng ml^{-1}	α: 5.17 h^{-1}	$t_{½\gamma}$ = 34.3 h
B:	99.6 ng ml^{-1}	β: 1.12 h^{-1}	V_γ = 149 l kg^{-1}
P:	7.8 ng ml^{-1}	γ: 0.020 h^{-1}	Cl_{tot} = 0.050 l min^{-1} kg^{-1}

It can be concluded that the method described in this paper is reliable, sensitive and easy to use in pharmacological practice and may give us further insight into the pharmacology of adriamycin and its active metabolite in the rat and other species.

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CHROMBIO. 1521

Note

High-performance liquid chromatographic procedure for the quantitation of propafenone in serum and tissues

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Propafenone is a new antiarrhythmic agent effective in controlling ventricular and atrial arrhythmias [1] but little is known about its metabolism or pharmacokinetics. In a clinical study dealing with the relationship between pharmacological effects of propafenone and its serum concentrations, Keller et al. [2] found a good correlation between serum levels and atrioventricular conduction times. Serum levels of propafenone were determined by high-performance liquid chromatography (HPLC) by the above authors [2]. In the present communication, we describe a simple HPLC procedure for the quantitation of propafenone in serum and tissue extracts. The method is appropriate for use in the study of clinical and experimental pharmacokinetics of propafenone.

EXPERIMENTAL

Propafenone in injectable form (Rytmonorm® I.V.) was obtained from Knoll (Ludwigshafen, G.F.R.) and was used without further dilution. The internal standard Li 6115, 2'(2-hydroxy-3-ethylaminopropoxy)-3-phenyl-propiophenone hydrochloride, was a gift from Dr. E.B. Kirsten, Knoll Pharmaceuticals (Whippany, NJ, U.S.A.). Diethyl ether and methanol were HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.) and were filtered and degassed before use. All other solvents and chemicals were of reagent grade.

A Waters HLC/GLC Model equipped with a Waters M 6000A pump, a U6K injector, a Model 450 variable-wavelength detector and a Hewlett-Packard HP 3380 A integrator was used. Separations were achieved in an Altex (Beckman, Irvine, CA, U.S.A.) 150 mm × 4.6 mm I.D. column with silica gel (particle size

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5 μm) and a mobile phase consisting of methanol—diethyl ether (30:70, v/v) and 0.02% perchloric acid at a flow-rate of 1.0 ml/min [3]. Propafenone and the internal standard were detected at 254 nm.

The extraction procedure for propafenone from rabbit, dog and human sera was the same as that described by Keller et al. [2]. Rabbit hearts (300—500 mg) were homogenized in 5 parts distilled water and aliquots of supernatant in duplicate equivalent to 120—150 mg wet tissue were taken for analysis. Standard curves were prepared for propafenone and Li 6115 internal standard either together or individually in methanol, serum and myocardial tissue extracts. Interassay and intra-assay standardizations were carried out with rabbit sera.

RESULTS AND DISCUSSION

Fig. 1 shows the separation and quantitation of propafenone (peak I) using internal standard Li 6115 (peak II) in rabbit serum. The retention times of propafenone and internal standard were 4.9 min and 5.8 min, respectively. Fig. 1A shows the chromatogram of blank rabbit serum before propafenone was injected. Fig. 1B and C show the quantitation of propafenone from serum and myocardial tissue 3 h after intravenous (i.v.) injection of propafenone (2 mg/kg) to the same rabbit.

Standard curves were prepared by adding known amounts of propafenone and internal standard to sera from control rabbits, dogs and to normal human serum, and analyzing the samples and determining the peak ratios of propa-

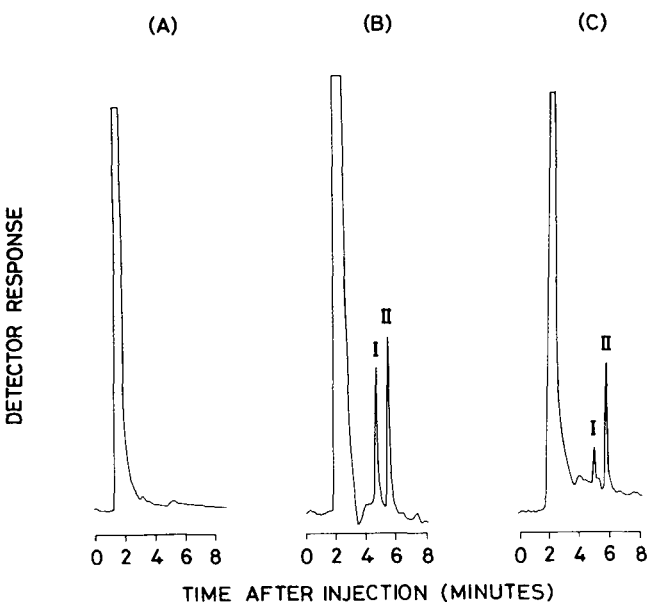

Fig. 1. Quantitation of propafenone from rabbit serum and myocardium. Chromatograms: (A) extracted blank serum sample; (B) and (C) are extracted serum and myocardium respectively from the same rabbit 3 h after i.v. injection of propafenone (2 mg/kg body weight). Peaks: I = propafenone and II = internal standard, Li 6115.

fenone to internal standard. The curves were linear from 0.025—2 μg/ml. Fig. 2 shows a calibration curve obtained using rabbit serum spiked with 0.1—2 μg/ml propafenone and 0.5 μg of Li 6115. The data points for each concentration represent mean ± S.E. from six determinations except for 0.1 and 0.25 μg (n = 4). A good correlation was found between propafenone added to rabbit serum and propafenone found. A linear curve passing through the origin was obtained for the given concentrations. A similar curve (not shown) was obtained for spiked serum samples from control dogs.

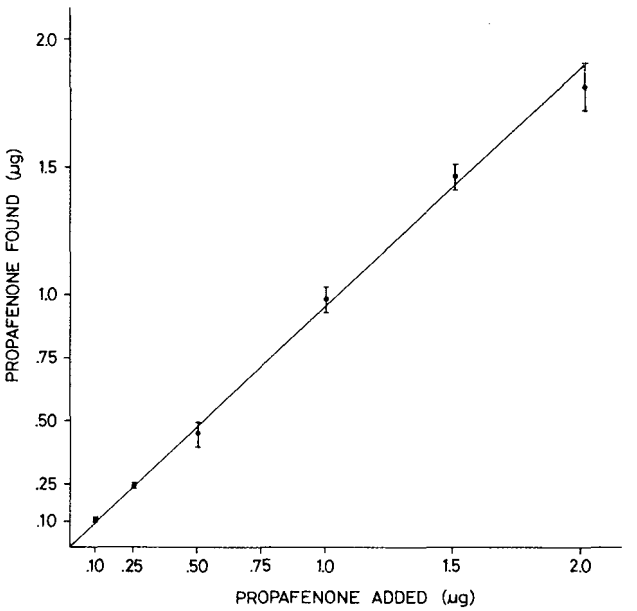

Fig. 2. Calibration curve for propafenone in rabbit serum. The data points represent mean ± S.E. from 4—6 determinations. Quantitation was done by measuring peak height ratios of propafenone to internal standard. Abscissa shows the amount of propafenone added to 1 ml rabbit serum and ordinate, amounts found by HPLC analysis.

The present method could quantitate up to 50 ng/ml propafenone with reasonable accuracy and the detection limit was 20 ng/ml. The intra- and inter-assay variations determined using spiked rabbit sera were 6.1% (n = 12) and 5.5% (n = 8), respectively.

The present method offers certain advantages over the recently published one of Brode et al. [4]. We have found that the Altex 5-μm normal-phase silica column gave better separations than the column used by previous authors [2, 4]. Separations were achieved using a methanol—ether mobile phase which is simple to prepare and which gives a steady baseline. In addition, this method has been successfully adapted for the analysis of tissue extracts such as from myocardial preparations. Experimental and clinical pharmacokinetics of propafenone are currently under study in our laboratory with the aid of this HPLC procedure for the quantitation of propafenone.

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Book Review

Plasma and cellular modulatory proteins, edited by D.H. Bing and R.A. Rosenbaum, Center for Blood Research Inc., Boston, MA, 1981, IX + 240 pp., price US$ 25.00.

The present volume summarizes the lectures presented at a conference held on 23—25 November, 1980, in Boston, MA. As indicated in the title, the conference dealt with blood proteins capable of stimulating cell growth: insulin-like growth factors, platelet-derived growth factor, factors controlling fibroblastic proliferation, factors controlling the growth of endothelial cells and muscle cells. Special chapters are devoted to proteolytic control of hormone function, characterization of thrombin and plasminogen activator binding sites, complement pathway proteins and characterization of glandular kallikreins.

From the mere listing of the problems discussed it is evident that solving such problems is based on a strong methodological background. The separation methods used for individual protein species are either directly described or appropriately referred to. In the main combinations (sometimes quite complex) of classical polyacrylamide gel electrophoresis and liquid column chromatography are dealt with. It is rather surprising that none of the contributors tried the more advanced separation procedures like high-resolution two-dimensional electrophoresis or modern chromatographic techniques. The volume is meticulously assembled and offers a good survey of the current separation and isolation procedures for the title proteins.

Prague (Czechoslovakia) Z. DEYL

Related articles published in *Journal of Chromatography*, Vols. 250—255

Volume 250

Analysis of haloperidol tablets by high-performance liquid chromatography — an inter-laboratory study
by A.R. Lea, D.M. Hailey and P.R. Duguid (Canberra City, Australia) 35

Titration curves by combined isoelectric focusing—electrophoresis on a thin layer of agarose gel
by C. Troungos, R. Krishnamoorthy, J. Elion and D. Labie (Paris, France) 73

Silica gel-impregnated paper chromatographic determination, by differential staining, of N-acyl lipids
by M.H. Hack (New Orleans, LA, U.S.A.) and F.M. Helmy (Dover, DE, U.S.A.) 80

Separation of prostaglandins and thromboxane B2 by high-resolution gas chromatography coupled to mass spectrometry or electron-capture detection
by C. Chiabrando, A. Noseda and R. Fanelli (Milan, Italy) 100

Separation of amino acids by charge-transfer interaction chromatography in aqueous systems
by K. Ishihara, T. Iida, N. Muramoto and I. Shinohara (Tokyo, Japan) 119

Detection of N-acetyl amino acids on paper and sugars on thin-layer chromatograms by a thermal—ultraviolet method
by M.S. Dubra, D.M. Alperin, A. Sagedahl, V.P. Idoyaga-Vargas and H. Carminatti (Buenos Aires, Argentina) 124

Separation of iodinated compounds of L-tyrosyl-L-tyrosine from iodothyronines by reversed-phase high-performance liquid chromatography
by J.C. Maurizis, C. Nicolas, J. Michelot and G. Meyniel (Clermont-Ferrand, France) 129

Separation of the enantiomers of (±)-norephedrine by rotation locular counter-current chromatography
by B. Domon and K. Hostettmann (Lausanne, Switzerland) and K. Kovačevic and V. Prelog (Zürich, Switzerland) 149

Isotachophoretic assay of aminoglycosides and lincomycins in pharmaceuticals
by H. Klein and R. Teichmann (Wiesbaden, G.F.R.) 152

Volume 251

Off-line combination of liquid chromatography and field desorption mass spectrometry: Principles and environmental, medical and pharmaceutical applications
by H.-R. Schulten (Bonn, G.F.R.) 105

Use of high-performance liquid chromatography—mass spectrometry for the study of the metabolism of ranitidine in man
by L.E. Martin, J. Oxford and R.J.N. Tanner (Ware, Great Britain) 215

Separation of steroid conjugates by high-performance liquid chromatography
by Sj. van der Wal (Tarrytown, NY, U.S.A.) and J.F.K. Huber (Vienna, Austria) 289

Volume 252

Comparison between binding analysis performed by equilibrium dialysis and partitioning in aqueous two-phase systems exemplified by the binding of Cibacron Blue to serum albumin
by T.G.I. Ling and B. Mattiasson (Lund, Sweden) 159

Single-column gas chromatographic analysis of amino acid oxazolidinones
by P. Hušek, V. Felt and M. Matucha (Prague, Czechoslovakia) 217

Determination of oxazolidine-2-thiones in biological fluids in the ppb range
by H.F. De Brabander and R. Verbeke (Ghent, Belgium) 225

Countercurrent chromatography of diaziquone by a horizontal flow-through coil planet centrifuge
by R.D. Friedman, H. Nakazawa, F.-T.E. Chou and N.R. Bachur (Baltimore, MD, U.S.A.) and Y. Ito (Bethesda, MD, U.S.A.) 283

Post-column air-segmentation reactor for the fluorometric detection of reserpine by liquid chromatography
by J.R. Lang, I.L. Honigberg and J.T. Stewart (Athens, GA, U.S.A.) 288

Identification and quantitation of 1-(2-pyrimidinyl)-piperazine, an active metabolite of the anxiolytic agent buspirone, in rat plasma and brain
by S. Caccia, S. Garattini, A. Mancinelli and M. Muglia (Milan, Italy) 310

Ethanol levels in postmortem body fluids
by R.D. Budd (Los Angeles, CA, U.S.A.) 315

Quantitative gas—liquid chromatographic determination of pemoline using trimethylanilinium hydroxide
by A.I. Krylov and N.S. Khlebnikova (Leningrad, U.S.S.R.). 319

Thin-layer chromatographic separation and quantitation of radioactively labelled 5-hydroxytryptamine, 5-hydroxytryptamine-O-sulfate, and 5-hydroxyindoleacetic acid
by D.D. Fay and J.L. Costa (Bethesda, MD, U.S.A.) and J. Launay (Paris, France). 338

Volume 253

Determination of urokinase activity by high-performance liquid chromatography with radioisotope detection
by T. Someno, K. Katoh, K. Niijima and H. Miyazaki (Tokyo, Japan) 81

Anion chromatography of carboxylic acids and keto acids using a hollow-fibre suppressor
by S. Rokushika, Z.L. Sun and H. Hatano (Kyoto, Japan). 87

Determination of optical isomers in diltiazem hydrochloride by high-performance liquid chromatography
by R. Shimizu, K. Ishii, N. Tsumagari, M. Tanigawa and M. Matsumoto (Osaka, Japan) and I.T. Harrison (Palo Alto, CA, U.S.A.). 101

Simple and rapid ion-pair high-performance liquid chromatographic assay for *p*-nitrophenyl glucuronide in rat-liver microsomal incubations
by M. Motohashi, H. Torii, T. Doi and S. Tanayama (Osaka, Japan) 129

Preparative separation of nucleosides and nucleotides on a non-ionic gel column
by S. Aoyagi, K. Hirayanagi, T. Yoshimura and T. Ishikawa (Chiba, Japan). 133

Evaluation of conventional and medium-performance anion exchange for the separation of proteins
by Y. Kato, K. Nakamura and T. Hashimoto (Yamaguchi, Japan) 219

Micro-bore high-performance liquid chromatography for the analysis of pharmaceutical compounds
by K. Tsuji and R.B. Binns (Kalamazoo, MI, U.S.A.). 227

Selective modification and purification of phosphopeptides for automated sequence analysis
by T.R. Soderling (Nashville, TN, U.S.A.) and K. Walsh (Seattle, WA, U.S.A.) 243

9-Anthryldiazomethane derivatives of prostaglandins for high-performance liquid chromatographic analysis
by M. Hatsumi, S.-I. Kimata and K. Hirosawa (Tokyo, Japan) 271

Dansylation and high-performance liquid chromatographic separation of phosphoserine, phosphothreonine and phosphotyrosine from ^{32}P-labeled protein hydrolyzates
by L.F. Congote (Montreal, Canada) 276

Volume 254

Liquid chromatography with crown ether-containing mobile phases. III. Retention of catecholamines and related compounds in reversed-phase high-performance liquid chromatography
by T. Nakagawa and A. Shibukawa (Kyoto, Japan) and T. Uno (Nishinomiya-shi, Japan). 27

Reversed-phase ion-pair chromatography of anthracyclines
by M.A. Van Lancker, H.J.C.F. Nelis and A.P. De Leenheer (Ghent, Belgium) 45

Prostaglandins and thromboxane: *tert.*-butyldimethylsilyl ethers as derivatives for their analyses by gas chromatography—mass spectrometry—selected ion monitoring
by J. Mai, B. German and J.E. Kinsella (Ithaca, NY, U.S.A.) 91

Chromatographic study of the reaction of mercury(II) and silver(I) ions with nickel dithiocarbamate complexes derived from some aromatic amine drugs
by G.K.C. Low, P.R. Haddad and A.M. Duffield (Kensington, Australia) 123

Simultaneous gas chromatographic analysis of drugs of abuse on two fused-silica columns of different polarities
by A. Alm, S. Jonson, H. Karlsson and E.G. Sundholm (Linköping, Sweden) 179

Gas—liquid chromatography of cholesteryl esters on non-polar and polar capillary columns following on-column injection
by N.B. Smith (London, Canada) 195

Gel filtration in sodium dodecylsulphate of hydrophobic muscle proteins on Sephacryl S-400 superfine
by V.V.A.M. Schreurs, H.A. Boekholt and R.E. Koopmanschap (Wageningen, The Netherlands). 203

Separation of myelin basic protein peptide 43—88 and its fragments by analytic and preparative high-performance liquid chromatography
by R.D. Gilliom, J.N. Withaker and J.M. Seyer (Memphis, TN, U.S.A.) 211

Rapid preparation and purification of alkaline phosphatase and ^{125}I-labeled antibody by minicolumn gel centrifugation chromatography
by A.J. Parkinson, E.N. Scott and H.G. Muchmore (Oklahoma City, OK, U.S.A.) 219

In situ quantitation of lipids by thin-layer chromatography using reflectance densitometry
by F.B. Banks (Liverpool, Great Britain). 247

Sensitive and specific radioenzymatic assay for norepinephrine, epinephrine and dopamine based on the thin-layer chromatographic separation of their Dns-O-methyl derivatives
by S.R. Philips and A.M. Robson (Saskatoon, Canada). 257

Relationship between R_M values and hydrophobicity. Chromatographic behaviour of crotonolactones
by K. Dadáková, J. Hartl and K. Waisser (Hradec Králové, Czechoslovakia) 277

Raman spectroscopic detection of haemoproteins in the eluate from high-performance liquid chromatography
by K. Iriyama, Y. Ozaki, K. Hibi and T. Ikeda (Tokyo, Japan) 285

Heterogeneity of human pituitary neurophysins by ampholyte displacement chromatography
by J.G. Verbalis (Pittsburgh, PA, U.S.A.) 309

Quantitative high-performance liquid chromatography of structurally similar nucleosides
by W. Schroeder, III, T.L. Cupps and L.B. Townsend (Ann Arbor, MI, U.S.A.). 315

Post-column oxidation of reduced biopterins for fluorescence detection
by K. Yazawa and Z. Tamura (Tokyo, Japan). 327

Volume 255

Retention behavior of amino acid enantiomers in reversed-phase liquid chromatography
by E. Grushka, R. Leshem and C. Gilon (Jerusalem, Israel) 41

High-performance liquid chromatography of amino acids, peptides and proteins. XXXIX. Reversed-phase high-performance liquid chromatographic analysis of Dns-amino acids: Comparison of several different elution systems
by B. Grego and M.T.W. Hearn (Fitzroy, Australia) 67

Automated determination of drugs in blood samples after enzymatic hydrolysis using precolumn switching and post-column reaction detection
by C.E. Werkhoven-Goewie, C. de Ruiter, U.A.Th. Brinkman and R.W. Frei (Amsterdam, The Netherlands), G.J. de Jong (Weesp, The Netherlands) and C.J. Little and O. Stahel (Ashford, Great Britain) 79

Structure—distribution relationships of radiopharmaceuticals. Correlation between the reversed-phase capacity factors for Tc-99m phenylcarbamoylmethyliminodiacetic acids and their renal elimination
by A.D. Nunn (New Brunswick, NJ, U.S.A.) 91

Effects of chain length and carbon load on the performance of alkyl-bonded silicas for protein separations
by N.H.C. Cooke and B.G. Archer (Berkeley, CA, U.S.A.), M.J. O'Hare (Sutton, Great Britain), E.C. Nice (Melbourne, Australia) and M. Capp (Iowa City, IA, U.S.A.) 115

High-performance liquid chromatography of amino acids, peptides and proteins. XL. Further studies on the role of the organic modifier in the reversed-phase high-performance liquid chromatography of polypeptides. Implications for gradient optimisation
by M.T.W. Hearn and B. Grego (Fitzroy, Australia) 125

Stationary phase effects on the reversed-phase liquid chromatographic retention behavior of purine and pyrimidine compounds
by M. Zakaria and P.R. Brown (Kingston, RI, U.S.A.) 151

Study on the rate of epimerization of amoxicillin β-penicilloic acid to its α-form in aqueous solutions using high-performance liquid chromatography
by G.W.K. Fong, R.N. Johnson and B.T. Kho (Rouses Point, NY, U.S.A.) 199

Quantitation of radiolabeled biological molecules separated by high-performance liquid chromatography
by M.J. Kessler (Houston, TX, U.S.A.) 209

Bioanalysis of cimetidine by high-performance liquid chromatography
by J.M. Bartlett and A.B. Segelman (Piscataway, NJ, U.S.A.) 239

Determination of conjugated compounds by liquid chromatography with electrochemical detection using post-column hydrolysis. Application to dopamine sulfate isomers
by M.A. Elchisak (West Lafayette, IN, U.S.A.) 475

Analysis of SK&F 82526 in plasma by high-performance liquid chromatography with electrochemical detection
by V.L. Osborne and W.C. Randolph (Philadelphia, PA, U.S.A.) 491

Simultaneous multiple electrode liquid chromatographic–electrochemical assay for catecholamines, indoleamines and metabolites in brain tissue
by G.S. Mayer and R.E. Shoup (West Lafayette, IN, U.S.A.) 533

Author Index

Aanonsen, L., see Verghese, C. 149
Amagaya, S., see Shimizu, K. 170
Andermann, G.
— and Dietz, M.
Dosage plasmatique et urinaire du 3,3,5-triméthylcyclohexanol-*cis* libre et conjugué 299
Arman, M.
— and Jamali, F.
High-performance liquid chromatographic determination of ethyl biscoumacetate in human plasma 406
Asada, H., see Niwa, T. 227
Assenza, S.P.
— , Brown, P.R. and Goldberg, A.P.
Isocratic reversed-phase liquid chromatographic separation of 3′,5′-cyclic ribonucleotides 373
Bain, R., see Brown, R.R. 351
Banes, A.J.
— , Yamauchi, M. and Moody, R.T.
Reversed-phase high-performance liquid chromatography method for separation of collagen tryptic peptides 336
Bareggi, S.R., see Zecca, L. 401
Bendt, J.S., see Tserng, K.-Y. 233
Bergqvist, Y.
— and Domeij-Nyberg, B.
Distribution of chloroquine and its metabolite desethyl-chloroquine in human blood cells and its implication for the quantitative determination of these compounds in serum and plasma 137
Bhat, P.V.
— and Lacroix, A.
Separation and estimation of retinyl fatty acyl esters in tissues of normal rat by high-performance liquid chromatography 269
Blain, Y., see Montay, G. 359
Bonini, L., see Zecca, L. 401
Bory, M., see Calaf, R. 385
Bots, A.M.B.
— , Van Oort, W.J., Noordhoek, J., Van Dijk, A., Klein, S.W. and Van Hoesel, Q.G.C.M.
Analysis of adriamycin and adriamycinol in micro volumes of rat plasma 421
Bouquet des Chaux, J.-L., see Vink, J. 87
Braun, D.G., see Rosenfelder, G. 21
Brittain, A.T., see Hoppel, C.L. 43
Brown, F.M., see Hoppel, C.L. 43
Brown, P.R., see Assenza, S.P. 373
Brown, R.R.
— , Bain, R. and Jordan, V.C.
Determination of tamoxifen and metabolites in human serum by high-performance liquid chromatography with post-column fluorescence activation 351
Buckingham, W., see Phipps, J.A. 392
Bury, R.W., see Kacprowicz, A.T. 417
Calaf, R.
— , Marie, P., Ghiglione, Cl., Bory, M. and Reynaud, J.
Dosage du diltiazem plasmatique par chromatographie en phase gazeuse 385
Campos Flor, S.
Determination of the calcium antagonist flunarizine in biological fluids by gas—liquid chromatography 315
Caradonna, P., see Castagnola, M. 51
Castagnola, M.
— , Caradonna, P., Salvi, M.L. and Rossetti, D.
Investigation of the heterogeneity of hemoglobin by cation-exchange chromatography on Bio-Rex 70 51
Chan, K.Y.
— , Lang, J.F. and Okerholm, R.A.
Quantitative determination of cardiotonic agent MDL 17,043 in plasma by reversed-phase high-performance liquid chromatography 396
Chang, J.-Y., see Rosenfelder, G. 21
Chang, S.F.
— , Welscher, T.M., Miller, A.M. and Ober, R.E.
High-performance liquid chromatography method for the quantitation of flecainide, a new antiarrhythmic, in human plasma and urine 341
Chang, T., see Hayes, A.G. 176
Chung, T.G., see Takagi, T. 279
Cook, N.J.
— and Jowett, D.A.
Determination of valproic acid in human serum by gas—liquid chromatography on OV-17 using nitrogen-specific detection 181
Danish, R.K., see Tserng, K.-Y. 233
Davis, C.M.
— and Fenimore, D.C.

Determination of fluphenazine in plasma by high-performance thin-layer chromatography 157
Dietz, M., see Andermann, G. 299
Dijk, A. van, see Bots, A.M.B. 421
Domeij-Nyberg, B., see Bergqvist, Y. 137
Farrell, B.M.
— and Jefferies, T.M.
An investigation of high-performance liquid chromatographic methods for the analysis of amphetamines 111
Fenimore, D.C., see Davis, C.M. 157
Flor, S. Campos, see Campos Flor, S. 315
Ghiglione, Cl., see Calaf, R. 385
Gibbons, A.P., see Hoppel, C.L. 43
Goldberg, A.P., see Assenza, S.P. 373
Grindel, J.M.
— , Hills, J.F. and Renzi, N.L.
Reversed-phase high-performance liquid chromatographic assay for zomepirac in urine 210
Hal, H.J.M. van, see Vink, J. 87
Hayes, A.G.
— and Chang, T.
Determination of indeloxazine, a new antidepressant agent, in human plasma by gas—liquid chromatography with electron-capture detection 176
Hersh, E.M., see Rosenblum, M.G. 200
Hills, J.F., see Grindel, J.M. 210
Hir, A. le, see Montay, G. 359
Hisamura, M., see Kaji, H. 166
Hoesel, Q.G.C.M. van, see Bots, A.M.B. 421
Hogan, E.L., see Traylor, T.D. 9
Honda, S., see Kakehi, K. 1
Hoppel, C.L.
— , Weir, D.E., Gibbons, A.P., Ingalls, S.T., Brittain, A.T. and Brown, F.M.
Determination of 6-N-trimethyllysine in urine by high-performance liquid chromatography 43
Ikeda, M.
— , Shimada, K. and Sakaguchi, T.
High-performance liquid chromatographic determination of free fatty acids with 1-naphthylamine 251
Ingalls, S.T., see Hoppel, C.L. 43
Ishimoto, M., see Kaji, H. 166
Jamali, F., see Arman, M. 406
Jefferies, T.M., see Farrell, B.M. 111
Jordan, V.C., see Brown, R.R. 351
Jowett, D.A., see Cook, N.J. 181
Jung, G., see Kuhnz, W. 333
Kacprowicz, A.T.
— , Shaw, P.G., Moulds, R.F.W. and Bury, R.W.
Determination of ketanserin in human plasma by high-performance liquid chromatography 417
Kågedal, B.
— and Pettersson, A.
Determination of urinary 5-S-cysteinyldopa by high-performance liquid chromatography 287
Kaji, H.
— , Saito, K., Saito, N., Hisamura, M., Ishimoto, M. and Kondo, H.
Simple gas chromatographic analysis of 3-methylthiopropionate in human urine 166
Kakehi, K.
— , Maeda, K., Teramae, M., Honda, S. and Takai, T.
Analysis of sialic acids by gas chromatography of the mannosamine derivatives released by the action of N-acetylneuraminate lyase 1
Kamada, S.
— , Maeda, M. and Tsuji, A.
Fluorescence high-performance liquid chromatographic determination of free and conjugated bile acids in serum and bile using 1-bromoacetylpyrene as a pre-labeling reagent 29
Kannan, R.
— , Tidwell, D. and Singh, B.N.
High-performance liquid chromatographic procedure for the quantitation of propafenone in serum and tissues 428
Kawahara, K.
— and Ofuji, T.
Gas chromatographic determination of the glucuronide of 2-(1-hydroxyethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)-benzofuran, a metabolite of befunolol, in human urine 187
Kawanishi, A., see Niwa, T. 227
Kawasaki, T.
— , Maeda, M. and Tsuji, A.
Immobilized 3α-hydroxysteroid dehydrogenase and dansyl hydrazine as a pre-labeling reagent for high-performance liquid chromatography with fluorescence detection of bile acids 261
Kennedy, S., see Maddock, J. 129
Kim, C.
Simple and sensitive method for the determination of propylthiouracil

in blood by high-performance liquid chromatography 376
Kinoshita, H., see Tanaka, E. 380
Klein, S.W., see Bots, A.M.B. 421
Klotz, U., see Okonkwo, P.O. 411
Kondo, H., see Kaji, H. 166
Koontz, D.A., see Traylor, T.D. 9
Kuhnz, W.
— , Zech, K., Lupp, R., Jung, G., Voelter, W. and Matzkies, F.
Quantitative determination of O-(β-hydroxyethyl)-rutosides in serum by high-performance liquid chromatography 333
Kuroiwa, Y., see Tanaka, E. 380
Kuye, J.O.
— , Wilson, M.J. and Walle, T.
Gas chromatographic analysis of chloroquine after a unique reaction with chloroformates 307
Lacroix, A., see Bhat, P.V. 269
Lamiable, D.
— and Millart, H.
High-performance liquid chromatographic determination of 4-aminopyridine and 3,4-diaminopyridine in rat cerebrospinal fluid and serum 221
Lang, J.F., see Chan, K.Y. 396
Le Hir, A., see Montay, G. 359
Lennard, M.S.
— and Silas, J.H.
Rapid determination of metoprolol and α-hydroxymetoprolol in human plasma and urine by high-performance liquid chromatography 205
Leroux, F., see Sioufi, A. 103
Lewis, P.A., see Maddock, J. 129
Loo, T.L., see Rosenblum, M.G. 200
Lupp, R., see Kuhnz, W. 333
Maddock, J.
— , Lewis, P.A., Woodward, A., Massey, P.R. and Kennedy, S.
Determination of isosorbide dinitrate and its mononitrate metabolites in human plasma by high-performance liquid chromatography—thermal energy analysis 129
Maeda, K., see Kakehi, K. 1
— , see Niwa, T. 227
Maeda, M., see Kamada, S. 29
— , see Kawasaki, T. 261
Mahuzier, G., see Prognon, P. 193
Marie, P., see Calaf, R. 385
Marzo, A.
— , Quadro, G. and Treffner, E.
Gas—liquid chromatographic evaluation of fenquizone in biological samples for pharmacokinetic investigations 95
Massey, P.R., see Maddock, J. 129
Matzkies, F., see Kuhnz, W. 333
Maurer, H.
— and Pfleger, K.
Screening procedure for detecting butyrophenone and bisfluorophenyl neuroleptics in urine using a computerized gas chromatographic—mass spectrometric technique 75
Millart, H., see Lamiable, D. 221
Miller, A.M., see Chang, S.F. 341
Montay, G.
— , Blain, Y., Roquet, F. and Le Hir, A.
High-performance liquid chromatography of pefloxacin and its main active metabolites in biological fluids 359
Moody, R.T., see Banes, A.J. 366
Moulds, R.F.W., see Kacprowicz, A.T. 417
Niwa, T.
— , Yamamoto, N., Asada, H., Kawanishi, A., Yokoyama, M., Maeda, K. and Ohki, T.
Ischemic change of organic acids in kidney 227
Noordhoek, J., see Bots, A.M.B. 421
Ober, R.E., see Chang, S.F. 341
Ofuji, T., see Kawahara, K. 187
Ogihara, Y., see Shimizu, K. 170
Ohki, T., see Niwa, T. 227
Ohya, K., see Takasu, A. 243
Okerholm, R.A., see Chan, K.Y. 396
Okonkwo, P.O.
— , Reimann, I.W., Woestenborghs, R. and Klotz, U.
High-performance liquid chromatographic assay with fluorometric detection of ketanserin, a new antihypertensive agent and serotonin S_2 antagonist in human plasma, blood and urine 411
Oort, W.J. van, see Bots, A.M.B. 421
Pettersson, A., see Kågedal, B. 287
Pfleger, K., see Maurer, H. 75
Phipps, J.A.
— , Sabourin, M.A., Buckingham, W. and Strunin, L.
Detection of picogram concentrations of fentanyl in plasma by gas—liquid chromatography 392
Pognat, J.-F., see Vink, J. 87
Pritchett, E.L.C., see Verghese, C. 149
Prognon, P.

— , Simon, G. and Mahuzier, G.
Dosage du méthoxy-5 psoralène dans le plasma par chromatographie en phase liquide et détection spectrofluorimétrique 193
Quadro, G., see Marzo, A. 95
Reidl, U.
Determination of acetylsalicylic acid and metabolites in biological fluids by high-performance liquid chromatography 325
Reimann, I.W., see Okonkwo, P.O. 411
Renzi, N.L., see Grindel, J.M. 210
Reynaud, J., see Calaf, R. 385
Roquet, F., see Montay, G. 359
Rosenblum, M.G.
— , Hersh, E.M. and Loo, T.L.
Determination of the biological response modifier MVE-2 (AD-022) in biological fluids by high-performance liquid chromatography 200
Rosenfelder, G.
— , Chang, J.-Y. and Braun, D.G.
Sphingosine determination at the picomole level using dimethylaminoazobenzene sulphonyl chloride 21
Rossetti, D., see Castagnola, M. 51
Sabourin, M.A., see Phipps, J.A. 392
Saito, A., see Takagi, T. 279
Saito, K., see Kaji, H. 166
Saito, N., see Kaji, H. 166
Sakaguchi, T., see Ikeda, M. 251
Salvi, M.L., see Castagnola, M. 51
Sandman, R.
Determination of 4-methylumbelliferone after separation from its conjugates by high-performance liquid chromatography. Application to lysosomal enzyme activity assays 67
Sandrenan, N., see Sioufi, A. 103
Sano, M., see Takasu, A. 243
Shand, D.G., see Verghese, C. 149
Shaw, P.G., see Kacprowicz, A.T. 417
Shimada, K., see Ikeda, M. 251
Shimizu, K.
— , Amagaya, S. and Ogihara, Y.
Analysis of corticosterone in the serum of mice and rats using high-performance liquid chromatography 170
Silas, J.H., see Lennard, M.S. 205
Simon, G., see Prognon, P. 193
Singh, B.N., see Kannan, R. 428
Sioufi, A.
— , Leroux, F. and Sandrenan, N.
Gas chromatographic determination of metoprolol in human plasma 103
Smith, M.S., see Verghese, C. 149
Strunin, L., see Phipps, J.A. 392
Takagi, T.
— , Chung, T.G. and Saito, A.
Determination of polyamines in hydrolysates of uremic plasma by high-performance cation-exchange column chromatography 279
Takai, T., see Kakehi, K. 1
Takasu, A.
— , Ohya, K. and Sano, M.
Determination of 20α-hydroxy-9β,10α-pregna-4,6-dien-3-one in plasma by selected ion monitoring 243
Tanaka, E.
— , Kinoshita, H., Yoshida, T. and Kuroiwa, Y.
Determination of trimethadione and its metabolite by gas chromatography with flame-thermionic detection and its application to pharmacokinetic studies of the drug in carbon tetrachloride-treated rats 380
Teramae, M., see Kakehi, K. 1
Tidwell, D., see Kannan, R. 428
Traylor, T.D.
— , Koontz, D.A. and Hogan, E.L.
High-performance liquid chromatographic resolution of *p*-nitrobenzyloxyamine derivatives of brain gangliosides 9
Treffner, E., see Marzo, A. 95
Tserng, K.-Y.
— , Danish, R.K. and Bendt, J.S.
Rapid simultaneous assay of serum estrone, estradiol, and estriol in pregnant women using methyl ether acetate derivatives by capillary gas chromatography and electron-impact mass spectrometry 233
Tsuji, A., see Kamada, S. 29
— , see Kawasaki, T. 261
Van Dijk, A., see Bots, A.M.B. 421
Van Hal, H.J.M., see Vink, J. 87
Van Hoesel, Q.G.C.M., see Bots, A.M.B. 421
Van Oort, W.J., see Bots, A.M.B. 421
Verghese, C.
— , Smith, M.S., Aanonsen, L., Pritchett, E.L.C. and Shand, D.G.
High-performance liquid chromatographic analysis of diltiazem and its metabolite in plasma 149
Vink, J.
— , Van Hal, H.J.M., Pognat, J.-F. and Bouquet des Chaux, J.-L.
Determination of the anti-ischaemic drug bepridil in human plasma using

gas chromatography with nitrogen-sensitive detection 87
Voelter, W., see Kuhnz, W. 333
Walle, T., see Kuye, J.O. 307
Weir, D.E., see Hoppel, C.L. 43
Welscher, T.M., see Chang, S.F. 341
Whelpton, R.
Analysis of plasma physostigmine concentrations by liquid chromatography 216
Wilson, M.J., see Kuye, J.O. 307
Woestenborghs, R., see Okonkwo, P.O. 411
Woodward, A., see Maddock, J. 129
Yamamoto, N., see Niwa, T. 227
Yamauchi, M., see Banes, A.J. 366
Yokoyama, M., see Niwa, T. 227
Yoshida, T., see Tanaka, E. 380
Zecca, L.
— , Bonini, L. and Bareggi, S.R.
Determination of dihydroergocristine and dihydroergotamine in plasma by high-performance liquid chromatography with fluorescence detection 401
Zech, K., see Kuhnz, W. 333

Subject Index

Acetylsalicylic acid
Determination of acetylsalicylic acid and metabolites in biological fluids by HPLC 325
Adriamycin
Analysis of adriamycin and adriamycinol in micro volumes of rat plasma 421
Adriamycinol
Analysis of adriamycin and adriamycinol in micro volumes of rat plasma 421
Amino acids
Determination of 6-N-trimethyllysine in urine by HPLC 43
Amino acids
Determination of urinary 5-S-cysteinyldopa by HPLC 287
4-Aminopyridine
HPLC determination of 4-aminopyridine and 3,4-diaminopyridine in rat cerebrospinal fluid and serum 221
Amphetamines
An investigation of HPLC methods for the analysis of amphetamines 111
Befunolol metabolite
GC determination of the glucuronide of 2-(1-hydroxy-ethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)-benzofuran, a metabolite of befunolol, in human urine 187
Bepridil
Determination of the anti-ischaemic drug bepridil in human plasma using GC with nitrogen-sensitive detection 87
Bile acids
Fluorescence HPLC determination of free and conjugated bile acids in serum and bile using 1-bromoacetylpyrene as a pre-labeling reagent 29
Bile acids
Immobilized 3α-hydroxysteroid dehydrogenase and dansyl hydrazine as a pre-labeling reagent for HPLC with fluorescence detection of bile acids 261
Butyrophenone
Screening procedure for detecting butyrophenone and bisfluorophenyl neuroleptics in urine using a computerized GC—MS technique 75
Chloroquine
Distribution of chloroquine and its metabolite desethyl-chloroquine in human blood cells and its implication for the quantitative determination of these compounds in serum and plasma 137
Chloroquine
GC analysis of chloroquine after a unique reaction with chloroformates 307
Corticosterone
Analysis of corticosterone in the serum of mice and rats using HPLC 170
3′,5′-Cyclic ribonucleotides
Isocratic reversed-phase LC separation of 3′,5′-cyclic ribonucleotides 373
5-S-Cysteinyldopa
Determination of urinary 5-S-cysteinyldopa by HPLC 287
Desethyl-chloroquine
Distribution of chloroquine and its metabolite desethyl-chloroquine in human blood cells and its implication for the quantitative determination of these compounds in serum and plasma 137
3,4-Diaminopyridine
HPLC determination of 4-aminopyridine and 3,4-diaminopyridine in rat cerebrospinal fluid and serum 221
Dihydroergocristine
Determination of dihydroergocristine and dihydroergotamine in plasma by HPLC with fluorescence detection 401
Dihydroergotamine
Determination of dihydroergocristine and dihydroergotamine in plasma by HPLC with fluorescence detection 401
1,3-Dihydro-4-methyl-5-[4-(methylthio)-benzoyl]-2H-imidazol-2-one
Quantitative determination of cardiotonic agent MDL 17,043 in plasma by reversed-phase HPLC 396
Diltiazem
Quantitative determination of diltiazem in plasma by GC 385
Diltiazem
HPLC analysis of diltiazem and its metabolite in plasma 149
5,5-Dimethyl-2,4-oxazolidinedione

Determination of trimethadione and its metabolite by GC with flame-thermionic detection and its application to pharmacokinetic studies of the drug in carbon tetrachloride--treated rats 380
Dydrogesterone
Determination of 20α-hydroxy-9α,10β-pregna-4,6-dien-3-one in plasma by selected ion monitoring 243
Ergot derivatives
Determination of dihydroergocristine and dihydroergotamine in plasma by HPLC with fluorescence detection 401
Estradiol
Rapid simultaneous assay of serum estrone, estradiol, and estriol in pregnant women using methyl ether acetate derivatives by capillary GC and electron-impact MS 233
Estriol
Rapid simultaneous assay of serum estrone, estradiol, and estriol in pregnant women using methyl ether acetate derivatives by capillary GC and electron-impact MS 233
Estrone
Rapid simultaneous assay of serum estrone, estradiol, and estriol in pregnant women using methyl ether acetate derivatives by capillary GC and electron-impact MS 233
Ethyl biscoumacetate
HPLC determination of ethyl biscoumacetate in human plasma 406
Fatty acids
HPLC determination of free fatty acids with 1-naphthylamine 251
Fenquizone
GLC evaluation of fenquizone in biological samples for pharmacokinetic investigations 95
Fentanyl
Detection of picogram concentrations of fentanyl in plasma by GLC 392
Flavonoids
Quantitative determination of O-(β-hydroxyethyl)-rutosides in serum by HPLC 333
Flecainide
HPLC method for the quantitation of flecainide, a new antiarrhythmic, in human plasma and urine 341
Flunarizine
Determination of the calcium antagonist flunarizine in biological fluids by GLC 315
Fluphenazine
Determination of fluphenazine in plasma by HPTLC 157
Gangliosides
HPLC resolution of *p*-nitrobenzyloxyamine derivatives of brain gangliosides 9
Gentisic acid
Determination of acetylsalicylic acid and metabolites in biological fluids by HPLC 325
Hemoglobins
Investigation of the heterogeneity of hemoglobin by cation-exchange chromatography on Bio-Rex 70 51
2-(1-Hydroxy-ethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)-benzofuran
GC determination of the glucuronide of 2-(1-hydroxy-ethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)-benzofuran, a metabolite of befunolol, in human urine 187
O-(β-Hydroxyethyl)-rutosides
Quantitative determination of O-(β-hydroxyethyl)-rutosides in serum by HPLC 333
α-Hydroxymetoprolol
Rapid determination of metoprolol and α-hydroxymetoprolol in human plasma and urine by HPLC 205
20α-Hydroxy-9β,10α-pregna-4,6-dien-3-one
Determination of 20α-hydroxy-9α-10β-pregna-4,6-dien-3-one in plasma by selected ion monitoring 243
Indeloxazine
Determination of indeloxazine, a new antidepressant agent, in human plasma by GLC with electron-capture detection 176
Isosorbide dinitrate
Determination of isosorbide dinitrate and its mononitrate metabolites in human plasma by HPLC—thermal energy analysis 129
Ketanserin
Determination of ketanserin in human plasma by HPLC 417
Ketanserin
HPLC assay with fluorometric detection of ketanserin, a new antihypertensive agent and serotonin S_2 antagonist in human plasma, blood and urine 411
Lysosomal enzymes
Determination of 4-methylumbelliferone after separation from its conjugates by HPLC. Application

to lysosomal enzyme activity assays 67

Maleic vinyl ether copolymers
Determination of the biological response modifier MVE-2 (AD-022) in biological fluids by HPLC 200

MDL 17,043
Quantitative determination of cardiotonic agent MDL 17,043 in plasma by reversed-phase HPLC 396

3-Methylthiopropionate
Simple GC analysis of 3-methylthiopropionate in human urine 166

4-Methylumbelliferone
Determination of 4-methylumbelliferone after separation from its conjugates by HPLC. Application to lysosomal enzyme activity assays 67

D-Methionine
Simple GC analysis of 3-methylthiopropionate in human urine 166

5-Methoxypsoralene
Dosage of 5-methoxypsoralene in plasma by LC and spectrofluorometric detection 193

Metoprolol
GC determination of metoprolol in human plasma 103

Metoprolol
Rapid determination of metoprolol and α-metoprolol in human plasma and urine by HPLC 205

Neuroleptics
Screening procedure for detecting butyrophenone and bisfluorophenyl neuroleptics in urine using a computerized GC—MS technique 75

Nucleotides
Isocratic reversed-phase LC separation of 3′,5′-cyclic ribonucleotides 373

Organic acids
Ischemic change of organic acids in kidney 227

Pefloxacin
HPLC of pefloxacin and its main active metabolites in biological fluids 359

Peptides, collagen tryptic
Reversed-phase HPLC method for separation of collagen tryptic peptides 366

Physostigmine
Analysis of plasma physostigmine concentrations by LC 216

Polyamines
Determination of polyamines in hydrolysates of uremic plasma by high-performance cation-exchange column chromatography 279

Propafenone
HPLC procedure for the quantitation of propafenone in serum and tissues 428

Propylthiouracil
Simple and sensitive method for the determination of propylthiouracil in blood by HPLC 376

Retinyl fatty acyl esters
Separation and estimation of retinyl fatty acyl esters in tissues of normal rat by HPLC 269

Salicylic acid
Determination of acetylsalicylic acid and metabolites in biological fluids by HPLC 325

Salicyluric acid
Determination of acetylsalicylic acid and metabolites in biological fluids by HPLC 325

Sialic acids
Analysis of sialic acids by GC of the mannosamine derivatives released by the action of N-acetylneuraminate lyase 1

Sphingosine
Sphingosine determination at the picomole level using dimethylaminoazobenzene sulphonyl chloride 21

Steroids
Analysis of corticosterone in the serum of mice and rats using HPLC 170

Steroids
Determination of 20α-hydroxy-9β,10α-pregna-4,6-dien-3-one in plasma by selected ion monitoring 243

Steroids
Rapid simultaneous assay of serum estrone, estradiol, and estriol in pregnant women using methyl ether acetate derivatives by capillary GC and electron-impact MS 233

Tamoxifen
Determination of tamoxifen and metabolites in human serum by HPLC with post-column fluorescence activation 351

Trimethadione
Determination of trimethadione and its metabolite by GC with flame-thermionic detection and its application to pharmacokinetic studies of the drug in carbon tetrachloride-

treated rats 380
cis-3,3,5-Trimethylcyclohexanol
Determination of free and conjugated *cis*-3,3,5-trimethylcyclohexanol in plasma and urine 299
6-N-Trimethyllysine
Determination of 6-N-trimethyllysine in urine by HPLC 43
Valproic acid
Determination of valproic acid in human serum by GLC on OV-17 using nitrogen-specific detection 181
Zomepirac
Reversed-phase HPLC assay for zomepirac in urine 210

Vol. 272, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

ANNOUNCEMENTS OF AWARDS AND PRIZES

DAL NOGARE AWARD

The Chromatography Forum of the Delaware Valley will present the 1983 Dal Nogare Award to John H. Knox at the 1983 Pittsburgh Conference in Atlantic City, NJ, U.S.A.

Dr. Knox was born in Edinburgh, Scotland, on October 21, 1927. In 1949 he received a B.S. Degree with honors from the University of Edinburgh and in 1953 a Ph.D. Degree in Chemistry from Cambridge. His thesis under the supervision of Professor R.G.W. Norrish was entitled "The Cool Flame Oxidation of Propane." In 1963 he received a D.Sc. Degree from the University of Edinburgh with a thesis entitled "The Kinetics and Mechanism of Oxidation and Halogenation Reactions in the Gas Phase."

Dr. Knox has had numerous appointments at the University of Edinburgh where he is currently personal Chair in Physical Chemistry and Director of the Wolfson Liquid Chromatography Unit. He has served as a consultant in gas chromatography to Bruce Peebles Ltd. and Beckman Instruments Ltd. and in high-performance liquid chromatography to the Du Pont Company, Rank Hilger, Shandon Southern Instruments and Kratos Scientific Instruments.

He has co-authored over 116 scientific publications and several books. Dr. Knox has received the Chemical Society Award for Chemical Analysis and Instrumentation sponsored by Perkin Elmer Ltd. and the Tswett 75th Anniversary Medal (Tallin, U.S.S.R.). His early work involved the application of liquid chromatography to the separation of key aldehyde intermediates, and gas chromatography to chlorination and combustion reactions, as well as gas kinetics. His interest was then drawn more to liquid chromatography where he worked with the development of spherical silica gel and several bonded derivatives. Current work at the Wolfson Unit involves the production of novel forms of silica gel and porous glassy carbon while his pure research interests concern the understanding and exploitation of electrophoresis and endosmotically generated chromatography.

BIOCHEMICAL ANALYSIS PRIZE 1984

The German Society for Clinical Chemistry awards the Biochemical Analysis Prize every two years at the Biochemische Analytik Conference in Munich, G.F.R. The prize of DM 10.000,- - is donated by Boehringer Mannheim GmbH for outstanding and novel work in the field of biochemical analysis or biochemical instrumentation or for significant contributions to advancement in experimental biology especially relating to clinical biochemistry. Competitors for the 1984 prize (to be awarded at the 1984 conference, which will take place on April 10–13, 1984) should submit papers concerning one theme (either published or accepted for publication between Oct. 1, 1981, and Sept. 30, 1983) before November 15, 1983, to: Prof. Dr. Dr. I. Trautschold, Secretary of the Biochemical Analysis Prize, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, 3000 Hannover 61, G.F.R.

MEETINGS

20th ANNIVERSARY – INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY

The 20th Anniversary of the International Symposium on Advances in Chromatography will be held on Oct. 3–6, 1983, in Amsterdam, The Netherlands. The scope of the meeting will cover papers and informal discussion groups by outstanding researchers from throughout the world in all fields of chromatography. In particular, new developments in gas, liquid, and high-performance thin-layer chromatography will be included. There will also be a commercial exhibition of the latest instrumentation and books. Participation in the symposium will be on the basis of invited papers as well as unsolicited contributions. Authors desiring to present papers must submit 200-word abstracts by April 1, 1983. Complete manuscripts of accepted authors will be due on Oct. 3, 1983, at the meeting in Amsterdam. Special separate intensive two-day short courses on high resolution capillary columns, HPLC, GC–MS, and computer chromatography will be available on Friday and Saturday, Sept. 30 and Oct. 1, just prior to the meeting. All correspondence pertaining to the symposium, short courses, and exhibition space should be directed to: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.

8th INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY

The 8th International Symposium on Column Liquid Chromatography will be held in New York City at the New York Statler Hotel on May 20–26, 1984. This will be the eighth of a symposium series that deals with recent developments in high-performance liquid chromatography and related techniques. These symposia are organized annually and alternately in the United States and a European country.

The scientific program of the symposium will consist of invited and contributed lectures and posters. Emphasis will be placed on the fundamentals and applications of HPLC in various areas of the life sciences and in industry. An exhibition of modern liquid chromatographic equipment and accessories will be held concurrently with the symposium.

Requests for the first circular and inquiries should be directed to the Chairman of the symposium, Professor Cs. Horváth, Yale University, Department of Chemical Engineering, P.O. Box 2159, Yale Station, New Haven, CT 06520, U.S.A. Tel: (203) 436-1271.

4th INTERNATIONAL SYMPOSIUM ON ISOTACHOPHORESIS – ITP 84

The above-mentioned symposium will be held on Sept. 2–5, 1984, at the Pharmaceutical Faculty of the Charles University, Hradec Králové, Czechoslovakia, under the auspices of the Czechoslovak Chemical Society, the Institute of Organic Chemistry and Biochemistry and Institute of Analytical Chemistry of the Czechoslovak Academy of Sciences, the Faculty of Natural Sciences of the Charles University and the Chemical Institute of the University of Komenský.

The scientific program will comprise plenary lectures and communicated papers and/or posters on the fundamental aspects of isotachophoresis and related methods, their application in biochemical, clinical, environmental, pharmaceutical, physical and industrial (agriculture, food industry, etc.) fields. An exhibition of isotachophoretic equipment will be included. In addition, an intensive course on isotachophoresis, covering fundamental and practical aspects, will be held during the symposium.

The deadline for registration is February 1, 1984. For further information contact: ITP 84, Dr. Z. Prusik, C.Sc., Institute of Organic Chemistry and Biochemistry ČSAV, Flemingovo nám. 2, CS-166 10 Praha 6, Czechoslovakia.

NEW BOOKS

Progress in medicinal chemistry, Vol. 19, edited by G.P. Ellis and G.B. West, Elsevier Biomedical Press, Amsterdam, New York, 1982, 353 pp., price US$ 93.50 (U.S.A. and Canada), Dfl. 220.00 (rest of world), ISBN 0-444-80415-3.

Immunopharmacology, edited by P. Sirois and M. Rola-Pleszczynski, Elsevier Biomedical Press, Amsterdam, New York, 1982, 836 pp., price US$ 97.75 (U.S.A. and Canada), Dfl. 230.00 (rest of world), ISBN 0-444-80416-1.

NEW PRODUCTS

N-1700

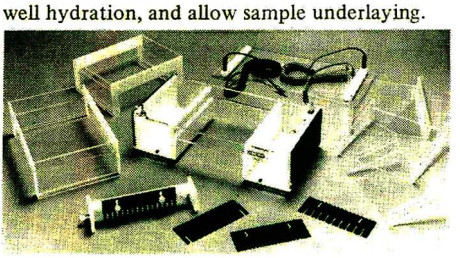
NEW ELECTROPHORESIS CHAMBER

Shandon have modified their Model 600 Electrophoresis Chamber with a Cellulose Acetate Electrophoresis System. The C.A.M. system is designed for simple operation so that laboratory staff can produce readable separations of serum proteins and haemoglobins with a minimum of training and practice. A feature of the system is the tensioning device for the Cellulose Acetate Membrane. This tensions the strip uniformly without variation between the strips. The C.A.M. systems can be used with existing 600 Electrophoresis Chambers.

N-1703

ELECTROPHORESIS CELL

The Bio-Rad Sub-Cell™ System is a horizontal DNA electrophoresis cell which is said to separate DNA restriction fragments and other nucleic acids efficiently. Although the cell is primarily meant for simple electrophoresis of submersible gels, it may also be used with agarose gel bridges. Submarine gels are easily cast, and dissipate heat which could lead to band distortion. The gels prevent the electrical field discontinuities caused by wicks or sample well hydration, and allow sample underlaying.

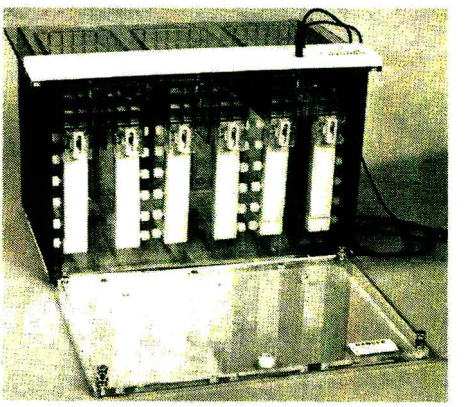


Agarose bridges are useful for casting the soft gels required for separating high-molecular-weight DNA, or for casting thicker preparative gels requiring high currents. Agarose gels are more conductive than paper wicks and prevent field strength discontinuities caused by uneven wetting.

N-1707

TWO-DIMENSIONAL ELECTROPHORESIS CELL

Bio-Rad's Multiple Gel Protean™ Cell offers the same convenience as the Dual Slab Cell, but is capable of running up to twelve 16 × 18 cm gels simultaneously under identical condi-

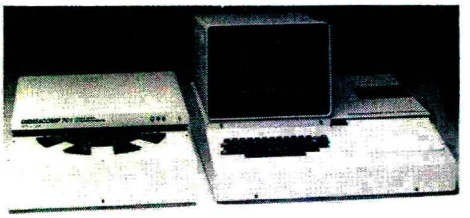


tions. Designed to meet the requirements of Anderson and Anderson ISO-DALT applications, the standard cell comes with enough spacers, plates, and accessories to run up to 12 two-dimensional slab gels. Three upper buffer chambers, which fit into a single large lower chamber, are used both to cast the gels and to support them during the electrophoresis. The large volume of the lower buffer is adequate for cooling two-dimensional SDS gels, so that no heat exchangers are needed.

N-1701

DENSITOMETER

Carlo Erba Strumentazione has introduced the Densicomb 701 Optical Reader and Data Processor for the automatic evaluation of electrophoresis strips. The instrument incorporates a numbered, eight-segment automatic sample charger, which can handle up to 64 serum pro-



teins in micro. The Optical Reader evaluates the strips in transmittance and may be used with all known electrophoresis support materials. The Densicomb 701 is compatible with most of the micro-computers used in laboratories nowadays. A video display allows the operator to decide the best parameters to quantify the strip.

N-1722

PREABSORBENT, CHANNELLED HPTLC PLATES

The Whatman LHP-KD/LHP-KDF plates incorporate several new features, making them very attractive in a wide variety of HPTLC-applications. The suffix 'F' indicates that a fluorescence indicator has been incorporated in the analytical layer. These plates belong to the Whatman Linear-K class of TLC plates, i.e., the surface incorporates a special inert preadsorbent strip 2.0-cm wide along the spotting edge contiguous with the analytical area. This makes it possible to use HPTLC without the need for special spotting apparatus; a conventional microcap is all that is required. This is a major advantage when larger volumes must be applied. The plates accept crude analytes; prepurification is often unnecessary.

N-1723

HPTLC PLATES

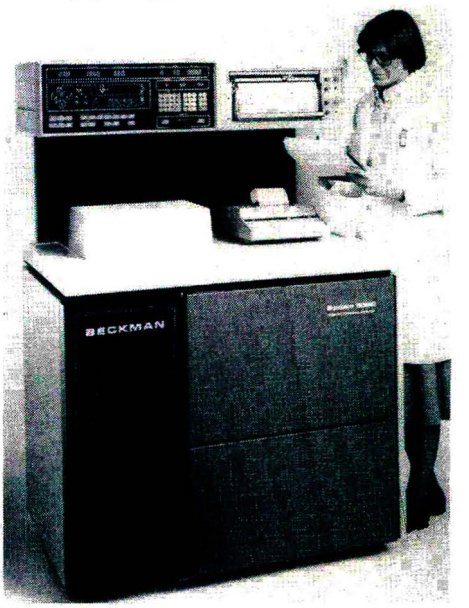
Whatman introduces the new type HP-K precoated glass plates for HPTLC. These plates are said to be able to separate and detect substances present in nanogram and, sometimes, in picogram amounts, resulting from a high efficiency and a very high speed (3–7 cm migration in 1–4 min). The reproducibility is said to be excellent. The HP-K surface is a thin 200-μm layer of special silica gel with an average particle size of 4.5 μm and of good purity. The silica gel formulation has been especially developed and is applied in layers of very good uniformity.

The new HP-K plates are suitable for conventional linear development techniques or with automated U-chamber radial (or circular) apparatus.

N-1709

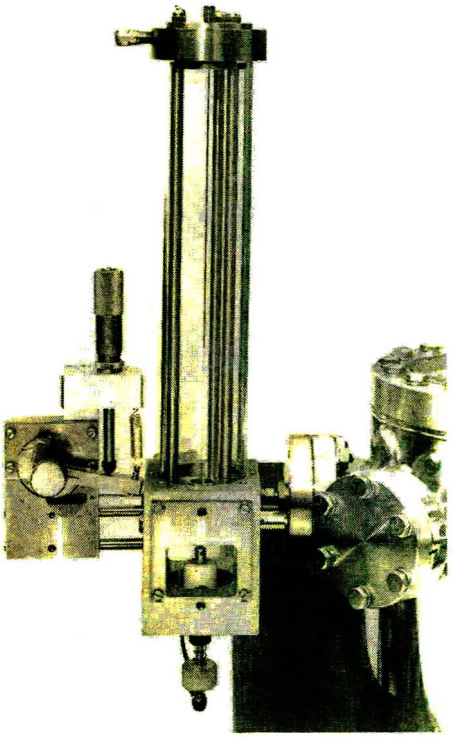
AMINO ACID ANALYZER

The Beckman System 6300 Amino Acid Analyzer is characterized by the company as having optimum speed, resolution, sensitivity, reproducibility, and operator convenience. The new ion-exchange chromatograph provides complete analysis of hydrolyzate fluids in 30 min and of physiological fluids in 2 h.



The System 6300 can quantitate samples of 20 μl or less, containing 50 pmol or less of each component. The instrument can detect components in concentrations as low as 10 pmol. A touch-control panel provides micro-processor control at the operator's fingertips. Automatic program linking eliminates manual adjustments of preparation of the instrument prior to operation or between analytical programs. Four different programs can be stored in the instrument's memory.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.



N-1706

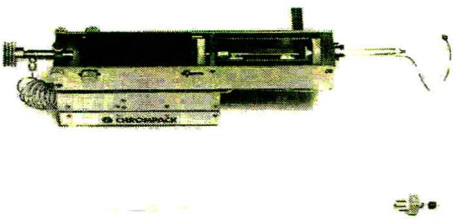
GC–MS INTERFACE

Extranuclear Labs. has introduced a stationary liquid chromatograph–mass spectrometer interface which continuously concentrates the effluent from a conventional HPLC system and delivers the concentrated solution to the mass spectrometer. The interface is combined with an HPLC system, the Extranuclear Simulskan mass spectrometer and a data system to an LC–MS–DS instrument, especially useful for analysis of complex and intractable biological, environmental and petrochemical samples.

N-1720

THERMODESORPTION COLD TRAP INJECTOR

Chrompack has introduced a Thermodesorption Cold Trap injector. The principle of this injector is a concentrating of diluted samples, followed by injection on a capillary column. The system consists of two major parts, one of which is the adsorption tube, a glass tube filled with a well known amount of adsorbent (Tenax or glass wool). The components concentrated in



this tube are desorbed thermally in an oven. The second part is the cold trap, consisting of a piece of fused silica that can be cooled. In this part of the injector the components, thermally desorbed from the first part, condense in a very small volume. The cold trap is heated instantly and the components are injected as a very small band on the following fused-silica capillary column of the GC system. The injector can be used with most gas chromatographs currently available.

N-1705

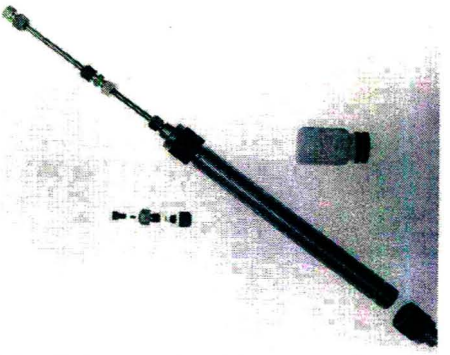
CAPILLARY COLUMN STARTER KIT

For those chromatographers who are not sure which column type would best meet their requirements, or when a range of different polarity columns is required, SGE offers a kit containing three flexible vitreous silica capillary columns with different polarities. The columns are 12 m × 0.2 mm I.D. with film thickness of 0.25 μm. The phases included are the polar Superox 0.1, the medium polarity OV 1701 and the non-polar SE 30. Each column is individually tested and the results are supplied with the kit.

N-1721

SLURRY PACKING KIT

Scientific Systems Inc. have introduced a packing kit for HPLC columns. The company claims that HPLC columns can be efficiently and reproducibly packed with the new kit. The rapid closure reservoir reduces settling of pack-



ing while in suspension giving good quality columns for chromatographers who wish to pack their own. Each Column Slurry Packing Kit is supplied with a reservoir, column assembly (250 X 4.6 mm) including fittings, a high-pressure on/off valve and a column packing guide.

N-1716

RAPID PROTEIN SEPARATIONS

The Beckman Series 341P Liquid Chromatography System offers a fast method of separating biochemical macromolecules on both semi-preparative and analytical scales by size-exclusion chromatography. The Series 341P includes an isocratic liquid chromatograph, the Model 160 Selectable Wavelength UV Detector with 254 and 280 nm filters, a mercury lamp and a Spherogel™ TSK 3000 SW column. The system can be expanded and updated. The Spherogel columns provide rapid elution; they separate globular proteins with molecular weights ranging from 2000 to 1,000,000. Recommended accessories include a Kipp BD-40 recorder, a zinc lamp, a 214 nm filter, a precolumn and a table top centrifuge.

N-1719

GEL FILTRATION COLUMNS

Kratos Analytical Instruments has introduced a complete line of gel filtration columns. Included are 30 and 60 cm columns (I.D. 7.5 mm). Packing materials are available for separations of enzymes, proteins, polysaccharides, nucleic acids, water soluble polymers, oligomers, etc. A wide range of packing material pore sizes accommodates a broad range of molecular weights. Kratos also offers a selection of precolumns compatible with the analytical columns.

N-1712

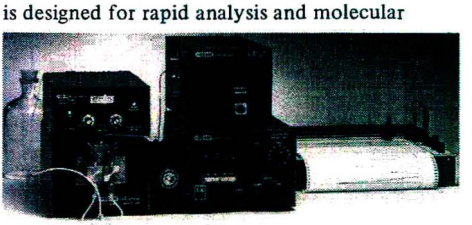
GEL FOR PROTEIN PURIFICATION

Bio-Rad's Affi-Gel Heparin is an affinity chromatography support for purifying a wide range of proteins. It is said to provide rapid one-step purification of coagulation factors and other plasma proteins, polynucleotide polymerase, nucleases, lipase, lipoproteins, and proteases. The Affi-Gel method is simple to use. The sample is applied, and the unbound protein is washed out in low salt buffer. The protein of interest is eluted with higher salt. These conditions are non-denaturing and favor high recovery of active material.

N-1711

HPLC GEL PERMEATION CHROMATOGRAPHY

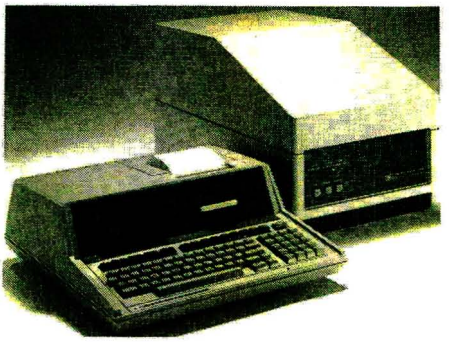
Bio-Rad's HPLC gel filtration system is said to separate proteins and other biopolymers in well under an hour with the efficiency and gentleness equal to classical gel filtration. The system is designed for rapid analysis and molecular



weight determination of samples such as human serum proteins, amino acids, peptides, RNAs, glycolipids and dairy product proteins. It can also be used for the recovering of enzymes and other fragile molecules in active form for further analysis and experiments.

N-1710

HPLC DETECTOR



Hewlett-Packard have introduced the HP 1040A high-speed spectrophotometric detector for use in HPLC systems. The HP 1040A is a stand-alone instrument that can be interfaced with most of the existing HPLC systems on the market. The detector uses a photodiode array detection system, made up of a series of 200 light-sensitive cells etched on silicon chips. These cells work in parallel, simultaneously monitoring all wavelengths over the range of 190–600 nm. The detector allows the operator to analyze quickly and easily a sample at eight independent wavelengths simultaneously without interrupting the chromatographic run.

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ELECTROPHORESIS

A Survey of Techniques and Applications

edited by Z. DEYL, *Czechoslovak Academy of Sciences, Prague*

JOURNAL OF CHROMATOGRAPHY LIBRARY, 18

PART A: TECHNIQUES

Z. DEYL *(editor)*
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PART B: APPLICATIONS

Z. DEYL *(editor)*
A. CHRAMBACH, F.M. EVERAERTS *and*
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Part B is an exhaustive survey of the present status of the application of electrophoretic techniques to many diverse compounds. Those categories of compounds most suited to these separations, such as proteins and peptides, are dealt with in detail, while the perspectives of the applications of these techniques to other categories of compounds less commonly electrophoresed are given. Special attention is paid to naturally occurring mixtures of compounds and their treatment. This is the first attempt to cover the field on such a broad scale and the book will be valuable to separation chemists, pharmacologists, organic chemists and those involved in biomedical research.

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