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CHROMBIO. 603

INVESTIGATIONS OF THE PERFORMANCE OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM WITH AN ELECTROCHEMICAL DETECTOR

GARY WENK* and ROBERT GREENLAND

University of Cincinnati, Institute of Environmental Health, Kettering Laboratory, Cincinnati, OH 45267 (U.S.A.)

(First received November 1st, 1979; revised manuscript received April 3rd, 1980)

SUMMARY

A procedure for the determination of norepinephrine and dopamine, based on high-performance liquid chromatography, is evaluated using an electrochemical detector system. The use of an inorganic mobile phase to provide resolution of low retention amines and extend column life is discussed. A high degree of correlation between estimations of endogenous catecholamine levels is reported using both electrochemical and fluorometric detector systems. Inter-assay reproducibility of the extraction method, and sensitivity and linearity of response of the electrochemical detector system are shown to be consistent across trials. The system described is determined to be accurate, sensitive, and reliable over time.

INTRODUCTION

The isolation and quantitation of catecholamines (CAs) in brain tissue are of major importance in neuroscience. Various methods have been developed for their detection, however some lack the necessary sensitivity, and others are too complicated for routine analysis. Several investigators have described procedures for CA determination based on high-performance liquid chromatography (HPLC) with either ultraviolet radiation detection [1], fluorescence detection after precolumn *o*-phthalaldehyde derivatization [2], or electrochemical detection (ED) [3—15]. Scratchley et al. [3] reported adequate separation and detection of 19 biologically active compounds using HPLC with various chromatographic conditions, including an ED system. A major difficulty mentioned by Scratchley et al. [3] was a shortened column life when using ion-pairing agents, e.g. sodium octyl sulfate (SOS). This problem is addressed later in the results section.

This report describes the performance of a HPLC system with an electrochemical detector. Determinations of sensitivity and linearity of response are

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made and compared across days to demonstrate the reliability of the detector system. A series of studies also examine (1) the use of various acids during the extraction procedure for eluting CAs from alumina, (2) the advantage of using an inorganic rather than organic mobile phase to improve resolution of low retention amines and extend column life, and (3) the correlation between electrochemical and fluorometric CA estimations of the same brain extract.

EXPERIMENTAL

Materials

A 15 cm × 3 mm I.D. HPLC stainless-steel column, packed with LiChrosorb RP-18, 10 μm (E. Merck, Elmsford, NY, U.S.A.) was equipped with an ED system consisting of a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model TL-7A detector cube and a Model LC-2A potentiastat with the electrochemical potential set at +0.72 V vs. Ag—AgCl reference electrode. The Model TL-7A uses a glassy carbon detector electrode, which improves the stability of the detector surface, and a facing auxilliary electrode, which extends the effective linear range of the detector system by two orders of magnitude. The mobile phase was 0.1 *N* nitric acid (pH 4.0 by sodium hydroxide), microfiltered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The ion pairing agent, SOS, was added (50 mg/l) and the solution was stirred, heated (40°C) and pumped by a Milton-Roy (Laboratory Data Control, Riviera Beach, FL, U.S.A.) mini-pump, Model 396-31 (1 ml/min), through a pressure gauge, pulse dampener, and a Rheodyne (Laboratory Data Control) loop injector, Model 7010. This instrument was assembled by the investigators and has been described previously by others [3, 4, 9]. The mobile phase consisted of triple-distilled water and analytical grade fuming nitric acid (Fisher Scientific, Cincinnati, OH, U.S.A.).

Sample preparation

The CA extraction method was from Felice et al. [16]. Rats were sacrificed by decapitation; the brains were excised and frozen immediately on dry ice. After sectioning and weighing, 100—500-mg samples were sonicated in 15-ml Pyrex centrifuge tubes which contained 1—2 ml of cold 0.05 *N* perchloric acid. Prior to sonication, some samples were spiked with the internal standard, 3,4-dihydroxybenzylamine (DHBA). Certain samples were also equally split and spiked with norepinephrine (N) and dopamine (D) (50 ng/100 μl). The standards were prepared by serial dilution of fresh standards (Sigma, St. Louis, MO, U.S.A.) dissolved in 0.1 *N* hydrochloric acid at a concentration of 1 mg/ml.

The homogenate was centrifuged at 15,000 *g* for 15 min; the supernatant was then poured into a 2-ml conical vial containing 90 mg of alumina and 1.0 ml of 3.0 *M* Tris buffer (pH 8.6). After addition of the supernatant, the vials were vigorously shaken for 10 min. The alumina was allowed to settle and the supernatant removed by aspiration. The alumina was then washed once with Tris buffer (6 m*M*, pH 8.6) and twice with triple-distilled water. The last rinse was gently aspirated from the alumina. A 1-ml volume of acid (usually 0.05 *N* $HClO_4$) was added before shaking again for 10 min. After the alumina settled, 100 μl of the eluent were applied to the column via the loop injector system.

Investigations of the detector's response characteristics

One factor determining the magnitude of the response of the detector system to the CAs is the oxidation potential applied to the TL-7A detector cube by the potentiastat. The relationship between applied potential and the output current due to the oxidation of the CAs at the detector electrode surface was investigated. The output current, measure in nanoamperes, was determined following the injection of a constant volume (100 μl) and amount (100 ng) of N. The input oxidizing potential was varied from +400 to +800 mV vs. Ag—AgCl reference electrode, and the output was monitored with a dual-beam storage oscilloscope (Tektronix, Model 564). Applied potentials greater than +800 mV produce background currents too great to offset electronically; therefore potentials in this range were not tested.

RESULTS

The response characteristics of the detector system described of applied potentials vs. nanoamperes of output current generated for the detection of N are shown in Fig. 1. The linearity of response and the consistency of the detector performance is shown in Fig. 2. Each data point in Fig. 2 represents ten determinations of serial dilutions of a standard on ten different occasions. The on-column detection limit was 500 pg per 100 μl injected volume with a signal-to-noise ratio of 2:1. Injected amounts greater than 1 μg saturate the detector and are not subject to quantitation.

Twenty-one estimations of percent recovery of CAs from spiked brain homogenate were made using the procedure out-lined above. Percent recovery was calculated according to the method by Felice et al. [16]. The values for

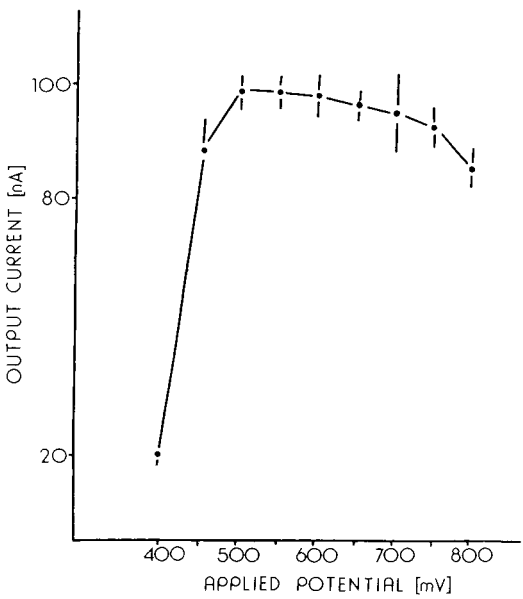

Fig. 1. Applied potential vs. output current. Each data point represents the $\bar{X}$ ± S.D. for five observations.

N, DHBA, and D were 84.3 ± 2.7, 65.4 ± 4.0 and 57.4 ± 5.3, respectively. These percent recovery values compare favorably with other reported values using more complicated extraction procedures [17, 18], and suggest a high degree of inter-assay consistency.

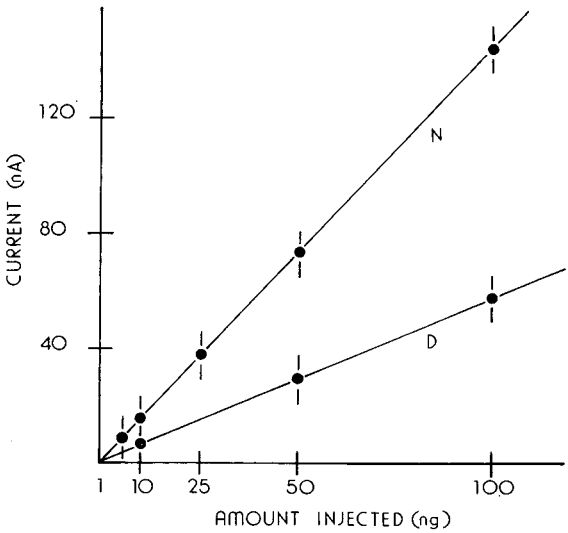

Fig. 2. Linearity of response to N and D. Each data point represents the $\bar{X}$ ± S.D. for ten observations. The various concentrations tested were made by serial dilution of a known standard. The figure also demonstrates a good inter-assay reproducibility.

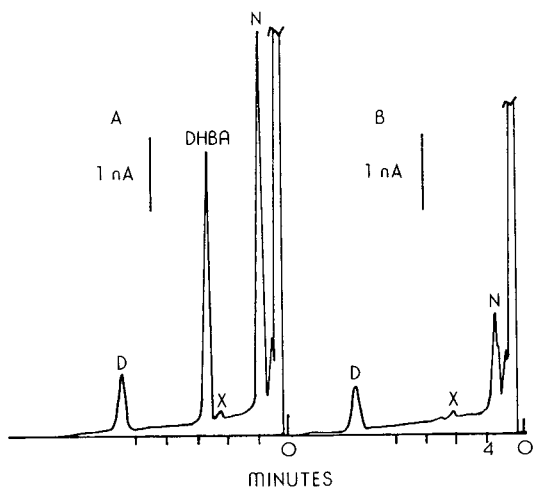

Fig. 3. Chromatograms of CAs from whole rat brains. (A) Spiked with 50 ng DHBA and 50 ng N: (B) endogenous levels. Chromatographic conditions: stationary phase, 30 cm × 3.9 mm I.D. LiChrosorb RP-18 (Merck); mobile phase, nitric acid—0.3 m*M* sodium octyl sulfate; flow-rate, 1 ml/min; temperature, ambient; electrode potential, +0.72 V vs. Ag—AgCl reference electrode; the CAs were eluted off the alumina in 0.05 *N* $HClO_4$; peak "x" is unknown.

Recovery from tissue: the effect of eluting acid

Felice et al. [16] reported that using perchloric acid to elute the CAs from alumina provides adequate recovery while not producing a large void volume response. However, the investigators were using a citrate—phosphate mobile phase. In this study the mobile phase was nitric acid. When phosphoric acid is used to elute the CAs off alumina, a smaller void volume response is produced than with perchloric acid (Figs. 3 and 4). The reduced response provides better resolution of low retention amines, such as N. Table I indicates that recovery is actually improved by using phosphoric acid.

At low levels N is usually overlapped by the void volume response. Generally, changing chromatographic conditions to increase N retention also greatly increases both D retention and analysis time. The system described thus provides increased sensitivity of N without the expense of time.

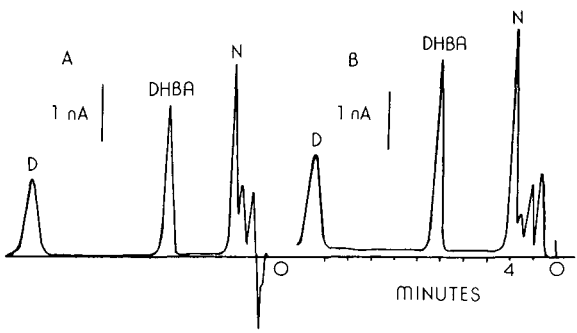

Fig. 4. Chromatograms of CAs from whole brain using perchlorid acid (A) and phosphoric acid (B) as the eluting vehicle. The chart speed was increased to demonstrate the components of the void volume response. Conditions as in Fig. 3. The homogenates were spiked with 50 ng of DHBA.

TABLE I

PERCENT RECOVERY FROM TISSUE: THE EFFECTS OF VARIOUS ELUTING ACIDS

Acid	Normality	Recovery (%)*		
		N	D	D**
Perchloric	0.05	84	75	75
Hydrochloric	0.01	9	5	—
Hydrochloric	0.10	0	55	—
Sulfuric	0.05	98	86	88
Phosphoric	0.05	98	92	97
Acetic	0.50	96	77	92

* % Recovery = $\frac{\text{ng (spike + endogenous)} - \text{ng (endogenous)}}{\text{ng (spiked amount)}}$ using 75—100 mg of tissue.

**Dopamine recovery values form Felice et al. [16]. Norepinephrine values were not reported.

Electrochemistry vs. fluorimetry: a comparison

Fifteen whole rat brains were homogenized and the CAs extracted and eluted in 0.05 *N* hydrochloric acid according to the procedures of Anton and Sayre [17, 18]. The amounts of N and D in the same extract were determined by HPLC—ED and by fluorimetry (Table II).

A paired *t*-test of dependent samples showed that the electrochemical and fluorimetric data do not differ significantly. A linear regression analysis was performed on the data and a correlation coefficient determined ($r = 0.81$).

TABLE II

ELECTROCHEMISTRY AND FLUORIMETRY: A COMPARISON

$\bar{X}$ ± S.D. of ng amounts of CAs/g wet weight tissue. All reported levels were for endogenous CAs.

Animal No.	Concentrations of CAs (ng/g tissue*)			
	Norepinephrine		Dopamine	
	Electrochemical	Fluorimetry	Electrochemical	Fluorimetry
1	403	418	964	878
2	447	395	1094	934
3	452	398	1243	1120
4	435	418	1245	1123
5	426	405	1613	—**
6	394	420	854	879
7	397	405	850	885
8	367	383	916	1012
9	419	452	955	977
10	451	425	867	929
11	408	408	925	880
12	410	410	930	884
13	431	436	960	943
14	447	459	1033	976
15	380	372	1029	915
$\bar{X}$ ± S.D.	417 ± 26	413 ± 23	1031 ± 202	925 ± 83
		$r = 0.81$		

*Wet weight.
**Lost sample.

DISCUSSION

In the present work, the HPLC—ED system is shown to be simple, sensitive, accurate, and reproducible; therefore suitable for the determination of small changes in CA concentrations in localized areas of the rat brain.

The HPLC system with an ED has been utilized by several investigators to detect CAs in various biological matrices. With appropriate modifications of the extraction procedure, and under varying chromatographic conditions, picogram levels of CAs have been analyzed in brain tissue [7], urine [5], and in small cell chambers containing brain slices following pharmacological manipulations [4]. The versatility of the method is due in part to its simplicity and primarily to its remarkable sensitivity.

The simplicity of the procedure encourages multiple sample analysis. Overnight storage may therefore become necessary if automatic sampling systems are not available. Storage of the eluant on alumina overnight at 4°C greatly reduces recovery. However, overnight storage of the homogenate at −20°C had no adverse effects on overall recovery.

Nitric acid is reported to improve column performance and extend column life. The LiChrosorb RP-18 (10-μm bead) used in this study performed adequately for 10 weeks and over 250 injections. The column was stored in the buffer and only rinsed with methanol—water (50:50, v/v) on weekends.

The method described is shown to be reliable, and to perform consistently with a minimum of maintenance. The detector is accurate when compared to existing methods, and is a versatile tool when used with the highly efficient microparticulate columns.

ACKNOWLEDGEMENTS

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REFERENCES

1 L.D. Mell and A.B. Gustafson, Clin. Chem., 23 (1977) 473.
2 L.D. Mell and A.B. Gustafson, J. Liquid Chromatogr., 1 (1978) 261.
3 G.A. Scratchley, A.N. Masoud, S.J. Stohs and D.W. Wingard, J. Chromatogr., 169 (1979) 313.
4 P.H. Plotsky, R.H. Wightman, W. Chey and R.N. Adams, Science, 197 (1977) 904.
5 R.E. Shoup and P.T. Kissinger, Clin. Chem., 23 (1977) 1268.
6 Y. Maruyama, N. Iida, M. Kusaka, A. Horikawa, J. Mori and K. Munekiyo, Prog. Neuro-Psychopharmacol., 2 (1978) 73.
7 C. Refshauge, P.T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R.N. Adams, Life Sci., 14 (1974) 311.
8 P.T. Kissinger, C. Refshauge, R. Dreiling and R.N. Adams, Anal. Lett., 6 (1973) 465.
9 R. Keller, A. Oke, I. Mefford and R.N. Adams, Life Sci., 19 (1976) 995.
10 P.T. Kissinger, C.S. Bruntlett, G.C. Davis, L.J. Felice, R.M. Riggin and R.E. Shoup, Clin. Chem., 23 (1977) 1449.
11 S. Sasa and C.L. Blank, Anal. Chem., 49 (1977) 354.
12 C.R. Freed and P.A. Asmus, J. Neurochem., 32 (1979) 163.
13 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren, J. Chromatogr., 162 (1979) 7.
14 M.J. Cooper, R.F. O'Dea and B.L. Mirkin, J. Chromatogr., 162 (1979) 601.
15 J. Wagner, M. Palfreyman and M. Zraika, J. Chromatogr., 164 (1979) 41.
16 L.J. Felice, J.D. Felice and P.T. Kissinger, J. Neurochem., 31 (1978) 1461.
17 A.H. Anton and D.F. Sayre, J. Pharmacol., 138 (1962) 360.
18 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 145 (1964) 326.

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CHROMBIO. 612

PREPARATION AND CHARACTERISATION OF PERMETHYLATED DERIVATIVES OF BILE ACIDS, AND THEIR APPLICATION TO GAS CHROMATOGRAPHIC ANALYSIS*

S. BARNES*

Comprehensive Cancer Center, University of Alabama Medical Center, University of Alabama in Birmingham, Birmingham, AL 35294 (U.S.A.)

D.G. PRITCHARD

Department of Microbiology, University of Alabama Medical Center, University of Alabama in Birmingham, Birmingham, AL 35294 (U.S.A.)

R.L. SETTINE

Gas Chromatography—Mass Spectrometry Center, University of Alabama in Birmingham, Birmingham, AL 35294 (U.S.A.)

and

M. GECKLE

Comprehensive Cancer Center, University of Alabama Medical Center, University of Alabama in Birmingham, Birmingham, AL 35294 (U.S.A.)

(First received January 25th, 1980; revised manuscript received April 4th, 1980)

SUMMARY

Permethylation of bile acids was achieved in a one-step reaction with sodium methylsulfinylmethanide and methyl iodide in dimethylsulfoxide. The products were characterised by proton nuclear magnetic resonance spectroscopy and by gas—liquid chromatography—mass spectrometry. The derivatives were separated by gas—liquid chromatography on a SP-2250 wall coated 30-m glass capillary column: formation of permethylated products was quantitative over the range 5—50 nmol for each bile acid.

*This work has been presented in part at the Annual Meeting of the American Gastroenterological Association, New Orleans, May, 1979 and published in abstract form in *Gastroenterology*, 76 (1979) 1095.

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INTRODUCTION

Analysis of unconjugated bile acids by gas–liquid chromatography (GLC) requires derivatisation of both carboxyl and hydroxyl groups. Previously this has been accomplished in two stages. Methyl esters are first formed in methanolic hydrochloric acid [1], or by the action of diazomethane [2]. After removal of these reagents, derivatisation of the hydroxyl groups is then carried out to form acetates [3] or trimethylsilyl ethers [4]. These methods can result in handling losses, e.g. extraction and transfer of each sample, and in the case of the trimethylsilyl ethers a susceptibility to hydrolysis.

In this study we report the formation of highly stable bile acid methyl ester methyl ether derivatives. These were prepared in a one-step reaction using the Hakomori reaction [5], a method that has been extensively used in the structural analysis of polysaccharides [5, 6].

MATERIALS AND METHODS

Bile acids were obtained from Research Plus Labs. (Denville, NJ, U.S.A.). Dimethylsulfoxide was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Silicic acid was obtained from Mallinckrodt (St. Louis, MO, U.S.A.) and sodium hydride from Ventron (Danvers, MA, U.S.A.). All other reagents were purchased from Fisher (Norcross, GA, U.S.A.).

Synthesis of permethylated bile acids

Bile acids were purified by silicic acid column chromatography using mixtures of hexane and ethyl acetate as the eluting solvent. The following reaction procedure was used for each bile acid. A specimen (up to 50 mg) of each was carefully dried in a 3-ml reacti-vial (Reliance Glassworks, Bensenville, IL, U.S.A.) in vacuo over phosphorus pentoxide. A triangular magnetic stirrer was placed in each vial which was then sealed with a septum cap closure. Using two 25-gauge needles, the air in each vial was flushed out with nitrogen. The nitrogen was an oxygen-free grade (Hamilton Oxygen, Birmingham, AL, U.S.A.) and was purified by passage through a molecular sieve filter. Anhydrous dimethylsulfoxide, distilled over calcium hydride, was injected through the septum, while maintaining the nitrogen flow. Sodium methylsulfinylmethanide, prepared by reaction between anhydrous dimethylsulfoxide and sodium hydride [5], was added to the vial, and mixing continued for 1 h. This formed the alkoxides of all the hydroxyl groups. Methyl iodide, purified by shaking with mercury, was added in small aliquots, 10% of that required to neutralise the sodium methylsulfinylmethanide, was added each time. Nitrogen flow was stopped during this period to prevent loss of methyl iodide prior to reaction with the reagent. Excess methyl iodide was added to consume all the reagent and mixing continued until the mixture was neutral. In some experiments the excess methyl iodide was blown off with nitrogen. Methylation was repeated with addition of more sodium methylsulfinylmethanide, and subsequently, more methyl iodide. The permethylated products were extracted by the addition of 1 ml hexane and vigorous shaking. The upper hexane phase was

removed and the extraction repeated. The combined hexane phases were evaporated to dryness under nitrogen.

Purification of the permethylated products was carried out by preparative silicic acid column (25 × 1 cm) chromatography. Mixtures of hexane—ethyl acetate were used as eluting solvent, with the ethyl acetate proportion varying between 5 and 20%. Fractions containing the permethylated material were combined and evaporated to dryness. The derivatives were recrystallised from hexane.

In order to recover all the products, including unreacted material, the reaction mixture was acidified with 4 *M* hydrochloric acid and extracted with 2 ml ethyl acetate twice. Iodine, which was also extracted was removed by sublimation after evaporation of the ethyl acetate. Uncorrected melting point determinations were performed using a Fisher—Johns melting point apparatus.

Evidence for permethylation

The term permethylated refers to a derivative in which all available hydroxyl groups (to form methyl ethers) and the carboxylic acid group (to form a methyl ester) have been methylated.

To measure the extent of methylation, two techniques were used.

For chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) 1 μCi of the 11,12-^{3}H-labelled material (Amersham-Searle, Arlington Heights, IL, U.S.A.) was added to the reaction. The permethylated material was analysed by thin-layer chromatography (TLC) on 0.25 mm thick silica gel G 20 × 20 cm plates (Rediplates, Fisher) using hexane—ethyl acetate (4:1, v/v) at 25°C. Duplicate tracks were sprayed with Usui's reagent [7], 3% (w/v) phosphomolybdic acid in glacial acetic acid—sulfuric acid (19:1, v/v), and heated at 100°C for 5 min to locate the bile acids. Each track was divided into 1-cm zones and the silica scraped into plastic scintillation vials. Radioactivity was measured following the addition of 0.5 ml methanol and 5 ml NE-260 scintillant (Nuclear Enterprises, San Carlos, CA, U.S.A.) using a Packard 2450 liquid scintillation counter (Downers Grove, IL, U.S.A.).

In the case of the other bile acids, the extent of methylation was determined by treatment of a portion of the ethyl acetate extract with pyridine—hexamethyldisilazane—trimethylchlorosilane (4:2:1, v,v) at 37°C for 30 min. The resulting derivatives were extracted into hexane after addition of 1 ml hexane and 0.5 ml of water. The hexane layer was evaporated to dryness after centrifugation and dissolved in toluene for analysis by GLC.

Gas—liquid chromatography

GLC analysis was performed using a 30 m × 0.2 mm wall-coated SP-2250 glass capillary (Scientific Glass Engineering, Houston, TX, U.S.A.) mounted in a Hewlett-Packard (Palo Alto, CA, U.S.A.) 5831 gas chromatograph. The conditions for analysis were as follows; helium carrier gas, 2 ml/min; inlet split ratio 10:1; make-up nitrogen gas, 30 ml/min, hydrogen, 40 ml/min and air 300 ml/min; temperatures: inlet, 300°C; column, 250°C and flame ionisation detector, 300°C. Additional studies were carried out using a splitless injector. The column was a 30 m × 0.2 mm wall-coated SP-2100 glass capillary. The temperature program used was 80°C for 0.5 min, 80—270°C at 20°C/min, and finally,

isothermal at 270°C. Acetate ester methyl ester derivatives of bile acids were prepared by the method of Roovers et al. [3].

Quantitative studies were carried out using mixtures of bile acids, with the mass of each varying from 5—50 nmol. The internal standard, 5 nmol 5α-cholestane, was added to the reaction mixture which was extracted with hexane. The hexane was evaporated to dryness and redissolved in 50 μl toluene. Aliquots (1 μl, 100—1000 pmol) were analysed on the SP-2250 capillary column. Linear regression analysis was used to relate the area response (relative to 5α-cholestane) and the mass of each bile acid being derivatised.

Mass spectrometry

Gas—liquid chromatography—mass spectrometry (GLC—MS) was performed on a Hewlett-Packard 5985 GC—MS instrument, with a 15 m × 0.2 mm SP-2250 wall-coated glass capillary. Splitless injection was used to introduce the samples and the temperature program 80°C for 0.5 min, 80—270°C at 20°C/min, and finally, isothermal at 270°C. The source temperature was maintained at 200°C. The ionising electron energy was 70 eV. Spectral data were acquired by an attached data system, and were processed to remove contributions from column bleed.

Nuclear magnetic resonance spectroscopy

Proton spectra were measured at 400 MHz using a Bruker WH-400 nuclear magnetic resonance spectrometer. Crystalline permethylated bile acids were kept in vacuo to remove solvents; then 5 mg (0.01 mmol) was dissolved in 1 ml deuterochloroform for measurement, tetramethylsilane being added as internal reference.

RESULTS

Methylation of each of the bile acids led to single products as judged by GLC and by TLC. An oily residue, probably arising from the mineral oil associated with the sodium hydride, was removed by passage over small silicic acid columns which were eluted with hexane—ethyl acetate (1:1, v/v). Preparative silicic acid chromatography for three of the bile acid derivatives yielded material which crystallised readily from hexane. Melting points were determined as follows: lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) 81—83°C, 3β-hydroxychol-5-enoic acid, 106—108°C and chenodeoxycholic acid, 127—128°C. The derivative of cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) only crystallised when kept in vacuo for one week. The crystalline product melted at 60°C, suggesting that it was highly solvated.

Nuclear magnetic resonance spectroscopy

Proton spectra of the crystalline derivatives at 400 MHz were determined in deuterochloroform. The methyl ether groups gave prominent resonances. The 3α-OCH_3 in the lithocholic acid derivative and the 3β-OCH_3 in 3β-hydroxychol-5-enoic acid derivative were 3.35 ppm downfield from the internal standard, tetramethylsilane. For the chenodeoxycholic acid derivative, the 3α-OCH_3 group was at 3.33 ppm and the 7α-OCH_3 group at 3.23 ppm. In the case of the

cholic acid derivative, the 3α-OCH_3 was at 3.33 ppm, the 7α-OCH_3 at 3.25 ppm and the 12α-OCH_3 at 3.19 ppm (Fig. 1). The methyl ester proton resonance was at 3.65 ppm.

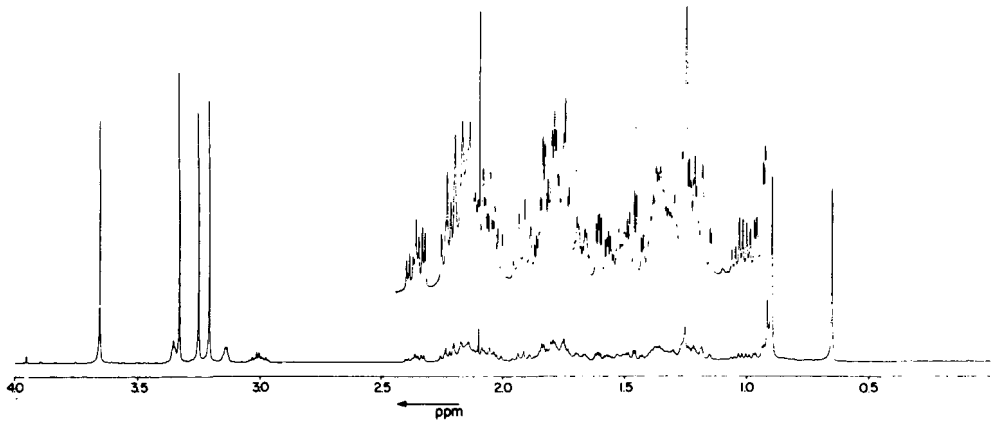

Fig. 1. Proton 400 MHz nuclear magnetic resonance spectrum of permethylated cholic acid. The methyl ether resonances are those at 3.33 ppm, 3.25 ppm and 3.19 ppm, the 3α-, 7α- and 12α-positions respectively. The resonance at 3.65 ppm is from the methyl ester protons. The large number of resonances between 1.0 and 2.5 ppm are from protons in the steroid nucleus.

Mass spectrometry

Electron ionisation mass spectra were obtained following GLC. These included the four crystalline materials and three other bile acids which were not purified prior to GLC. Fragments previously characterised for other bile acid derivatives [8, 9] were observed which represented loss of functional groups (*m/z* 368, 370, 372) and the additional loss of the side chain, C_{20-24} (*m/z* 253, 255, 257). For permethylated bile acids, fragments 32 mass units larger, representing retention of a methyl ether group, were also found. Permethylated derivatives of lithocholic acid (*m/z* 404), 3β-hydroxychol-5-enoic acid (*m/z* 402), and ursodeoxycholic (3α,7β-dihydroxy-5β-cholan-24-oic acid) and hyodeoxycholic acids (3α,6α-dihydroxy-5β-cholan-24-oic acid) (*m/z* both at 434) gave molecular ions (Fig. 2).

Quantitative aspects

The completeness of methylation was investigated using [11,12-^{3}H]chenodeoxycholic acid and 3 mg unlabelled chenodeoxycholic acid. When the necessary amount of methyl iodide was added in one addition, methylation was only 50—80% complete as evidenced by local heating and evolution of gas. In contrast, addition of methyl iodide in small amounts gave a yield in excess of 99.5%, as assessed by TLC analysis. This result was confirmed by treating the hexane fraction to form trimethylsilyl ethers of any unreacted hydroxyl groups. A small amount of 3α-methoxy-7α-trimethylsilyl ether of methyl chenodeoxycholate (0.2%) was found. As noted by others [6], there is no substitute for scrupulous attention to maintaining dry and oxygen-free conditions in order to obtain complete methylation.

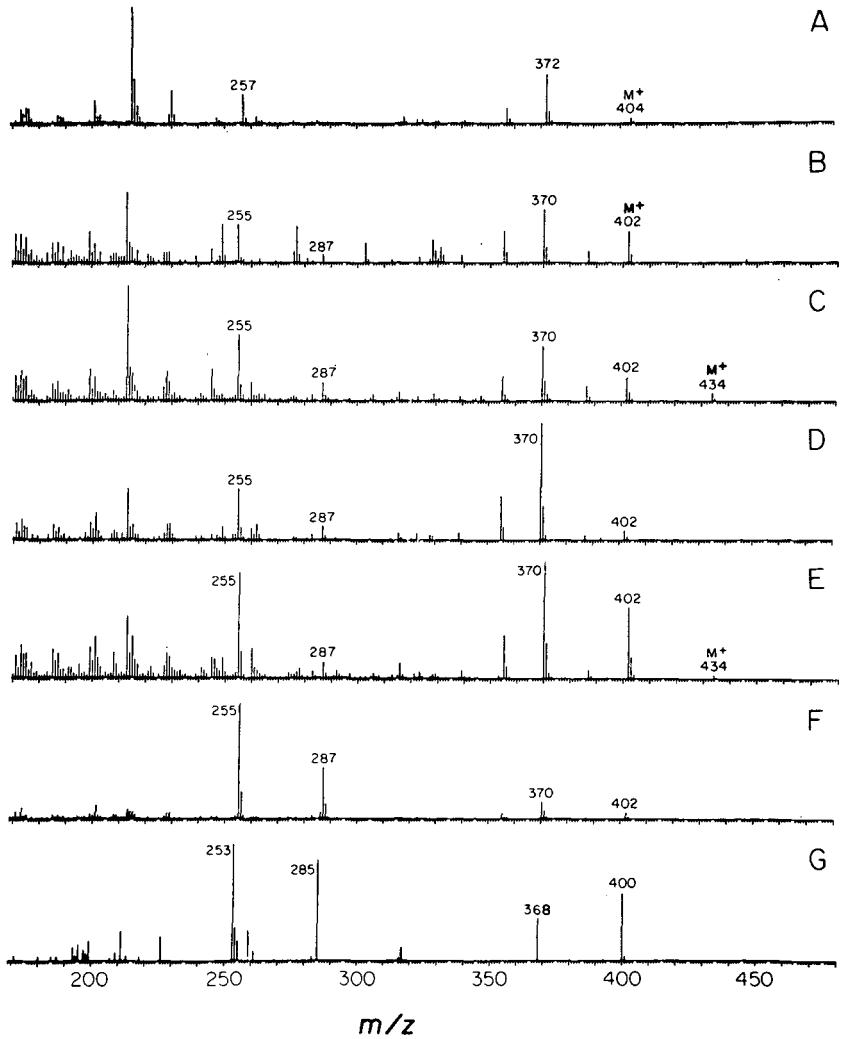

Fig. 2. Electron ionisation mass spectra of seven permethylated bile acids. Identification: A = lithocholic acid; B = 3β-hydroxychol-5-enoic acid; C = hyodeoxycholic acid; D = chenodeoxycholic acid; E = ursodeoxycholic acid; F = deoxycholic acid (3α-12α-dihydroxy-5β-cholan-24-oic acid) and G = cholic acid.

The applicability of the method was investigated in the range 5—50 nmol, using a mixture of seven bile acids. The response curve (by area relative to that of the internal standard 5α-cholestane) was linear for each bile acid as shown for chenodeoxycholic acid in Fig. 3. The relative molar responses (in arbitary units) were close and ranged from 0.113 to 0.120.

Using splitless injection, 50 pmol (20 ng) of each bile acid could be detected with a stable baseline (Fig. 4). For comparison, acetate ester methyl ester derivatives of each bile acid were added to the sample (Fig. 4).

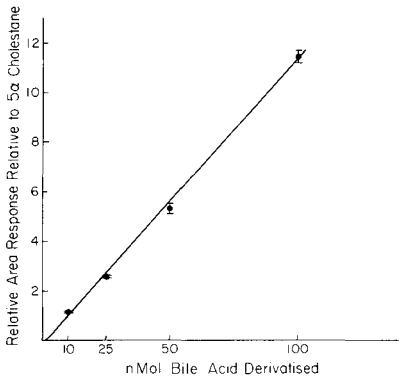

Fig. 3. Response curve of permethylated chenodeoxycholic acid. Response was determined on basis of peak area relative to the internal standard, 5α-cholestane. Data shown are mean ± S.D. (n = 4).

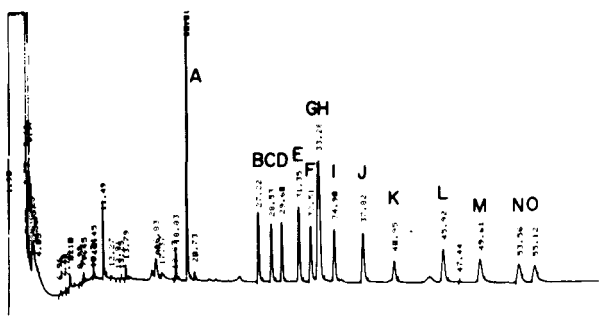

Fig. 4. Capillary gas—liquid chromatogram of bile acid derivatives. Each of the bile acids (50 pmol) was analysed by splitless injection on a wall-coated SP-2100 30 m × 0.2 mm glass capillary column. The temperature program 80°C for 0.5 min, 80—270°C at 20°C/min, and finally, isothermal at 270°C was used. Also included in the sample were the corresponding acetate ester methyl ester derivatives. Peaks: A = 5α-cholestane; B,I = lithocholic acid; C,J = 3β-hydroxychol-5-enoic acid; D,K = deoxycholic acid; E,L = chenodeoxycholic acid; F,O = ursodeoxycholic acid; G,N = hyodeoxycholic acid; H,M = cholic acid. The first letter of each pair corresponds to the permethylated derivative, the second letter to the acetate ester methyl ester derivative.

DISCUSSION

Using the Hakomori reagent [5], sodium methylsulfinylmethanide, and methyl iodide, bile acids are readily converted in a one-step reaction to permethylated derivatives. This type of derivative has been prepared previously [10—13], but by inferior methods. Partially methylated derivatives of chenodeoxycholic acid were produced as unwanted by-products during methyl ester formation using ethereal diazomethane [10]. The permethylated form of 3β-7α-dihydroxychol-5-enoic acid has been prepared using methanol—hydrochloric acid in order to overcome the problems of measuring this bile acid [11, 12]. The permethylated derivative of lithocholic acid has been made recently using potassium *tert.*-butoxide and methyl iodide [13].

The permethylated derivatives are very stable and can be readily separated by capillary GLC. Electron impact ionisation mass spectra gave rise to fragments specific for the presence of the methyl ether group. Similar fragments have been found for the 3α-methoxy-7α,12α-diacetoxy derivative of cholic acid methyl ester [8]. Permethylated derivatives of several bile acids gave rise to readily discernible mass ions (M^+), indicating the increased stability of the molecular ion of this type of derivative over the acetate esters and trimethylsilyl ether derivatives.

The formation of the permethylated derivatives was quantitative and reproducible on the micro scale, the response curve being linear over the range 5—50 nmol. Other studies, not presented here, have extended the linearity into the pmol range. Using splitless injection techniques, amounts as little as 1—5 pmol (injected) can be satisfactorily separated and quantified. Thus without resort to electron-capture detection, sensitivity which approximates that of the bile acid radioimmunoassays [14, 15] can be achieved.

The high degree of stability of permethylated derivatives and the advantages of their single-step formation makes these derivatives excellent candidates for quantitative analysis. In an analogous manner to structural analysis of carbohydrates, methyl ether methyl ester derivatives can be used to determine the position and orientation of sulfate groups in the bile acid molecule [16].

ACKNOWLEDGEMENTS

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REFERENCES

1 S. Barnes, B.H. Billing and J.S. Morris, Proc. Soc. Exp. Biol. Med., 152 (1976) 292.
2 H. Schlenk and J.L. Gellerman, Anal. Chem., 32 (1960) 1412.
3 R.E. Roovers, E. Evrard and H. Vanderhaeghe, Clin. Chim. Acta, 19 (1968) 449.
4 M. Makita and W.W. Wells, Anal. Biochem., 5 (1963) 523.
5 S. Hakomori, J. Biochem. (Tokyo), 55 (1964) 205.
6 D.P. Sweet, P. Albersheim and R.H. Shapiro, Carbohyd. Res., 40 (1975) 199.
7 T. Usui, J. Biochem. (Tokyo), 54 (1963) 283.
8 P.A. Szczepanik, D.L. Hachey and P.D. Klein, J. Lipid Res., 17 (1976) 314.
9 B. Almé, A. Bremmelgaard, J. Sjövall and P. Thomassen, J. Lipid Res., 17 (1977) 339.
10 R. Shaw and W.H. Elliott, J. Lipid Res., 19 (1978) 783.
11 T. Harano, K. Harano and K. Yamasaki, Steroids, 32 (1978) 73.
12 M. Ikeda and K. Yamasaki, Steroids, 32 (1978) 85.
13 R. Shaw and W.H. Elliott, Biomed. Mass Spectrom., 5 (1978) 433.
14 W.J. Simmonds, M.G. Korman, V.L.W. Go and A.F. Hofmann, Gastroenterology, 65 (1973) 705.
15 J.G. Spenney, B.J. Johnson, B.I. Hirschowitz, A.A. Mihas and R. Gibson, Gastroenterology, 72 (1977) 305.
16 S. Barnes, D.G. Pritchard and R.L. Settine, Gastroenterology, 76 (1979) 1095.

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND QUANTIFICATION OF INDIVIDUAL HUMAN BILE ACIDS

G. MINGRONE* and A.V. GRECO

Istituto di Patologia Medica, Catholic University, Rome (Italy)

and

S. PASSI

Ospedale S. Gallicano, Rome (Italy)

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SUMMARY

The reversed-phase high-performance liquid chromatographic separation and quantification of individual bile acids is described.

Taurine- and glycine-conjugated bile acids were separated and detected directly by an ultraviolet absorbance detector operating at 200 nm. Simultaneous quantitation of at least 100 ng of each conjugated bile acid is possible.

Carboxylic (free and glycine-conjugated) bile acids were esterified with *p*-bromophenacylbromide. The reaction, using N,N-diisopropylethylamine as catalyst, yields quantitatively the strongly absorbing *p*-bromophenacyl esters which can be determined by absorbance measurement at 254 nm. Simultaneous quantitation of less than 20 ng of each bile acid is possible.

The present method is applied to the quantitation of individual bile acids in ten human gallbladder bile samples.

INTRODUCTION

Current methodology available for measurement of bile acids* shows

*Abbreviations used in the figures and tables: CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid. The prefixes glyco- (G) and tauro- (T) are used for bile acids having glycine or taurine in amide linkage at C-24.

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differences in the data produced by each procedure. Determination of individual bile acids in biological fluids by thin-layer chromatography (TLC) and fluorimetry has been reported [1]. TLC requires the use of time-consuming elution procedures; besides, the resolution of glycine and taurine conjugates is, in practice, not satisfactory. Total bile acids can be quantified by the use of 3α-hydroxysteroid-dehydrogenase. This method can not differentiate between the individual bile acids [2—4]. A specific radioimmunoassay method has been used to determine cholyl and chenyl conjugates [5—10]. Despite the improvement of gas—liquid chromatography (GLC) for the measurement of free bile acids [11, 12], it is not possible to separate individual conjugated bile acids by this method. The preparation of a sample for GLC shows inherent disadvantages, including the destruction of conjugates, formation of artefacts during vigorous alkaline hydrolysis, and incomplete hydrolysis [13]. However, a quantitative enzymatic hydrolysis of bile acid conjugates has been reported [14].

Modern high-performance liquid chromatography (HPLC) offers the possibility of separating and quantifying individual bile acids. Detection by non-destructive refractive index or ultraviolet (UV) detectors also permits recovery of the injected samples for further analysis. In this connection, numerous studies have been reported [15—21].

In the present work, a reversed-phase HPLC separation and quantification of individual bile acids is reported.

EXPERIMENTAL

Reagents

Acetonitrile Lichrosolv, methanol Lichrosolv, *p*-bromophenacylbromide, phosphoric acid, acetic acid and diisopropyl ether were all from Merck (Darmstadt, G.F.R.) and were used as received. N,N-Diisopropylethylamine, dioxane, chloroform, 2,2,4-trimethylpentane and isopropanol (all from Merck) were redistilled before use.

Silica gel (high purity grade) precoated plates without organic binder (Stratocrom SI-AP, Carlo Erba, Milan, Italy) were used for TLC.

Cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, glycocholic acid (sodium salt), glycochenodeoxycholic acid (sodium salt), glycolithocholic acid (sodium salt), taurodeocycholic acid (sodium salt) and taurolithocholic acid (sodium salt) were obtained from Calbiochem, Lucerne, Switzerland. Ursodeoxycholic acid was kindly supplied by Zambon Pharmaceuticals, Milan, Italy. Their purity was checked by TLC prior to use, as described by Cass et al. [22] for conjugated bile acids and by Panveliwalla et al. [1] for free bile acids. All were found to be 96—98% pure.

HPLC

Analyses were conducted using the 1084 B liquid chromatograph (Hewlett-Packard) equipped with either a single-wavelength (254 nm) UV detector or a scanning spectrophotometer with a wavelength range of 190—540 nm. The liquid chromatograph includes an integrator given areas and times for each peak in the chromatogram.

A reversed-phase Brownlee Labs column (Santa Clara, CA, U.S.A.), 25 cm × 4 mm I.D., RP-18, particle size 5 μm, was used for analysing all bile acids. Chromatographic column and solvents were operated at 40 ± 1°C.

HPLC analysis of standard taurine- and glycine-conjugated bile acids

The conditions were as follows. Mobile phase A was methanol—water (70:30). Phosphoric acid was used to adjust the pH of the mobile phase to 3.1. Flow-rate was 0.5 ml/min, the detector UV (200 nm). Sensitivity was a.u. from 8.0 to 128.0 × 10^{-4}/cm (depending on the amount of injected substances). Chart speed was 0.4 cm/min. Standards were all dissolved in mobile phase A before injection.

HPLC analysis of standard free and glycine-conjugated bile acids

Derivatization procedure. The reaction of free and glycine-conjugated bile acids with *p*-bromophenacylbromide, using N,N-diisopropylethylamine as catalyst, yields quantitatively strongly absorbing *p*-bromophenacyl esters that can be determined by HPLC with absorbance measured at 254 nm.

Briefly, 1 mg of bile acid is dissolved in 5 ml of anhydrous acetonitrile—methanol (9:1) containing 2.5 mg of *p*-bromophenacylbromide; 5 μl of N,N-diisopropylethylamine are added to catalyze the reaction which is complete in about 2 h at 25°C. Heated to 50—60°C, the reaction is complete in about 15 min. For 0.5—20 μg of bile acids, which are the amounts we used for calibration curves, a three-fold molar excess of *p*-bromophenacylbromide and a six-fold molar excess of N,N-diisopropylethylamine must be maintained. The *p*-bromophenacyl esters can be purified from excess reagents by TLC using benzene—dioxane (70:30) as eluent. The excess reagent and its degradation products migrate with the solvent front, *p*-bromophenacyl esters are revealed by a shortwave UV lamp (254 nm) and extracted three times with 5 ml of acetonitrile.

HPLC analysis. Mobile phase B was acetonitrile—water (70:30) at pH 3.10 (with phosphoric acid). The run was isocratic for 5 min, then gradient elution from 70% to 100% acetonitrile in 50 min. Detector: UV (254 nm). Flow-rate: 1 ml/min. Sensitivity: a.u. from 4.0 to 128.0 × 10^{-4}/cm (depending on the amount of injected substances). Chart speed: 0.25 cm/min.

With this mobile phase, if the excess derivatizing reagent and its degradation products are not eliminated by TLC (as is the case when derivatizing bile acids from biological fluids), they interfere with the peak of glycocholic *p*-bromophenacyl ester. Another mobile phase consisting of 100% acetonitrile (flow-rate 0.5 ml/min) is used for separation of glycocholic ester from the interfering derivatizing reagent excess.

Isolation of bile acids from human gallbladder bile samples

The samples were drawn during the removal of the gallbladders from subjects with cholesterin gallstones. Samples of the bile (0.2 ml) were deproteinized by treatment with 1 ml of ethanol (kept at —20°C overnight) and centrifuged. The residue was washed twice with ethanol and centrifuged. The supernatant and combined washes were divided into two parts. One half (for the investigation of conjugated bile acids) was evaporated to dryness and redissolved in 1 ml of mobile phase A and filtered through a Swinney filter prior to injection. The

remaining part (for the analysis of carboxylic bile acids) was acidified to pH 2.5—3.0 with 1 *N* HCl and evaporated to dryness. Nitrogen was blown through the vessel until the odour of HCl was gone. The residue was redissolved in chloroform—methanol (1:2) and purified by TLC with chloroform as solvent.

Bile acids remained at the origin, while the less polar lipids, including cholesterol, free fatty acids, triglycerides, cholesterol esters, etc., moved with or near the solvent front [1]. Areas corresponding to the origin were scraped off from the plates and extracted three times with chloroform—methanol (1:2). The combined chloroform—methanol extracts were dried under nitrogen and used for the derivatization procedure.

The esterification procedure was carried out as reported above. *p*-Bromophenacylbromide and N,N-diisopropylethylamine were added on the basis of the amounts of glycoconjugates present in the bile and determined directly at 200 nm with mobile phase A.

RESULTS

Fig. 1 shows the linear detection response to quantities of eight standard conjugated bile acids between 0.1 and 2.0 μg, with the use of a UV absorbance detector operating at 200 nm. The detection limit at a sensitivity of 8.0×10^{-4} a.u./cm is of the order of 10 ng. The detectable level is based on a

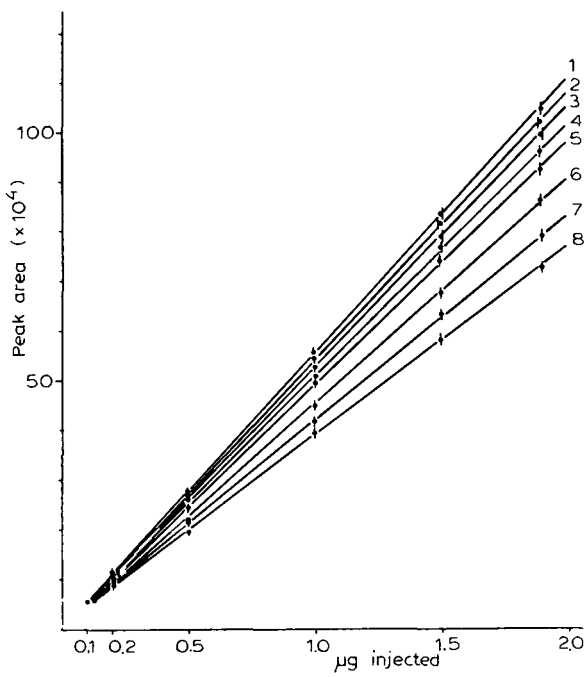

Fig. 1. UV (200 nm) detector response. Conditions are as described under Experimental. Each point is the mean ± S.D. of five determinations carried out for each conjugate dissolved in elution solvent mixture. 1, TCA; 2, TDCA; 3, TLCA; 4, TCDCA; 5, GCA; 6, GDCA; 7, GLCA; 8, GCDCA.

response of twice the noise level. At 210 nm, a response about three times lower is obtained.

Fig. 2 shows the linear detection response to quantities of carboxylic bile acids as *p*-bromophenacyl esters between 0.05 and 2.0 μg. Levels from 0.1 to 20 μg of standard free and glycine-conjugated bile acids were derivatized as

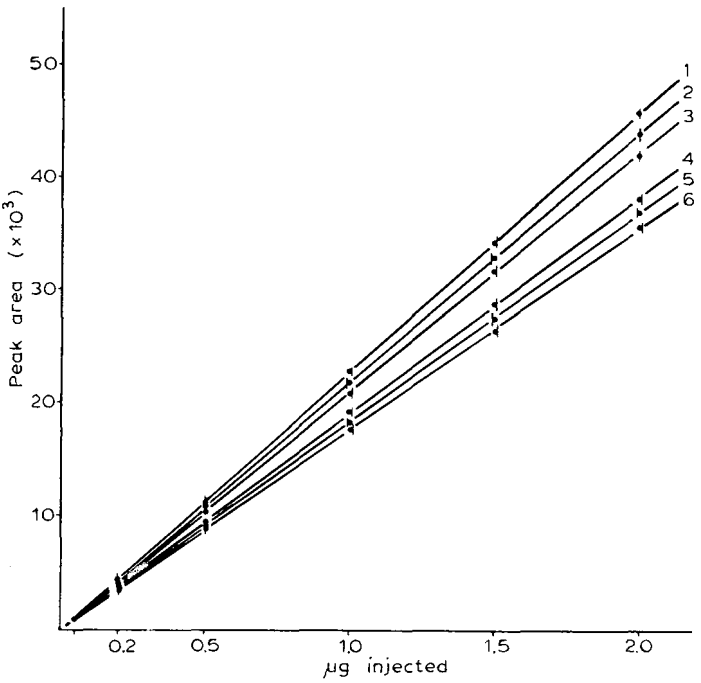

Fig. 2. UV (254 nm) detector response. Conditions are as described under Experimental. Each point is the mean ± S.D. of five determinations carried out for each *p*-bromophenacyl ester. 1, LCA; 2, DCA—CDCA; 3, CA; 4, GLCA; 5, GDCA; 6, GCA.

TABLE I

RECOVERY OF FREE AND GLYCINE-CONJUGATED BILE ACIDS AFTER TLC AND DERIVATIZATION

Each result represents the average of five experiments.

Bile acids	μg deposited on TLC plates	μg measured by HPLC after extraction from TLC plates and esterification procedures (mean ± S.D.)
CA	10	9.51 ± 0.36
DCA	10	9.55 ± 0.38
CDCA	10	9.60 ± 0.33
LCA	10	9.38 ± 0.44
UDCA	10	9.75 ± 0.29
GCA	10	9.48 ± 0.51
GDCA	10	9.50 ± 0.46
GCDCA	10	9.58 ± 0.41
GLCA	10	9.46 ± 0.39

described under Experimental. The reaction volume was always 1 ml and 100 μl were injected into the column. The *p*-bromophenacyl esters are strictly correlated to their molecular weights: the lower the molecular weight, the higher the peak area. λ_{max} for these esters is 255 nm with log ϵ values of about 4.5.

At the maximum detector sensitivity (1×10^{-4} a.u./cm), 1 ng represents the detection limit.

It was then ascertained that the carboxylic bile acids could be recovered from the plates (chloroform was used as eluent) and quantified. After extraction from the plates and esterification, the free and glycine-conjugated bile acids were measured by HPLC. Table I reports these results. Fig. 3 demonstrates the separation of a synthetic mixture of nine carboxylic bile acids as *p*-bromophenacyl esters.

Fig. 4 shows the separation of a synthetic mixture of conjugated bile acids.

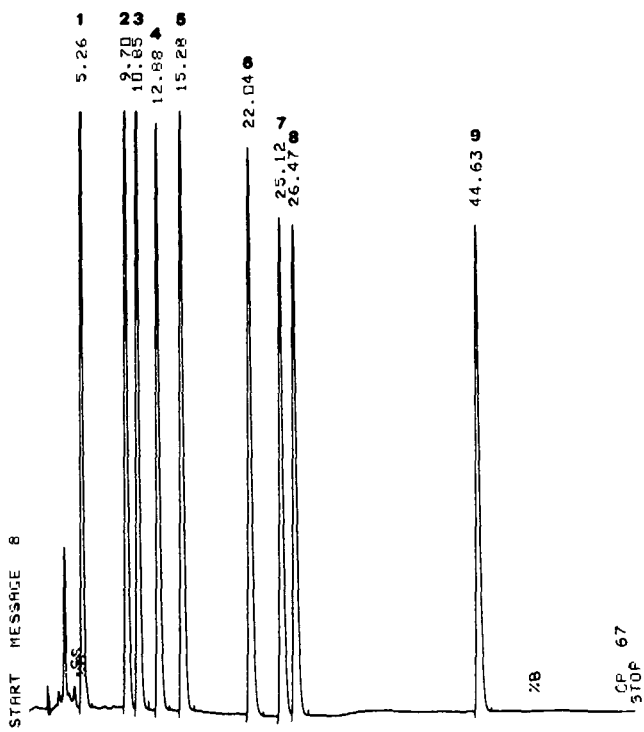

Fig. 3. Separation of standard free and glycine-conjugated bile acids as *p*-bromophenacyl esters, separated from excess derivatizing reagent by TLC. Conditions are as described under Experimental with a UV detector (254 nm). Amount injected was 1 μg of each standard. Peak identification: 1, GCA; 2, GCDCA; 3, GDCA; 4, CA; 5, UDCA; 6, GLCA; 7, CDCA; 8, DCA; 9, LCA.

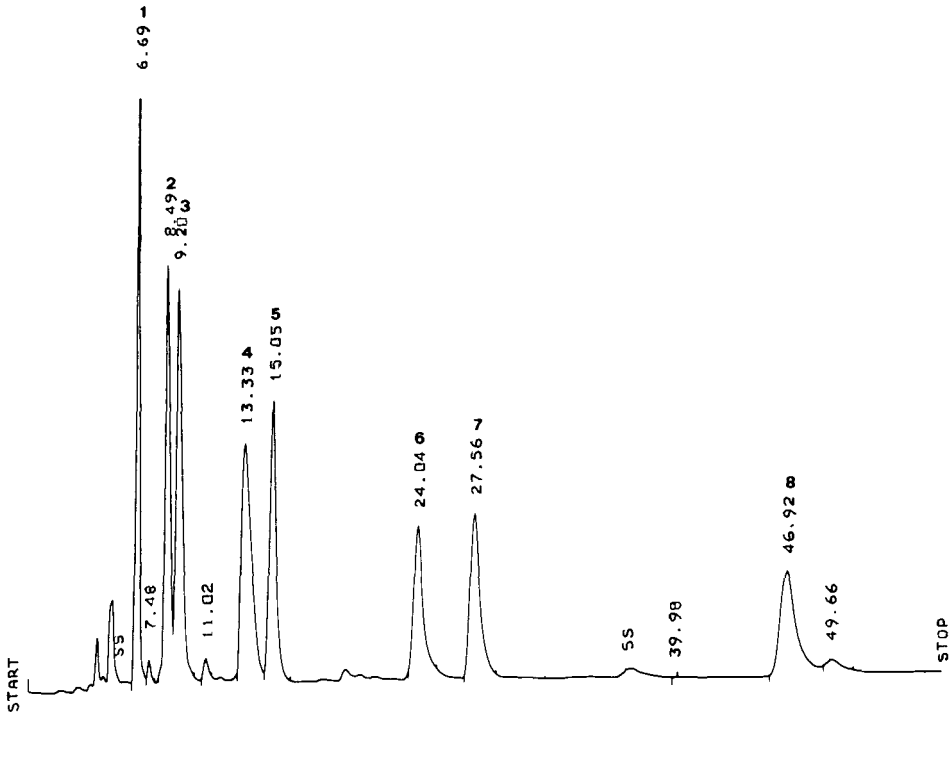

Fig. 4. Separation of a synthetic mixture of conjugated bile acids. Conditions are as described under Experimental, with a UV detector (200 nm). Amount injected was 2 μg of each standard. Peak identification: 1, TCA; 2, TCDCA; 3, TDCA; 4, TLCA; 5, GCA; 6, GCDCA; 7, GDCA; 8, GLCA. Unidentified peaks were observed. These were not investigated further.

TABLE II

AMOUNTS OF BILE ACIDS IN TEN HUMAN GALLBLADDER BILE SAMPLES

Values are given in μg/ml.

Sample	Cholate		Chenodeoxy-cholate		Deoxycholate		Lithocholate	
	T*	G**	T	G	T	G	T	G
I	1196	1724	2009	3501	311	1980	270	350
II	3960	2033	1850	1580	tr***	430	tr	tr
III	1820	1360	1205	1870	tr	520	tr	tr
IV	3850	6733	5012	6036	1125	2148	tr	tr
V	2146	1993	2630	1836	386	990	258	1036
VI	1285	963	865	830	89	225	tr	385
VII	2376	2467	2261	2625	318	750	tr	420
VIII	2040	2180	2520	1850	427	780	tr	tr
IX	1680	1640	2040	1320	tr	1215	tr	tr
X	2950	2350	3050	2010	217	853	tr	tr
Mean ± S.D.	2330 ± 974	2344 ± 1608	2344 ± 1138	2346 ± 1484	288 ± 337	298 ± 633	54 ± 111	220 ± 339

*T, taurine conjugates.
**G, glycine conjugates.
***traces (<0.5 μg/ml).

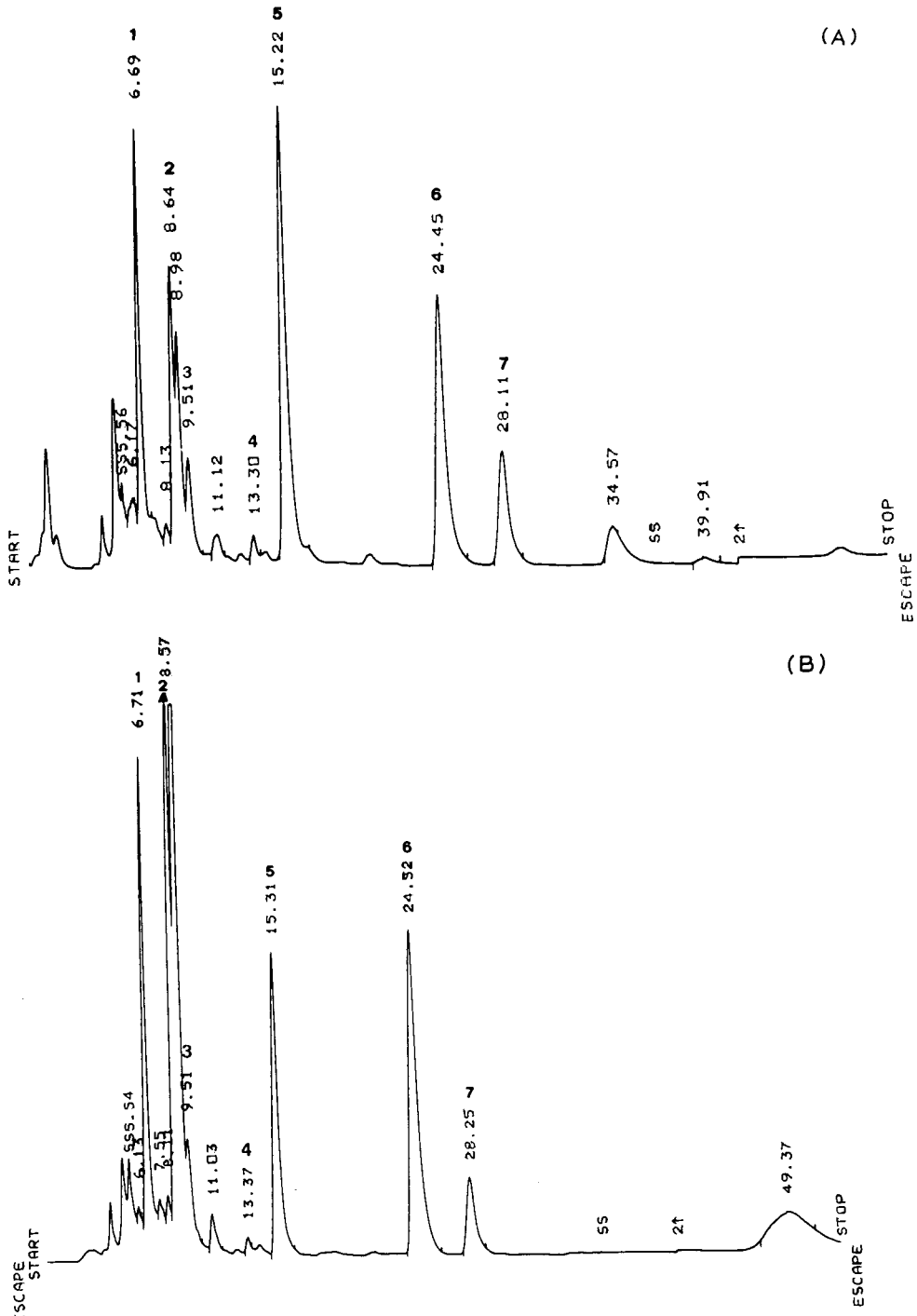

Fig. 5. Separation of conjugated bile acids from two samples of human gallbladder bile after deproteinization of the bile. For further details and peak identification see Fig. 4 and the text.

Only conjugated bile acids were found in ten samples studied, free bile acids were not found to be present.

Fig. 5A and B demonstrates the separation of conjugated bile acids from two samples of human gallbladder bile after deproteinization of the bile. Finally, Table II shows the amounts (μg/ml) of bile acids found in ten human gallbladder bile samples.

DISCUSSION

The recent use of reversed-phase HPLC coupled to a UV detector [16, 17, 21] or to a refraction index detector [18, 19, 21] for the analysis of conjugated bile acids prompted us to inquire into the possibility of improving the reversed-phase system and testing it on human bile samples. The pH of the mobile phase we used (methanol–water, 70:30) significantly affected the elution of conjugated bile acids and the shape of the eluting peaks. Acidification of the mobile phase at pH 3.1 improved both the separation of the acids and the shape of the eluting peaks by reducing tailing.

Because of our interest in measuring in the lower concentration range, the use of a UV detector provided a much greater sensitivity than that afforded by a refractive index detector. In our studies the operating wavelength of the UV detector was fixed at 200 nm. In fact, in the mobile phase A, 203 nm was the wavelength for maximal absorption of both taurocholate and glycocholate, with 200 nm or less for other conjugates, as determined directly by the scanning spectrophotometer connected to the liquid chromatograph. The sensitivity of the detection decreases progressively as the wavelength of the detector is increased. At 210 nm, about a three-times lower response is obtained; at 254 nm the response is minimal. This imposes the use of pure solvents in the preparation of the mobile phase in order to avoid high noise levels, above all when working at low attenuations. Linear relationships were found between peak areas and quantities of the conjugated acids between 0.1 and 2.0 μg, as shown in Fig. 1.

With the carboxylic bile acids, strongly UV-absorbing (254 nm) derivatives were prepared. The *p*-bromophenacyl esters proved to be particularly useful in this respect. The maximum for these esters is 255 nm with log ϵ values of about 4.5. This is more than sufficient to provide nanogram sensitivities.

Simultaneous determination of glycine- and taurine-conjugated bile acids were carried out on ten human gallbladder bile samples. No free bile acids were found in any of the ten samples. Appreciable differences were found among total bile acids and also among individual bile acids with each sample. Further studies on a large number of samples are necessary to elucidate this biological variability and its importance in relation to physiological and/or pathological events. In this connection, because of its sensitivity and reproducibility, the present method facilitates a much more accurate study of bile acids, allowing the investigation of a possible relationship between states of liver function and bile acid patterns.

REFERENCES

1 D. Panveliwalla, B. Lewis, I.D.P. Wootton and S. Tabaqchali, J. Clin. Pathol., 23 (1970) 309.
2 G.M. Murphy, B.H. Billing and D.N. Baron, J. Clin. Pathol., 23 (1970) 594.
3 H.P. Schwarz, K.V. Bergman and G. Paumgartner, Clin. Chim. Acta, 50 (1974) 197.
4 O. Fausa, Scand. J. Gastroenterol., 10 (1975) 747.
5 W.J. Simmonds, M.G. Corman, V.L.M. Go and A.F. Hofmann, Gastroenterology, 65 (1973) 705.
6 L. Barbara, A. Roda, E. Roda, R. Aldini, G. Mazzella, D. Festi and C. Sama, Rend. Gastroenterol., 8 (1976) 194.
7 A. Roda, E. Roda, R. Aldini, D. Festi, G. Mazzella, C. Sama and L. Barbara, Clin. Chem., 23 (1977) 2107.
8 J.W.O. van den Berg, M. van Blankenstein, E.P. Bosman-Jacobs, M. Frenkel, P. Hörchner, O.I. Oost-Harwig and J.H.P. Wilson, Clin. Chim. Acta, 73 (1976) 277.
9 L.M. Demers and G. Herpner, Clin. Chem., 22 (1976) 602.
10 S.W. Schalm, N.F. La Russe, A.F. Hofmann, N.E. Hofman, G.P. van Berge Henegouwen and M.G. Korman, Gut, 19 (1978) 1006.
11 J. Roovers, E. Eurard and H. van der Haeghe, Clin. Chim. Acta, 19 (1968) 449.
12 G.P. van Berge Henegouwen, A. Ruben and K.H. Brandt, Clin. Chim. Acta, 54 (1974) 249.
13 W.S. Harris, L. Marai, J.J. Myher and M.T. Ravi Subbiah, J. Chromatogr., 131 (1977) 437.
14 P.E. Ross, C.R. Pennington and I.A.D. Bouchier, Anal. Biochem., 80 (1977) 458.
15 R. Shaw and W.H. Elliot, Anal. Biochem., 74 (1976) 273.
16 S. Okuyama, D. Uemura and Y. Hirata, Chem. Lett., 9 (1976) 679.
17 N.A. Parris, J. Chromatogr., 133 (1977) 273.
18 C.A. Bloch and J.B. Watkins, J. Lipid Res., 86 (1978) 450.
19 J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 87 (1978) 141.
20 R.W.R. Baker, J. Ferrett and G.M. Murphy, J. Chromatogr., 146 (1978) 137.
21 R. Shaw, J.A. Smith and W.E. Elliot, Anal. Biochem., 86 (1978) 450.
22 O.W. Cass, A.F. Owen, A.F. Hofmann and S.B. Coffin, J. Lipid Res., 16 (1975) 159.

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CHROMBIO. 622

QUANTITATIVE DETERMINATION OF BILE ACIDS IN BILE WITH REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

FUMIO NAKAYAMA* and MITSURU NAKAGAKI

Department of Surgery 1, Kyushu University Faculty of Medicine, Fukuoka (Japan)

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SUMMARY

Separation and quantitation of glycine and taurine conjugates of commonly occurring bile acids in bile, i.e. lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids in their naturally occurring states have been successfully accomplished using high-performance liquid chromatography. No preliminary purification of bile acids is required except ethanol extraction of bile. A μBondapak C_{18} column and acetonitrile—methanol—phosphate buffer and ultraviolet detector at 200 nm were used. Detection limit was 0.05 μg and linearity was observed in the range up to 16 μg. Bile acid composition of ten randomly chosen normal human gallbladder bile samples is given. A large difference in bile acid composition between glycine and taurine conjugates was found to be present.

INTRODUCTION

Rapid expansion of our knowledge on bile acid metabolism in recent years is only made possible by the refinement of analytical techniques for bile acids, such as the introduction of column chromatography, gas—liquid chromatography, thin-layer chromatography (TLC), gas chromatography—mass spectrometry (GC—MS), radioimmunoassay and high-performance liquid chromatography (HPLC). The last has several advantages over the preceding methods, i.e. minimal sample preparation, requiring only ethanol extraction, no hydrolysis, good separation and sensitivity of detection. In the present communication, successful separation of glycine and taurine conjugates of commonly occurring bile acids in bile such as lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids has been accomplished in a single run using HPLC. Quantitative application to human bile is presented.

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MATERIALS AND METHODS

Samples

Human bile was obtained by puncture aspiration of gallbladder content during laparotomy from patients suffering from diseases other than those of the hepatobiliary tree. The bile samples obtained were kept frozen at −20°C until analyzed.

Reagents

The solvents were reagent grade and redistilled prior to use. Those for HPLC were analytical grade reagents prepared specifically for the purpose. Glycine and taurine conjugates of lithocholic, deoxycholic, chenodeoxycholic and cholic acids were obtained from Calbiochem (San Diego, CA, U.S.A.) or from Steraloid (Wilton, NH, U.S.A.). Glyco- and tauroursodeoxycholic acids were kindly supplied by Tokyo Tanabe (Tokyo, Japan).

Chromatography

The HPLC equipment used was a Waters ALC/GP C 202 equipped with a Model 6000 solvent delivery system and a U6K universal injector. The detector used was a Shimadzu variable ultraviolet spectrometer Model SPD-1 (Shimadzu Seisakusho, Kyoto, Japan) which had variable wavelengths between 190 and 750 nm. The μBondapak C_{18} column (30 cm × 3.9 mm I.D.) (CN 27324) and the guard column containing 400 mg of Bondapak C_{18}/Corasil (CN 27248) were supplied by Waters Assoc. (Milford, MA, U.S.A.). Peak area was computed with the use of Shimadzu Chromatopac E1A (Shimadzu Seisakusho). Bile samples were extracted with 20 volumes of ethanol, brought to boiling in a hot water bath, cooled to room temperature, left overnight and filtered through Toyo Roshi 5A filter paper, Toyo Roshi (Tokyo, Japan) (comparable to Whatman No. 43). The filtrate was passed through a Millipore filter, pore size 0.45 μm. To an aliquot of the final filtrate the internal standard of testosterone acetate was added in a form of ethanol solution 200 μg/ml and used for HPLC. An aliquot of 5—10 μl of the ethanol extract of bile containing 5—20 μg of solid and 1.0 μg of internal standard was injected. The solvent system used was a mixture of acetonitrile—methanol—0.03 *M* phosphate buffer, pH 3.40 (10:60:30, v/v/v), prepared just before use and the pH was adjusted exactly to three digits. The flow-rate was 0.5 ml/min with 600 p.s.i. isobaric flow. Detection was made at 200 nm.

RESULTS

Separation of conjugates of commonly occurring bile acids in bile

Baseline separation was obtained between the glycine and taurine conjugates of lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids as shown in Fig. 1. Ursodeoxycholic acid conjugates emerge before cholic acid conjugates. Taurine conjugates always precede glycine conjugates. However, the conjugates of each bile acid were grouped together except deoxycholic and chenodeoxycholic acid conjugates, i.e. in the order of taurochenodeoxycholic, taurodeoxycholic, glycochenodeoxycholic and glycodeoxycholic acids.

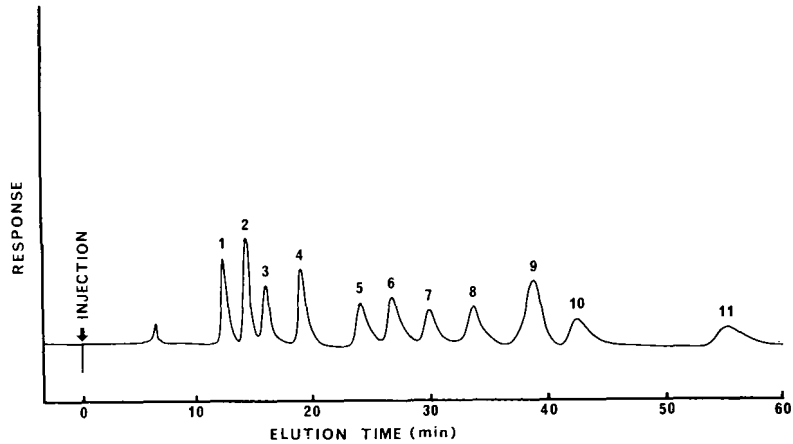

Fig. 1. Separation of standard mixture of conjugated bile acids. Peaks: 1, tauroursodeoxycholic; 2, glycoursodeoxycholic; 3, taurocholic; 4, glycocholic; 5, taurochenodeoxycholic; 6, taurodeoxycholic; 7, glycochenodeoxycholic; 8, glycodeoxycholic; 9, testosterone acetate (internal standard); 10, taurolithocholic; 11, glycolithocholic.

TABLE I

RELATIVE ELUTION VOLUME OF BILE ACID CONJUGATES

Bile acid	Relative elution volume*
Tauroursodeoxycholic acid	0.46
Glycoursodeoxycholic acid	0.53
Taurocholic acid	0.60
Glycocholic acid	0.71
Taurochenodeoxycholic acid	0.90
Taurodeoxycholic acid	1.00
Glycochenodeoxycholic acid	1.12
Glycodeoxycholic acid	1.26
Taurolithocholic acid	1.59
Glycolithocholic acid	2.07
Testosterone acetate (internal standard)	1.45

*Relative to taurodeoxycholic acid = 1.00.

The elution volume for each conjugate is given in Table I. In order to effect good separation it is important to adjust the pH exactly to three digits.

Quantitation of bile acid in bile

The calibration curve for each bile acid conjugate is shown in Fig. 2. Linearity was observed up to 16 μg. Good reproducibility was obtained (Table II). Recovery experiments carried out by adding a known amount of each bile acid conjugate to the bile of known bile acid composition gave good results (Table III). The detection limit was found to be 0.05 μg.

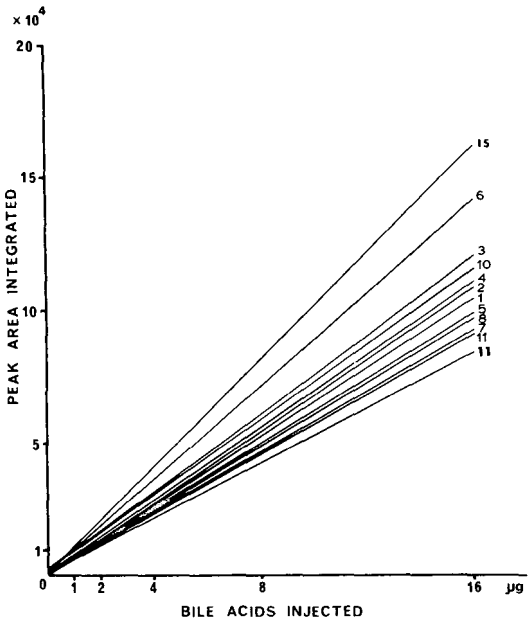

Fig. 2. Calibration curve for each conjugated bile acid and testosterone acetate, used as internal standard. Correlation coefficients of linear regression ranged from 0.996 to 0.999. Designations as in Fig. 1.

TABLE II

REPRODUCIBILITY OF DETERMINATION OF BILE ACID IN HUMAN GALLBLADDER BILE BY HPLC

n = 5. Coefficient of variation expressed in per cent of total bile acid.

Bile acid	Mean ± S.D. (mg/ml)	Coefficient of variation (%)
Glycolithocholic	—	—
Taurolithocholic	—	—
Glycodeoxycholic	45.0 ± 2.2	4.8
Taurodeoxycholic	7.1 ± 0.4	5.9
Glycochenodeoxycholic	40.6 ± 2.0	4.8
Taurochenodeoxycholic	13.4 ± 0.5	3.9
Glycoursodeoxycholic	2.9 ± 0.1	4.5
Touroursodeoxycholic	1.0 ± 0.04	3.9
Glycocholic	29.2 ± 1.9	6.4
Taurocholic	7.5 ± 0.3	4.3

Bile acid composition of normal human gallbladder bile

Ten human gallbladder bile samples were subjected to HPLC. Representative separation of bile acid from human gallbladder bile is shown in Fig. 3. Their bile acid composition is presented in Table IV. The major bile acids in bile were chenodeoxycholic acid and cholic acid conjugates in concordance to the earlier reports. Minor unidentified peaks appeared occasionally between glycoursodeoxycholic and taurocholic and between taurocholic and glycocholic acid. The bile acid composition of glycine and taurine conjugates showed considerable variation in individual cases.

TABLE III

RECOVERY OF BILE ACID ADDED TO HUMAN GALLBLADDER BILE

$n = 3$.

Compound added	Originally present (mg/ml)	Added (mg/ml)	Calculated (mg/ml)	Found (mg/ml)	Recovery of added compound (%)
Glycolithocholic	0.0	10.2	10.2	9.2	90.4
		51.0	51.0	52.0	102.0
Taurolithocholic	0.0	10.2	10.2	9.0	89.1
		50.8	50.8	47.3	93.2
Glycodeoxycholic	45.0	9.7	54.7	57.8	86.4
		48.5	93.5	105.0	112.3
Taurodeoxycholic	7.1	9.8	16.9	14.4	85.4
		49.0	56.1	47.8	85.2
Glycochenodeoxycholic	40.6	9.7	50.3	41.8	83.1
		48.5	89.1	96.3	108.1
Taurochenodeoxycholic	13.4	9.4	22.8	18.7	81.9
		47.0	60.4	54.3	89.9
Glycoursodeoxycholic	2.9	9.9	12.8	11.9	92.7
		49.5	52.4	55.8	106.4
Tauroursodeoxycholic	1.0	9.9	10.9	11.6	106.2
		49.5	50.5	53.6	106.1
Glycocholic	29.2	9.6	38.8	37.3	95.9
		48.0	77.2	65.5	84.8
Taurocholic	7.5	10.0	17.5	17.1	97.5
		50.0	57.5	54.2	94.1

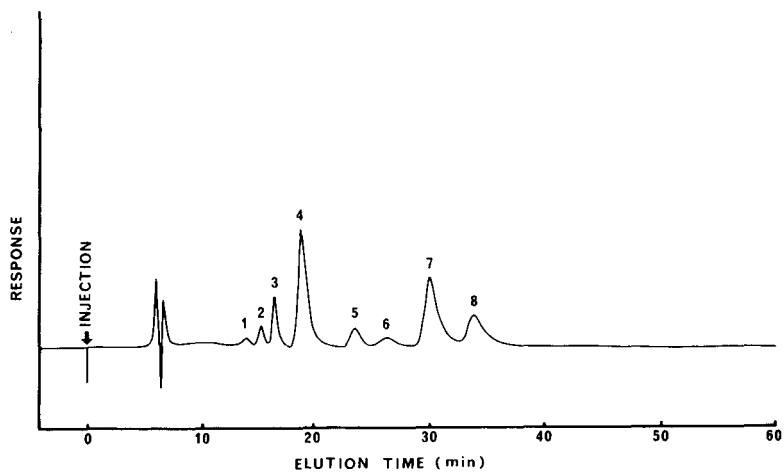

Fig. 3. Separation of bile acids from human gallbladder bile. Identification of peaks as in Fig. 1.

TABLE IV

BILE ACID COMPOSITION OF HUMAN GALLBLADDER BILE

Expressed in per cent of total bile acid. Unidentified peaks were calculated as having the same peak area response as glycodeoxycholic.

Bile acid	Case No.									
	1	2	3	4	5	6	7	8	9	10
Tauroursodeoxycholic	0	0	0.8	0.6	0	0.2	0	0.7	0	0
Glycoursodeoxycholic	0.9	0	1.1	3.6	3.6	0.1	0.2	0.1	0	0
Taurocholic	9.2	18.3	13.8	2.3	2.4	12.1	6.4	17.5	10.2	10.3
Glycocholic	30.8	27.4	21.3	31.3	23.7	41.0	32.7	36.6	43.5	39.6
Taurochenodeoxycholic	6.1	12.0	12.8	1.9	1.6	5.5	3.7	13.4	6.7	6.4
Glycochenodeoxycholic	25.2	42.2	31.8	46.3	32.9	22.9	32.9	31.5	34.5	32.8
Taurodeoxycholic	3.1	0	3.4	0.3	0.9	2.6	1.7	0.1	0	1.2
Glycodeoxycholic	18.9	0.1	15.0	12.2	32.9	14.6	20.3	0	5.1	10.2
Taurolithocholic	0	0	0	0	0	0	0	0	0	0
Glycolithocholic	2.2	0	0	0	0	0	0	0	0	0
Unidentified peak 1*	0	0	0	0.1	0.1	0	0.2	0.1	0	0
Unidentified peak 2**	3.6	0	0	1.4	2.2	1.0	1.9	0	0	0

*Appearing between glycoursodeoxycholic and taurocholic.

**Appearing between taurocholic and glycocholic.

DISCUSSION

The GC methods used for the analysis of bile acids commonly occurring as conjugates of glycine and taurine, usually require prior hydrolysis to enhance their volatility and to simplify the analytical procedure. The inclusion of hydrolysis steps has inherent disadvantages, i.e. it may remain incomplete and may produce artefacts. Furthermore, our knowledge on conjugate forms in which bile acids occur may be important in view of the differences in micelle formation [1] and during the intestinal passage [2]. Recent introduction of HPLC made it possible to analyse bile acids conjugates without prior hydrolysis [3—16]. However, the methods thus far proposed do not permit clear cut separation of glycoursodeoxycholic acid either from taurodeoxycholic and glycocholic acid [11] or from taurodeoxycholic acid [16], or require two runs for late emerging lithocholic acid conjugates [7]. The present method gives a baseline separation of glycine and taurine conjugates of all commonly occurring bile acids in bile, i.e. lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids. Since unconjugated bile acids occur in bile only in rare cases and in only minute proportions [17], prior separation of unconjugated from conjugated bile acids is usually not necessary when analyzing bile acid in bile.

Determination of the glycine and taurine conjugation ratio depends on either determination of bile acid after the separation by TLC [18, 19], or lipophilic gel column chromatography [7, 20] or determination of amino acid moieties after hydrolysis of conjugated bile acid separated [21]. These methods suffer from disadvantages, i.e. from the possibility of incomplete recovery during the chromatographic procedure or incomplete hydrolysis of conjugates. The

present HPLC method permits fingerprinting of bile acid conjugates in their naturally occurring state. Large differences were found in bile acid composition of glycine and taurine conjugates (Table IV).

The advantages offered by HPLC of bile acids are the ease of preparation of the samples, i.e. circumventing hydrolysis of bile acid conjugates as well as preliminary separation of phospholipids and cholesterol which usually emerge with the solvent front and does not interfere with the determination of the later emerging bile acids, and the sensitivity of detection of bile acid conjugates comparable to GC with flame ionisation detection.

The bile acid concentration in icteric sera comes well within the range of detection. However, at the present state of development of the method, the determination of bile acid sulfates requires their preliminary separation [22]. Determination of unconjugated bile acid poses another difficulty, i.e. ultraviolet absorption of bile acid in the unconjugated form is much less than in the conjugated form, approximately one tenth. To enhance detectability, pre- [3, 9, 14] and post-labelling of bile acids have been reported. So far pre-labelling of taurine-conjugated bile acid has been unsuccessful. Post-labelling of bile acids with 3α-hydroxysteroid dehydrogenase coupled with resazurin has been successful but the expense involved limits its routine use [23].

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REFERENCES

1 D.E. Earnest and W.H. Admirand, Gastroenterology, 60 (1971) 772.
2 A.F. Hofmann, Clin. Gastroenterology, 6 (1977) 3.
3 S. Okuyama, Chem. Lett., 7 (1976) 679.
4 R. Shaw and W.H. Elliott, Anal. Biochem., 74 (1976) 273.
5 N.A. Parris, J. Chromatogr., 133 (1977) 273.
6 C.A. Bloch and J.B. Watkins, J. Lipid Res., 19 (1978) 510.
7 J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 87 (1978) 141.
8 Y. Setoguchi and T. Katsuki, Kanzo (Liver), 63 (1978) 593 (short communication in Japanese).
9 B. Shaikh, N.J. Pontzer, J.E. Molina and M.E. Kelsey, Anal. Biochem., 85 (1977) 47.
10 R. Shaw and W.H. Elliott, Lipids, 13 (1978) 971.
11 R. Shaw, J.A. Smith and W.H. Elliott, Anal. Biochem., 86 (1978) 450.
12 K. Shimada, M. Hasegawa, J. Goto and T. Nambara, J. Chromatogr., 152 (1978) 431.
13 S.J. Soldin and D.G. Gall, Clin. Biochem., 11 (1978) 106.
14 F. Stellaard, D.L. Hachey and P.D. Klein, Anal. Biochem., 87 (1978) 359.
15 R.W.R. Baker, J. Ferrett and G.M. Murphy, J. Chromatogr., 146 (1978) 137.
16 M.S. Sian, A.J. Harding Rains, Clin. Chim. Acta, 98 (1979) 243.
17 F. Nakayama, M. Oishi, N. Sakaguchi and H. Miyake, Clin. Chim. Acta, 10 (1964) 544.
18 O. Fausa and B.A. Skålhegg, Scand. J. Gastroenterol., 9 (1974) 249.
19 M. Nakagaki and F. Nakayama, J. Chromatogr., 177 (1979) 343.
20 B. Almé, A. Bremmelgaard, J. Sjövall and P. Thomassen, J. Lipid Res., 18 (1977) 339.
21 H.G. Tiselius and R. Heuman, Clin. Chim. Acta, 83 (1978) 49.
22 J. Goto, H. Kato and T. Nambara, Lipids, 13 (1978) 908.
23 S. Baba, K. Suminoe, R. Uenoyama and Y. Kameno, Igaku to Seibutsugaku (Medicine and Biology), 97 (1978) 219 (in Japanese).

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CHROMBIO. 604

SEMI-AUTOMATED FLUORIMETRIC METHOD FOR THE ESTIMATION OF URINARY CATECHOLAMINES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

LINDSAY M. NELSON*,* and MALCOLM CARRUTHERS

Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF (Great Britain)

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SUMMARY

A high-performance liquid chromatographic method for the quantitation of adrenaline and noradranaline in urine is described, using fluorescence detection. The effluent from the liquid chromatograph is led directly into an analyser to produce the fluorescent trihydroxyindoles from the catecholamines. The method is more reliable and specific than conventional fluorescence techniques. Both catecholamines can be detected at levels of 0.5 ng on the column.

INTRODUCTION

The trihydroxyindole fluorescence method has been widely used for the determination of catecholamines in biological fluids [1—10]. This method, although very sensitive, has the disadvantage that it detects agents other than the catecholamines. L-Dopa, α-methyldopa [11], and labetolol [12] are documented as interfering, but there are other possible interfering agents. Carruthers et al. [13] found that ampicillin, promethazine, protamine, sulphadimidine, vitamin B complex, as well as coffee, tea and cocoa produced considerable interference in vitro. Whether these drugs and dietary components interfere in vivo is not known. Very high blanks are often associated with urine specimens, and quantitation becomes difficult in such cases.

Using high-performance liquid chromatography (HPLC) it is possible to separate the catecholamines, but there is a problem in measuring the physiological levels. Ultraviolet (UV) absorption is a commonly used detection system in HPLC, but is not really sensitive enough — Mell and Gustafson [14]

*Present address: Department of Neurology, King's College Hospital Medical School, Rayne Institute, 123, Coldharbour Lane, London SE5 9NU, Great Britain.

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had to use 200 ml of urine in order to detect noradrenaline (NA) and dopamine (DA) in urine by UV absorption. They were not able to measure adrenaline (A). Similarly, Knox and Jurand [15], using a UV detector, were unable to quantify normal levels of the catecholamines.

Since the fluorimetric methods are so sensitive, it seemed reasonable to use such a detection technique for the catecholamines. Davies et al. [16] prepared fluorescent *o*-phthalaldehyde derivatives of NA and DA and separated these by HPLC. The derivatisation only works for primary amines so A cannot be analysed by this technique. Similarly, in the post-column *o*-phthaladehyde derivatisation method of Froehlich and Cunningham [17], no A can be measured. Schwedt and Bussemas [18] describe 5-dimethylamino-1-naphthalinesulphonyl (dansyl) derivatives of NA, A, and DA, but they do not describe the detection of physiological levels nor any urine analyses.

Ueda et al. [19] describe an HPLC method using an automated trihydroxyindole reaction for the detection of NA, A and DA. The autoanalyser is extremely complicated, using five pumps, and although the authors quantify DA, in fact the system is relatively insensitive to this amine. Schwedt [20] described an HPLC trihydroxyindole method for NA and A using isoprenaline (IP) as an internal standard. In 1978 [21] he described its application to urinary catecholamines. The chromatograms shown in the paper are, however, rather noisy. The work described in this paper is similar but uses ascorbic acid rather than sodium sulphite as the fluorophore stabiliser, and produces clearer chromatograms. Using the method described in this paper, we are able to combine the high sensitivity of the trihydroxyindole detection with the separating power of HPLC. This permits A and NA to be resolved on a liquid chromatography column and introduced separately into the fluorimetric analyser.

METHODS

Sample collection

Twenty-four-hour urine specimens were collected over 25 ml of 6 *M* hydrochloric acid. The volume of each was recorded and an aliquot stored at −20°C until assayed.

Extraction procedure

A 5-ml sample of urine was added to a test-tube containing 5 ml of 0.2 *M* sodium acetate buffer (pH 8.4), 1 ml of 5% ethylenediamine tetraacetate, three drops of thymol blue (1% in ethanol), 0.2 g of activated alumina, and, as an internal standard, 0.5 μg of IP. The contents were taken to pH 8.4—8.6 with 0.5 *M* sodium carbonate. At this point the indicator has turned blue. The test-tube was stoppered and the contents shaken for 5 min. After allowing the alumina to settle, the supernatant was aspirated and the alumina washed with distilled water. The wash water was removed and the catecholamines were eluted by shaking the alumina for 5 min with 0.5 ml of 0.25 *M* acetic acid.

Chromatography

A 10-μl aliquot of the acetic acid eluant was injected onto a 25 cm × 5 mm

column of ODS-coated silica (Whatman Partisil PXS 10/25 ODS) with a mobile phase of 1% acetic acid, 0.0001% sodium dodecyl sulphate and about 10% methanol. The flow-rate of 1 ml/min was provided by a Milton Roy pump with a pulse dampener (Phase Separations). The effluent from the column was fed directly into an automated system producing the fluorescent trihydroxindole compounds from NA, A and IP. Fig. 1 shows the arrangement of this auto-analyser system. The autoanalysis equipment was from Technicon Instruments, and the fluorimeter was a Locarte Mk4 fitted with a 0.15-ml flow-cell and an LF2 filter on the excitation side, and LF7 and LF14 filters on the emission side. The recorder was a Linseis.

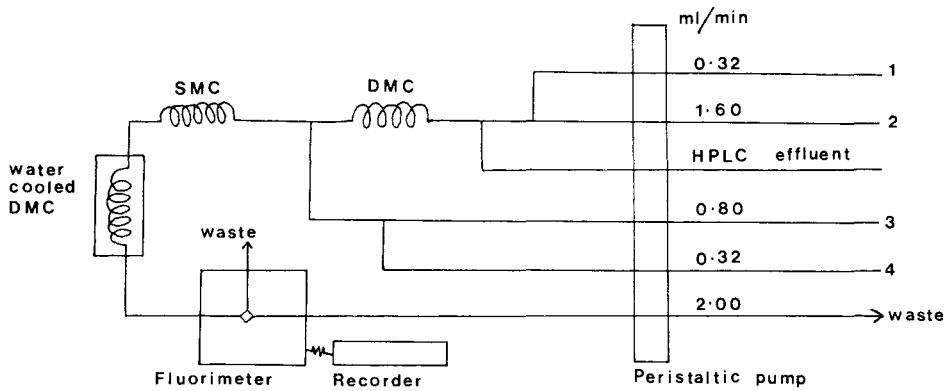

Fig. 1. Diagram of the autoanalyser system used to produce the trihydroxyindoles from NA and A after their separation by HPLC. SMC, single mixing coil; DMC, double mixing coil; 1, air; 2, 1.5 *M* sodium acetate and 0.01% potassium ferricyanide; 3, 2.5 *M* sodium hydroxide; 4, 0.1% ascorbic acid.

Quantitative work

Pure standards of NA, A and IP were made up to 10 μg/ml in 0.1 *M* hydrochloric acid and injected onto the column to determine the retention times. A standard curve was prepared by extracting and analysing solutions containing 0—2 μg of NA and A, and 0.5 μg of IP. Curves were plotted of concentration vs. NA/IP peak height ratios and A/IP peak height ratios. The reliability of the chromatography and the detection was determined by injecting the same specimen six times. The reliability of the whole procedure was determined by extracting six replicate samples of the same urine specimen. The precision was determined by adding known amounts of NA and A to six samples of the same urine taken through the procedure and determining the levels. The results of these tests were compared with equivalent tests on the trihydroxyindole method with prior HPLC.

In order to check that the peaks measured did correspond to those of the three catecholamines, the following standards were injected for possible interference: dopamine, L-dopa, dihydroxyphenylacetic acid, and dihydroxymandelic acid. Any contribution from A in the NA peak, and NA in the A peak, was checked by analysing urine for NA and A. The analysis was repeated twice, first with the addition of 0.2 μg of NA, and second with the addition of

0.2 μg of A. In the former case A was estimated and in the latter NA was estimated. The blank was determined by taking 5 ml of distilled water through the procedure.

The urine from eight children with neuroblastoma, and eight age-matched children in hospital for other complaints were analysed. Urine from three adults suspected of having a phaeochromocytoma were analysed. Urine from twelve of the sixteen children was analysed by the trihydroxyindole procedure without prior HPLC.

RESULTS AND DISCUSSION

The trihydroxyindole reaction involves oxidation of A and NA to adrenochrome and noradrenochrome, respectively, then rearrangement to the respective fluorescent trihydroxyindoles. The fluorescent products are very unstable and have to be protected from oxidation by the presence of a reducing agent. In this laboratory the normal procedure involves stabilisation of both products with ascorbic acid or stabilisation of only the NA product with dithioerythritol. Thus it is necessary to perform two assays — one for total catecholamines and one for NA. A is determined by subtraction, which is liable to compound any errors. An additional "blank" determination, in which the fluorescent products are not stabilised, attempts to compensate for interfering compounds. Thus at least three determinations are necessary to assay NA and A in urine by this conventional method.

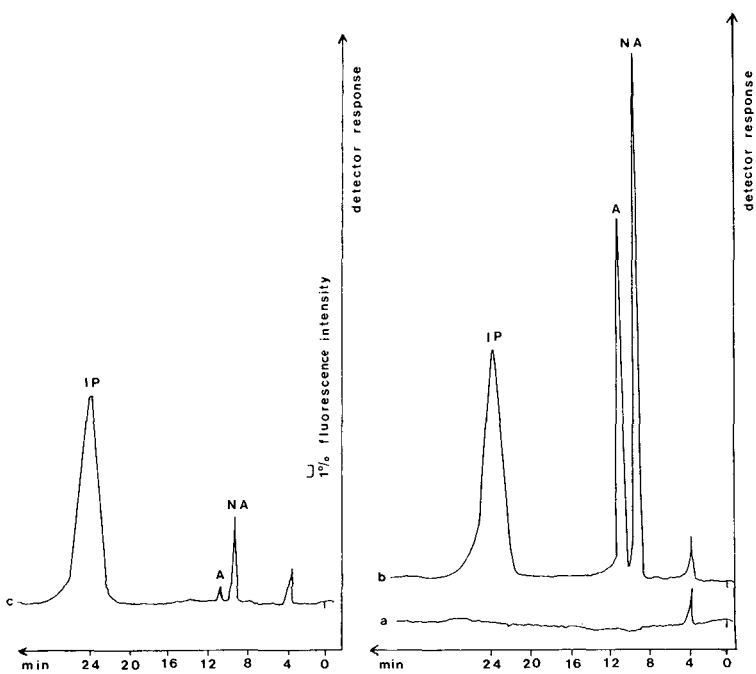

Fig. 2. Typical chromatograms. (a) Blank extraction of 5 ml of distilled water. (b) Standards: 0.2 μg of NA, 0.2 μg of A and 0.5 μg of IP in 500 ml of acetic acid. (c) Urine extract containing 0.05 μg of NA, 0.01 μg of A, and 0.5 μg of IP in 500 μl of acetic acid.

In the method described, both fluorophores can be stabilised by ascorbic acid and a single chromatogram obtained for quantitation of both catecholamines, eliminating the problems associated with blank determinations and, in many cases, interfering compounds. Introduction of an internal standard further improves the reliability of the method.

There is a tendency for the reversed-phase column to change its properties slightly with use [22], and therefore the methanol content of the mobile phase may require slight alterations from time to time in order to maintain the retention times.

The mean recovery of 1 μg of the catecholamines added to urine samples in ten extractions was: NA 72%; A 70%; IP 60%. Since they did not all extract equivalently, it was necessary to prepare an extracted standard curve as described in Methods.

The blank determination shown in Fig. 2 (curve a) shows that NA and A are estimated as 0.0 ng. The chromatograms for pure standards and a urine extract are shown in curves b and c (Fig. 2), respectively. Table I shows the retention times and relative responses of NA, A, IP, and various possible interfering substances. The relative responses show that only α-methyldopa and L-dopa are likely to interfere, and their retention times are well clear of the compounds of interest.

TABLE I

RETENTION TIMES OF NA, A, IP AND POSSIBLE INTERFERING SUBSTANCES

Compound	Retention time (min)	Relative response
Noradrenaline (NA)	9.2	100
Adrenaline (A)	11.0	67
α-Methyldopa	13.2	88
Isoprenaline (IP)	24.0	44
Dopamine (DA)	15.4	1
L-Dopa	10.1	100
Dihydroxyphenylacetic acid	10.4	2
Dihydroxymandelic acid	6.7	0.04

TABLE II

RELIABILITY OF THE METHOD

Aliquots of a single urine specimen were extracted and analysed by HPLC—fluorimetry and aliquots of another urine specimen were extracted and analysed by fluorimetry alone. Replicate injections of a urine extract demonstrated the reliability of the HPLC step. Values are given as the mean ± S.E.M.

Method	*n*	NA	A
HPLC—fluorimetry (μg per 24 h)	6	54.8 ± 3.5	6.2 ± 0.0
Fluorimetry alone (μg per 24 h)	6	37.3 ± 1.5	8.2 ± 1.2
Replicate injections (μg)	5	0.44 ± 0.0	0.10 ± 0.0

TABLE III

PRECISION OF THE METHOD

Method	*n*	Amount recovered (mean ± S.E.M.)	
		NA	A
HPLC—fluorimetry			
Added: 1 μg of NA, 0.2 μg of A	6	0.99 ± 0.04 μg	0.21 ± 0.01 μg
Fluorimetry alone			
Added: 10 ng of NA, 2 ng of A	7	10.04 ± 0.73 ng	1.80 ± 0.65 ng

Comparison of the reliability and precision of the HPLC method and the trihydroxyindole method without prior HPLC shows that the former is much more reliable, especially for A. Tables II and III show the results.

Additions of NA made no difference to the A determination and the same applied to A in the NA determination. Table IV gives the results of this test.

TABLE IV

CONTRIBUTION OF NA TO A DETERMINATIONS, AND A TO NA DETERMINATIONS

Sample	NA (μg)	A (μg)
Urine 1 alone	0.03	0.01
Urine 1 + 0.2 μg of NA	0.24	0.01
Urine 2 alone	0.05	0.01
Urine 2 + 0.2 μg of A	0.05	0.21

The urine estimations are shown in Table V. The neuroblastoma samples PD, JH, MC, and GN have much lower catecholamine levels when measured by the HPLC technique than when assayed by the less specific fluorimetric method. Other peaks in the chromatogram were observed, separated chromatographically from NA and A; these compounds had obviously interfered in the less specific method. None of the possible interfering compounds tried (Table I) seemed to cause the observed peaks in these samples. Possibly we are looking at drug effects.

The sensitivity of this method is such that 0.5 ng of NA or A on the column can be detected and quantified. This is clearly not sensitive enough for plasma catecholamine determinations except in cases of phaeochromocytoma where the amines are drastically raised. NA and A in a plasma sample from such a patient were measured; the catecholamines were found to be thirty times higher than in normal subjects.

The method is used in this laboratory for the routine measurement of NA and A in urine. It is quick, permitting one analysis in place of the usual three by conventional techniques. Also it is more specific and precise than the trihydroxyindole procedure alone. The method is thus more widely applicable to clinical studies where patients are heavily medicated, for example in open heart surgery, where potentially interfering antibiotics are given. It can be used to determine urinary catecholamines in patients on Aldomet and indeed can be used to estimate the drug.

TABLE V

ANALYSIS OF URINE SAMPLES

Subject	Age	Diagnosis	Concentration* in urine (μg per 24 h)			
			Fluorimetry alone		HPLC—fluorimetry	
			NA	A	NA	A
JL	14	Neuroblastoma	52	74	29	n.d.
RS	11	Neuroblastoma	35	16	60	12
GN	9½	Ganglioneuroblastoma	Interference		70	10
PD	9	Neuroblastoma	214	54	74	5
JH	6½	Abdominal ganglio-neuroblastoma	93	115	85	20
MC	6	Ganglioneuroblastoma	102	6	131	18
MC	4	Ganglioneuroblastoma	59	9	26	4
MY	3	Neuroblastoma	37	8	25	5
GF	14	Neurofibromatosis	32	1	137	11
EW	10¾	Abdominal pain	17	10	14	6
CK	9¾	Vomiting	n.e.	n.e.	79	37
KA	9	Pyrexia of unknown origin	73	0.3	21	17
JH	6	Vomiting	36	n.d.	23	5
JP	6	Enlarged liver	21	4	10.3	n.d.
BB	15	Optic glioma	19	4	8	1
AR	3½	Bobbing eyes	n.e.	n.e.	37	90
MG	?	?Phaeochromocytoma	95	18	49	6
MG	?	?Phaeochromocytoma	79	20	83	10
NF	20	?Phaeochromocytoma	n.e.	n.e.	72	17
HC	18	?Phaeochromocytoma	n.e.	n.e.	128	14
HC	18	?Phaeochromocytoma	n.e.	n.e.	116	22
HC	18	?Phaeochromocytoma	n.e.	n.e.	175	24
IW	?	?Phaeochromocytoma	Interference		107	19
IW	?	?Phaeochromocytoma	Interference		96	24

*n.e. = not estimated; n.d. = not detected.

REFERENCES

1 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360.
2 H.C. Campuzano, J.E. Wilkerson and S.M. Horvath, Anal. Biochem., 64 (1975) 578.
3 V. Fiorca and R. Moses, Biochem. Med., 5 (1971) 483.
4 J.C. Griffiths, F.Y. Leung and T.J. McDonald, Clin. Chim. Acta, 30 (1970) 395.
5 V. Renzini, C.A. Brunori and C. Valori, Clin. Chim. Acta, 39 (1970) 587.
6 J.R. Crout, J.J. Pisano and A. Sjoerdsma, Amer. Heart J., 61 (1961) 375.
7 J. Häggendal, Acta Physiol. Scand., 59 (1963) 242.
8 J.D. Sapira, T. Klaniecki and K. Riz, Clin. Chem., 17 (1971) 486.
9 H. McCullough, J. Clin. Pathol., 21 (1968) 759.
10 J.F. O'Hanlon, H.C. Campuzano and S.M. Horvath, Anal. Biochem., 34 (1970) 568.
11 B. Andersson, S. Homvöller, C.-G. Karlsson and S. Svensson, Clin. Chim. Acta, 51 (1974) 13.
12 C.A. Hamilton, D.H. Jones, H.J. Dargie and J.L. Reid, Brit. Med. J., 2 (1978) 800.
13 M. Carruthers, P. Taggart, N. Conway, D. Bates and W. Somerville, Lancet, ii (1970) 62.
14 L.D. Mell and A.B. Gustafson, Clin. Chem., 23 (1977) 473.
15 J.H. Knox and J. Jurand, J. Chromatogr., 125 (1976) 89.

16 T.P. Davies, C.W. Gehrke, C.W. Gehrke, Jr., T.D. Cunningham, K.C. Kuo, K.O. Gerhardt, H.D. Johnson and C.H. Williams, Clin. Chem., 24 (1978) 1317.
17 P.M. Froehlich and T.D. Cunningham, Anal. Chim. Acta, 97 (1978) 357.
18 C. Schwedt and H.H. Bussemas, Z. Anal. Chem., 283 (1977) 23.
19 E. Ueda, N. Yoshida, K. Nishimura, T. Joh, S. Antoku, K. Tsukada, S. Ganno and T. Kokubu, Clin. Chim. Acta, 80 (1977) 447.
20 G. Schwedt, J. Chromatogr., 143 (1977) 463.
21 G. Schwedt, Z. Anal. Chem., 293 (1978) 40.
22 M. Riggin and T. Kissinger, Anal. Chem., 49 (1977) 2109.

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CHROMBIO. 617

SEPARATION AND QUANTIFICATION OF ANGIOTENSINS AND SOME RELATED PEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J.A.D.M. TONNAER*, J. VERHOEF, V.M. WIEGANT and W. DE JONG

Rudolf Magnus Institute for Pharmacology, University of Utrecht, Medical Faculty, Vondellaan 6, Utrecht (The Netherlands)

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SUMMARY

A high-performance chromatographic technique for the separation of angiotensins and some related peptides is described. Complete separation of angiotensin I, angiotensin II, tetradecapeptide and the tetrapeptide Leu-Val-Tyr-Ser is achieved in a single step, using reversed-phase high-performance liquid chromatography. The application of this technique for the detection of renin activity in crude biological samples, employing the artificial renin substrate tetradecapeptide, is demonstrated.

INTRODUCTION

Extensive literature exists on the involvement of the peripheral renin—angiotensin system (RAS) in the regulation of blood pressure and aldosterone secretion [1]. Following the discovery of pharmacological effects of centrally administered components of the RAS, as well as the presence of RAS components and angiotensin receptor sites in the central nervous system [2], evidence has been given for the physiological role of a central RAS [3]. To further characterize the significance of a central RAS, the identification and subsequent quantification of all RAS components is important. Radioimmunoassay systems for angiotensins I and II (ANG I and ANG II), as well as radiochemical and fluorescence assays for renin have been described [4—7]. Dependent on the specificity of the antisera used in a radioimmunoassay, however, separation of angiotensins is essential in order to determine specifically the individual peptides. In addition, radiochemical and fluorescence assays for renin [5—7] require more or less extensive purification of the enzyme since crude biological

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preparations contain additional proteolytic activities which interfere with the renin activity in these assays.

In the present study the high resolving power of reversed-phase high-performance liquid chromatography (HPLC) is used to separate angiotensins and some related peptides [ANG I, ANG II, tetradecapeptide (TDP) and tetrapeptide (TP); see Fig. 1]. Analysis by HPLC appears to be a promising method for assaying renin activity. Using TDP as artificial renin substrate, the accumulation of the various reaction products can be easily quantified by measuring peak heights in the UV absorbance profile of the HPLC eluate.

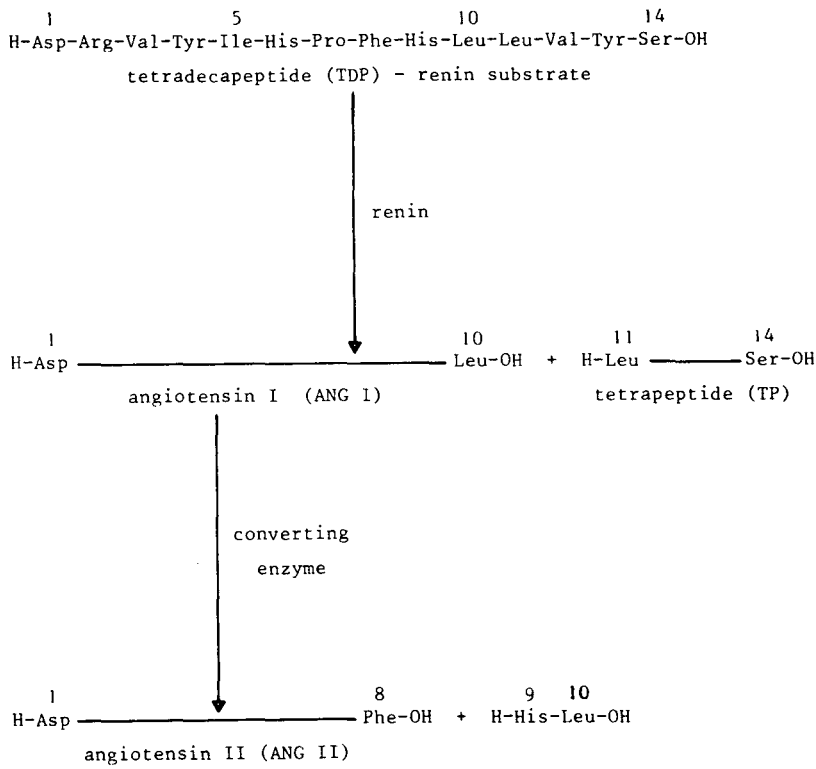

Fig. 1. Pathway of angiotensin II formation from tetradecapeptide (artificial renin substrate) by renin and converting enzyme.

MATERIALS

Synthetic ANG I (peptide content 88%) and ANG II (86%) were purchased from UCB (Brussels, Belgium). TDP (85%) was obtained from Beckman Bioproducts (Geneva, Switzerland), TP (81%) from Bachem Feinchemikalien (Bubendorf, G.F.R.), EDTA (disodium salt) from BDH Chemicals (Poole, Great Britain), 8-hydroxyquinoline (8-HQ) from Merck (Darmstadt, G.F.R.) and methanol (HPLC grade) from Baker (Deventer, The Netherlands). All other chemicals were reagent grade.

METHODS

HPLC of angiotensins and some related peptides

Separation of peptides was performed using HPLC equipment as described previously by Loeber et al. [8]. Samples were applied to a reversed-phase μBondapak C_{18} column (30 cm × 0.39 cm I.D.; 10 μm particle size; Waters Assoc., Milford, MA, U.S.A.). Elution (flow-rate 2 ml/min) was carried out at room temperature with a 45-min linear gradient of 0.01 *M* ammonium acetate, (pH 4.15) (*X*) and methanol containing 1.5 ml of acetic acid per liter (*Y*); initial conditions *X*/*Y* = 4:1, final conditions *X*/*Y* = 1:3. Eluted peptides were detected by UV absorbance which was monitored continuously at 210 nm.

Purification of renin

Hog renin, purified according to the method of Corvol et al. [9], was kindly provided by Dr. J. Menard (Paris, France). The specific activity was 280 GU*/mg protein.

Rat renin was partially purified following a method described by De Jong et al. [10]. The specific activity of the renin preparation thus obtained amounted to 2 GU/mg protein.

Renin assay

Synthetic TDP renin substrate (14.2 nmoles) was incubated with 20 mGU of renin for 0, 30 and 90 min at 37°C in 500 μl of 20 m*M* citrate—phosphate buffer (pH 6.0). The reaction was terminated by heating at 100°C for 10 min. Subsequently the incubation mixtures were centrifuged for 20 min at 20,000 *g* and the supernatants were subjected to HPLC fractionation as described above. Quantification of the peptides generated was done by measuring the peak heights above background in the HPLC profile. Control incubations (0 min) were carried out by boiling the reaction mixture immediately. Incubations with hog renin were done in the absence of angiotensinase inhibitors and incubations with the partially purified rat renin preparation without or with 10.0 m*M* EDTA and 4.3 m*M* 8-HQ to inhibit angiotensinase activity.

RESULTS

Separation and quantification of angiotensins and some related peptides

A typical elution pattern of a mixture of synthetic TP, ANG II, ANG I and TDP using HPLC is shown in Fig. 2. All peptides gave sharp, well-separated peaks. The retention times of the peptides were 6.6, 23.0, 30.2 and 37.2 min, respectively. The position of the various peaks in the elution pattern was not affected by the amount of peptides (up to 10 μg of each peptide) nor the sample volume (up to 1 ml) loaded on the column. In addition, application of the peptides in acetate, citrate or phosphate buffer did not affect the HPLC profile.

For each peptide tested a linear correlation exists between the amount of peptide (0.5—10.0 μg) applied to the column and the UV absorbance at 210 nm of the eluted peptides, as measured by peak heights.

*GU = Goldblatt Unit.

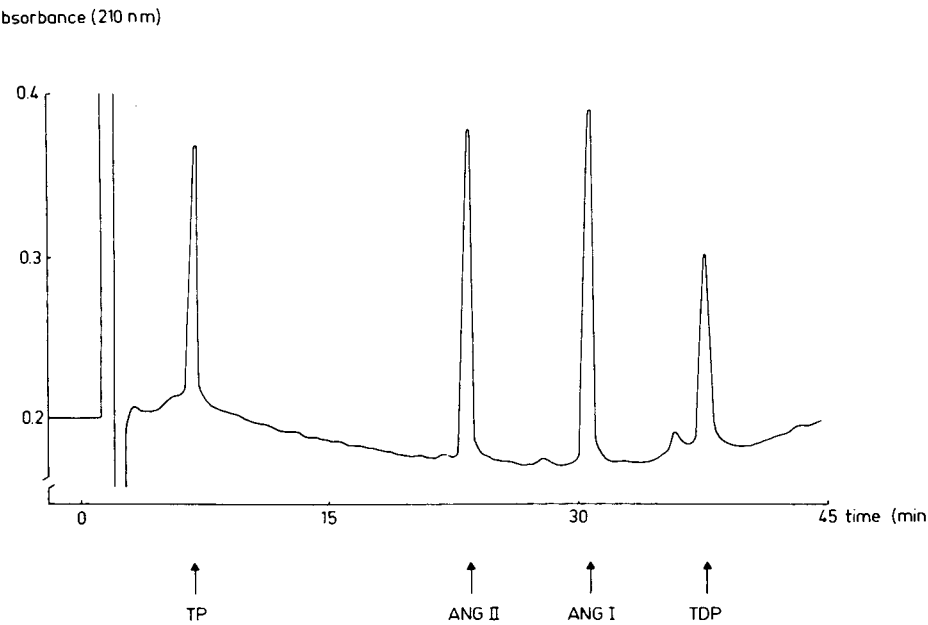

Fig. 2. UV absorbance profile of an HPLC eluate, showing the separation of a mixture containing synthetic ANG I, ANG II, TDP and TP (10 μg of each peptide) on a reversed-phase μBondapak C_{18} column. UV absorbance was monitored continuously at 210 nm.

Determination of renin activity using HPLC

Incubation of TDP (artificial renin substrate) with pure hog renin for 30 or 90 min resulted in the partial disappearance of this peptide, and the equimolar formation of ANG I and TP (Fig. 3B and Table I). Upon incubation for 30 min with partially purified rat renin, TDP was hydrolyzed completely, while the formation of ANG I and TP was not equimolar (Table I). A number of other, unidentified reaction products accumulated (Fig. 3C), suggesting non-specific enzymatic breakdown of peptides under these circumstances. Prolonged incubation up to 90 min resulted in further enzymatic breakdown of TP and ANG I (Table I). Addition of angiotensinase inhibitors (EDTA and 8-HQ) to the incubation mixture enhanced the accumulation of ANG I as expected, but also of some other digestion products (Fig. 3D and Table I).

DISCUSSION

Classical separation techniques like polyacrylamide gel electrophoresis, isoelectric focusing [11], paper chromatography [6, 12] and thin-layer chromatography [13] are generally used to separate ANG I and ANG II. Although the angiotensins have different mobilities and R_F values in these systems, separation of a peptide mixture will often result in an overlap of the components. A good separation of TDP and angiotensins by polyacrylamide gel electropheresis, isoelectric focusing or ion-exchange chromatography is hampered by close physicochemical similarities (unpublished results). Paper chromatography can be used for the separation of TDP, ANG I and TP [6], but is not useful to sepa-

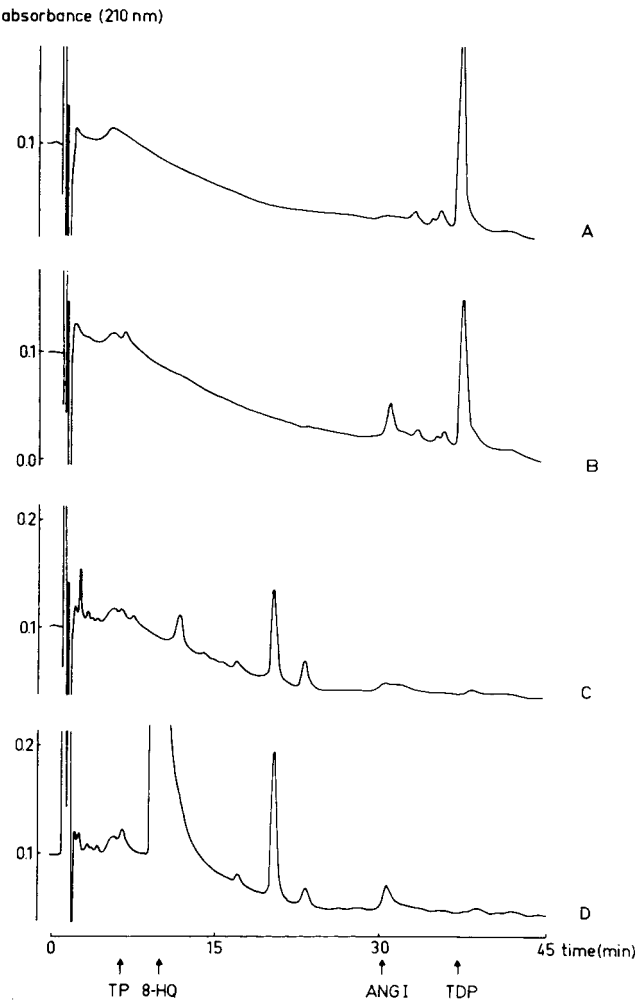

Fig. 3. UV absorbance profiles of HPLC separation of the reaction products of renin—TDP incubations. Incubations of 14.2 nmoles of TDP with 20 mGU of renin were made for 0 min (control incubation) or 30 min at 37°C in 500 μl of 20 m*M* citrate-phosphate buffer (pH 6.0). EDTA (10.0 m*M*) and 8-HQ (4.3 m*M*) were used as angiotensinase inhibitors. Conditions: control incubation (A), incubation with pure hog renin (B), partially purified rat renin (C), and rat renin in combination with angiotensinase inhibitors (D). The two small peaks preceding the TDP peak (see condition A) are caused by impurities in the TDP preparation.

rate ANG I and ANG II. Reversed-phase HPLC offers the possibility to separate complex mixtures, and to analyse the components qualitatively and quantitatively in a single and rapid step [14]. Retention on the reversed-phase columns is highly dependent on both molecular weight and polarity of the applied compounds. Application of this technique for various peptide mxitures has been described [8, 15]. In this study we demonstrate a complete separation of angiotensins and some related peptides by HPLC.

As expected from the small chain length, TP has a very short retention time on the reversed-phase column. Due to the apolar C-terminal dipeptide His-Leu,

TABLE I

TETRADECAPEPTIDE HYDROLYSIS BY HOG RENIN OR RAT RENIN

Incubation was carried out of 14.2 nmoles of TDP with 20 mGU of renin at 37°C in 500 µl of 20 m*M* citrate—phosphate buffer (pH 6.0). Rat renin was incubated in the absence and presence of angiotensinase inhibitors (10.0 m*M* EDTA/4.3 m*M* 8-HQ). Products were analysed by HPLC fractionation.

Enzyme	Incubation time (min)	Amount of peptides formed (nmoles)	
		TP	ANG I
Hog renin	0	–*	–
	30	1.4	1.3
	90	1.5	1.4
Rat renin	0	–	–
	30	0.9	0.3
	90	0.7	–
Rat renin + inhibitors	0	–	–
	30	2.2	1.1
	90	2.2	0.4

*Not detectable by UV monitoring of the HPLC eluate.

ANG I is less polar than ANG II. This, together with the difference in molecular weight, explains the complete separation of these peptides. Lengthening of ANG I with the apolar tetrapeptide Leu-Val-Tyr-Ser, yielding TDP, results in an increased retention time. Thus, with reversed-phase HPLC complete resolution of a mixture ANG I, ANG II, TP and TDP could be achieved. The elution pattern was very reproducible and independent of sample volume up to 1 ml, or of application buffers tested. The UV absorbance of the separated components in the eluate appeared to be linear with the amount of peptide applied to the column and thus enabled simple quantification in the microgram range. Thus, it appears that the procedure described here provides a simple and reliable method for the purification and quantification of angiotensins and related peptides in samples of various composition. Combination of appropriate radioimmunoassays with HPLC fractionation will increase the sensitivity of the peptide assay. Such a combination will also benefit the specificity of the radioimmunoassays since HPLC fractionation implicates separation of cross-reacting compounds (see ref. 8).

The determination of renin in biological samples without purification of the enzyme is problematic because these samples generally contain additional proteolytic activities. Radiochemical and fluorescence assays for renin [5—7] cannot distinguish these activities since the artificial renin substrates used in these assays will be hydrolyzed at several sites by the peptidases. This leads to the formation of multiple peptides, thereby causing serious interference in the assay. The use of these methods therefore requires extensive purification of renin. HPLC, on the other hand, offers the possibility to study directly the accumulation of all reaction products upon TDP incubation with unpurified

renin preparations. By this technique we have observed the formation of ANG I and TP as the only digestion products of TDP hydrolysis by pure hog renin. Following incubation of TDP with partially purified rat renin, in which other proteolytic enzymes are still present, the accumulation of other peptides could also be demonstrated. HPLC profiles also gave direct information about the inhibition of these additional proteolytic activities by angiotensinase inhibitors. Similarly, HPLC can prove to be a useful technique for studying the metabolism of angiotensins in biological systems (plasma, cerebrospinal fluid, tissue homogenates). In comparison to radiochemical and fluorescence enzyme assays, analysis of enzymatic reactions by HPLC fractionation enables a more specific detection of the reaction products, while possible accompanying co-products can be analysed properly.

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REFERENCES

1 M.J. Peach, Physiol. Rev., 57 (1977) 313.
2 D. Ganten and G. Speck, Biochem. Pharmacol., 27 (1978) 2379.
3 M.I. Phillips, J. Weyhenmeyer, D. Felix, D. Ganten and W.E. Hoffman, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 2260.
4 A.E. Freedlender and T.L. Goodfriend, in B.M. Jaffe and H.R. Behrman (Editors), Methods of Hormone Radioimmunoassay, Academic Press, New York, 1979, p. 889.
5 F.X. Galen, C. Devaux, P. Grogg, J. Menard and P. Corvol, Biochim. Biophys. Acta, 523 (1978) 485.
6 F.A. Mendelsohn and C.I. Johnston, Biochem. J., 121 (1971) 241.
7 D.A. Ontjes, J. Majstoravich and J.C. Roberts, Anal. Biochem., 45 (1972) 374.
8 J.G. Loeber, J. Verhoef, J.P.H. Burbach and A. Witter, Biochem. Biophys. Res. Commun., 86 (1979) 1288.
9 P. Corvol, C. Devaux, T. Ito, P. Sicard, J. Ducloux and J. Menard, Circ. Res., 41 (1977) 616.
10 W. de Jong, A.L. Frankhuyzen and A. Witter, Proc. Soc. Exp. Biol. Med., 132 (1969) 71.
11 P. Corvol, D. Rodbard, J. Drouet, K. Catt and J. Menard, Biochim. Biophys. Acta, 322 (1973) 392.
12 P.F. Semple, A.S. Boyd, P.M. Dawes and J.J. Morton, Circ. Res., 39 (1976) 671.
13 S. Cohen, J.M. Taylor, K. Murakami, A.M. Michelakis and T. Inagami, Biochemistry, 11 (1972) 4286.
14 P.R. Brown and A.M. Krstulovic, Anal. Biochem., 99 (1979) 1.
15 J.E. Rivier, J. Liquid Chromatogr., 1 (1978) 343.

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Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 605

TWO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATIONS FOR MEBENDAZOLE AND ITS METABOLITES IN HUMAN PLASMA USING A RAPID SEP PAK C18 EXTRACTION

R.J. ALLAN, H.T. GOODMAN and T.R. WATSON*

Department of Pharmacy, University of Sydney, Sydney, N.S.W. 2006 (Australia)

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SUMMARY

A rapid extraction procedure for mebendazole and its metabolites from plasma using Sep Pak C18 is described. This method eliminates the need for solvent extractions as such.

Two reversed-phase high-performance liquid chromatographic determinations for these extracts, one isocratic elution and the other gradient elution, using an analytical wavelength of 254 nm are also presented.

The gradient elution system provides superior resolution of these compounds and consequently has improved determination limits. For mebendazole the determination limits are 20 ng/ml (isocratic system) and 10 ng/ml (gradient system).

INTRODUCTION

Mebendazole is a broad spectrum anthelmintic drug which is tolerated well in animals [1]. There is growing evidence that mebendazole could be a useful chemotherapeutic agent in the management of hydatid disease in man, especially in cases where the cyst is surgically inaccessible or where spillage has occurred during surgical removal [2—8].

The very low water solubility of mebendazole (ca. 0.7 mg/l) prevents its intravenous administration and renders it poorly absorbed when administered orally. As a consequence the blood levels of mebendazole achieved after oral dosing are often difficult to quantify. High-performance liquid chromatography (HPLC) can detect and quantify mebendazole and its metabolites in human biological fluids in sub-microgram per ml concentrations. It is the only practical analytical system available for this determination.

Metabolism and pharmacokinetics studies with mebendazole during high level chronic dosing (ca. 40 mg/kg/day), as would be necessary in the treatment of hydatid disease, have not been reported in the literature, however in vitro metabolism studies have been performed in pig, rat and dog liver fractions [9].

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The major metabolite of mebendazole (methyl 5-benzoylbenzimidazole carbamate, I, Fig. 1) was found to be methyl 5-(α-hydroxybenzyl)-2-benzimidazole carbamate (III). A second metabolite was identified as 2-amino-5-benzoylbenzimidazole (II) and a minor metabolite was tentatively identified as 2-amino-5-(α-hydroxybenzyl)-benzimidazole (IV). An in vivo study [10] found compound II to be the major metabolite of mebendazole in man.

Two HPLC analyses for mebendazole in human plasma have been reported recently. One method [11] involved multiple extractions, washes and pH adjustments and used an analytical wavelength of 313 nm. The other method [12] was a simple solvent extraction which used an analytical wavelength of 307 nm. Compounds III and IV have very low molar extinction coefficients in the region of 310 nm and therefore these wavelengths are not suitable for their detection.

This work describes a rapid extraction procedure which eliminates solvent extractions as such but still produces a clean extract from human plasma suitable for HPLC analysis. Two HPLC systems are described which separate and quantitate mebendazole and its metabolites using an analytical wavelength of 254 nm and ethyl 5-benzoylbenzimidazole carbamate (V) as an internal standard.

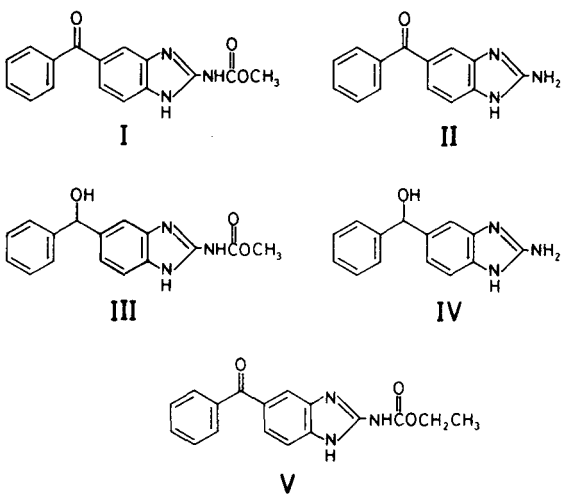

Fig. 1. Mebendazole (I), its metabolites (II, III and IV) and the internal standard (V).

EXPERIMENTAL

Materials

Mebendazole (I) was supplied by Ethnor (Sydney, Australia). Metabolite II was made by alkaline hydrolysis of I. Metabolites III and IV were made by sodium borohydride reduction of I and II respectively. The internal standard (V) was supplied by Mr E. Lacey of this department. Purity was confirmed by thin-layer chromatography (TLC), HPLC and mass spectrum analyses. Methanol and dimethyl sulphoxide (DMSO) (Ajax Chemicals, Sydney, Australia) were of analytical reagent grade. Phosphoric acid, hydrochloric acid

ammonia solution and sodium carbonate were all of laboratory reagent grade and were used without further purification. Plasma was obtained from drug-free healthy male volunteers.

Apparatus

The instruments used were an Altex Model 322 MP high-performance liquid chromatograph (Altex Scientific, Berkeley, CA, U.S.A.) equipped with a fixed-wavelength detector (254 nm, 8-μl flow cell), a Rheodyne (Berkeley, CA, U.S.A.) Model 7120 injection valve fitted with a 20-μl sampling loop, and a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3380A integrator recorder.

Extractions were performed using Sep Pak C18 cartridges (Waters Assoc., Milford, MA, U.S.A.).

Column

The chromatographic column (250 × 4.6 mm I.D.) contained LiChrosorb RP-8 10-μm reversed-phase packing (E. Merck, Darmstadt, G.F.R.) and was supplied packed by Brownlee Labs. (Santa Clara, CA, U.S.A.). A precolumn (35 × 3.2 mm I.D.), dry-packed with Corasil C18 (Waters Assoc.) was fitted to the system.

Mobile phase

The mobile phase consisted of methanol—distilled water (55 : 45, pump A) and methanol—aqueous ammonium phosphate 0.05 *M*, pH 5.5 (55 : 45, pump B).

Operating conditions

The flow-rate was 1.7 ml/min which produced a column pressure of 1200—1300 p.s.i. (ca. 8.2—9.0 MPa). Isocratic elution was performed with pump A providing 67% of the mobile phase and pump B the balance. Gradient elution commenced with pump A supplying 100% of the mobile phase. At 7 min a curved gradient began which brought pump B up to 45% at 13.2 min and then held these conditions up to 20 min (Fig. 2).

The detector sensitivity was set at 0.005 a.u.f.s. The chromatograms were attenuated at the integrator when necessary.

All operations were carried out at room temperature (ca. 20°C).

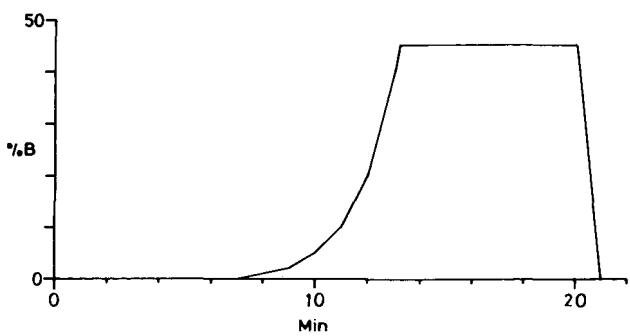

Fig. 2. The gradient elution profile. The contribution of pump B (%B) to the flow-rate (1.7 ml/min) versus time is plotted.

TABLE I

DEVELOPMENT OF EXTRACTION METHOD

Methanol fraction		Recovered (%)				
No.	Volume (ml)	I	II	III	IV	V
1,2,3,4	0.1	–	–	–	–	–
5	0.1	0.02	–	0.3	–	–
6	0.5	71.2	70.1	71.3	65.6	63.0
7	0.5	16.3	12.7	11.3	20.6	16.7
8	0.5	–	–	–	3.4	–
9	2.0	–	–	–	–	–
Σ5,6,7,8	1.6	87.52	82.8	82.9	89.6	79.7

TABLE II

RECOVERY STUDY

N (number of assays) = 3 in each case.

Compound	Theoretical content (μg)	Recovery mean ± S.D. (μg)	Recovery (%)
I	5.20	4.55 ± 0.128	87.5
	1.97	1.75 ± 0.064	88.8
	0.49	0.41 ± 0.025	83.7
	0.20	0.18 ± 0.013	90.0
II	4.90	4.05 ± 0.123	82.7
	1.97	1.45 ± 0.021	73.6
	0.49	0.31 ± 0.005	63.3
	0.20	0.15 ± 0.020	75.0
III	4.95	4.10 ± 0.006	82.8
	1.83	1.58 ± 0.063	86.3
	0.46	0.36 ± 0.013	78.3
	0.18	0.14 ± 0.034	77.8*
IV	5.18	4.64 ± 0.279	89.6
	2.06	1.75 ± 0.080	85.0
	0.52	0.30 ± 0.077	57.7*
	0.21	0.32 ± 0.059	152.4*
V	4.50	3.61 ± 0.049	80.2
	1.67	1.32 ± 0.076	79.0
	0.42	0.27 ± 0.025	64.3
	0.17	0.11 ± 0.009	64.7

*These values, when converted to μg/ml, are below the determination limits defined in Table III.

Extraction procedure and standard curve preparation

Standard solutions and serial dilutions of compounds I, II, III, IV and V were made in DMSO. A standard solution containing all five compounds was used to calibrate the integrator.

Plasma samples (5 ml) were spiked with varying amounts of the five compounds for the recovery studies (Table I, Table II). The standard curves were prepared by spiking 5-ml plasma samples with varying amounts of compounds I, II, III and IV, and with 5.0 μg of compound V as internal standard, in the range 30 μg to 10 ng (equivalent to concentrations of 6 μg/ml to 2 ng/ml). Samples were adjusted to pH 6 with dilute hydrochloric acid or sodium carbonate solution and extracted by passing through a Sep Pak C18 cartridge fitted to a Luer Lok glass syringe.

The Sep Pak C18 cartridge was prepared by flushing with 5 ml of methanol followed by 5 ml of aqueous ammonium phosphate 0.017 *M*, pH 5.5. After the spiked plasma was passed through the cartridge it was washed with 20 ml of distilled water, 0.5 ml of 40% methanol in distilled water and 0.4 ml of methanol. The next 1.6 ml of methanol eluted the five compounds from the cartridge. After washing with a further 10 ml of methanol the cartridge was ready for re-use.

The 1.6-ml methanol fractions were evaporated to dryness in pointed glass tubes on a water bath and then the residues were redissolved in 100 μl of DMSO. Aliquots of 20 μl were injected into the HPLC system.

Calculation of results

The extraction development and recovery results were calculated using the HP3380A external standard routine which computed the amount of each compound injected from the calibration data. The amounts present in 100 μl were then expressed as percentages of the amounts initially added. Results for the standard curves were calculated using the HP3380A internal standard routine which computed peak area ratios with respect to the internal standard and then multiplied the result by the amount of internal standard added to give the amount of each compound in the entire sample. A control plasma extract containing only the internal standard was used to estimate the background levels associated with each compound. These values were used as corrections for the data obtained before any statistical analyses were carried out.

RESULTS AND DISCUSSION

The Sep Pak C18 extraction development is detailed in Table I. In subsequent extractions the fractions containing compounds I, II, III, IV and V were collected as a single fraction. Details of the extraction efficiency of this method are recorded in Table II. The use of the Sep Pak C18 cartridge for plasma extractions had several advantages over the solvent extraction methods. It provided chromatographically cleaner extracts because of the partial separation which it produced, it removed any materials which otherwise might have adsorbed irreversibly to the chromatographic column, and it effected a significant time saving per extraction because it was a single operation. The extraction efficiency of this method was at least equal to that of the solvent extraction methods.

Contrary to the manufacturer's recommendation we found that a Sep Pak C18 cartridge could be re-used a number of times without any loss of performance.

Linear regression analyses were performed on the data from the two chromatographic systems. The regression lines were constructed from a minimum of 28 data points. Most points were duplicates but larger numbers of replicates were prepared in the region of the minimum determinable concentrations (S.D. $\leqslant$ 10%). Table III presents the correlation coefficients for mebendazole and its metabolites in the two systems and their minimum determinable concentrations. Typical chromatograms are shown in Fig. 3 and the gradient profile used is presented in Fig. 2.

The retention times and resolution of compounds II and IV in these systems were very dependent on the mobile phase electrolyte concentration, as indicated in Table IV. Variations to the percentages of mobile phases A and B in the isocratic system could be made, if necessary, to optimize the resolution of compounds II, III and IV.

The gradient elution separation was based on the observation that in the absence of electrolytes, compounds II and IV travelled through this system very slowly while compounds I, III and V were unaffected. By maintaining a constant methanol concentration in the mobile phase throughout the gradient, the problems associated with baseline shifts were avoided. Because the resolution in the gradient elution system was particularly electrolyte dependent, it was necessary to adequately re-equilibrate the column before each run. Re-equilibration was achieved by pumping 25 column (plus pre-column) volumes of electrolyte-free mobile phase through the system. The flow-rate was increased during this operation to shorten the re-equilibration time.

TABLE III

CORRELATION COEFFICIENTS AND DETERMINATION LIMITS

S.D. $\leqslant$ 10%.

Compound	Correlation coefficient	N^*	Determination limit (μg/ml)	N^{**}
Isocratic elution				
I	0.9982	42	0.02	4
II	0.9926	39	0.06	3
III	0.9975	28	0.06	3
IV	0.9787	30	0.56	4
Gradient elution				
I	0.9979	39	0.01	3
II	0.9914	41	0.03	4
III	0.9986	30	0.06	4
IV	0.9959	30	0.10	4

*Number of points in regression analysis.

**Number of points at determination limit.

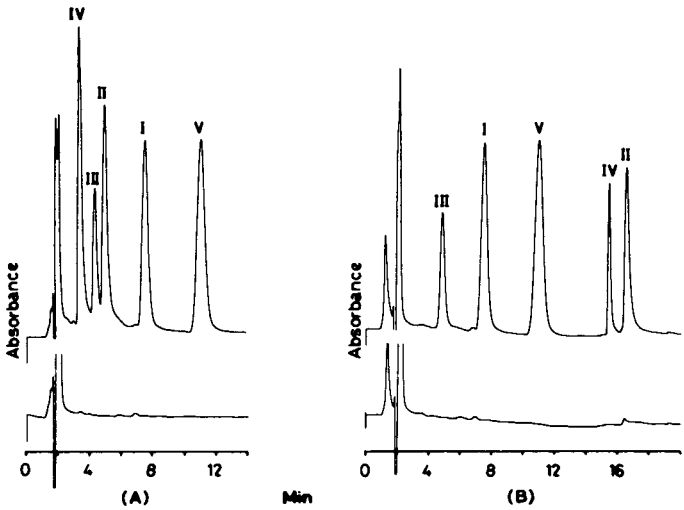

Fig. 3. Typical chromatograms. A, isocratic elution; B, gradient elution. The chromatograms represent plasma spiked with 0.65 μg/ml of I, 0.72 μg/ml of II, 1.29 μg/ml of III, 3.30 μg/ml of IV and 0.92 μg/ml of V (upper traces) and control plasma extracts (lower traces). Sensitivity, 0.005 a.u.f.s. and attenuation × 16.

TABLE IV

ELECTROLYTE CONCENTRATION EFFECT ON THE ELUTION OF COMPOUNDS I, II, III, IV AND V

V = 1.00 for all determinations.

Electrolyte concentration (*M*)	Retention relative to compound V			
	I	II	III	IV
0.0225	0.69	0.45	0.40	0.28
0.0180	0.69	0.46	0.40	0.29
0.0135	0.69	0.47	0.40	0.31
0.0101	0.69	0.48	0.40	0.33
0.0068	0.69	0.50	0.40	0.37
0.0045	0.69	0.52	0.40	0.43
0.0023	0.69	0.60	0.40	0.61
0.0011	0.69	0.69	0.41	1.01
0.0006	0.69	0.96	0.41	1.77
0.0000	0.69	7.93	0.40	*

*Value greater than 10.00, not determined.

Two distinct separatory mechanisms appeared to be operating in the gradient elution system. Compounds I, III and V were separated by reversed-phase partition whilst compounds II and IV seemed to be separated by an ion-exchange mechanism. The following observations supported this theory. The nature of the electrolyte was relatively unimportant. Sodium phosphate and sodium chloride produced separations almost identical to ammonium phosphate under the conditions described. Compounds II and IV eluted in a similar

manner from a cation-exchange column when subjected to the same gradient, but compounds I, III and V eluted with the solvent peak. The pK_a values of compounds II and IV were such that at the pH of the mobile phase, in the range 6.3 to 6.6, they would have been approximately 50% ionized. The pK_a values of compounds I, III and V were such that they would have been undissociated in this pH range and therefore unable to interact with any ion-exchange sites.

The two HPLC systems presented here had similar sensitivities for mebendazole and its metabolites. Of the two methods the isocratic elution system was favoured when the biological background levels were low and when adequate resolution existed between the metabolites. In cases where the biological background produced a tailing "solvent" peak which extended under the metabolites, the gradient system was found to be superior. The gradient system eluted the biological material more quickly than the isocratic system, provided increased separation of the metabolites and gave increased resolution of the minor metabolite (IV).

These systems are being used to estimate plasma levels of mebendazole and metabolites in patients suffering from hydatid disease. Fig. 4 presents typical chromatograms from one such patient.

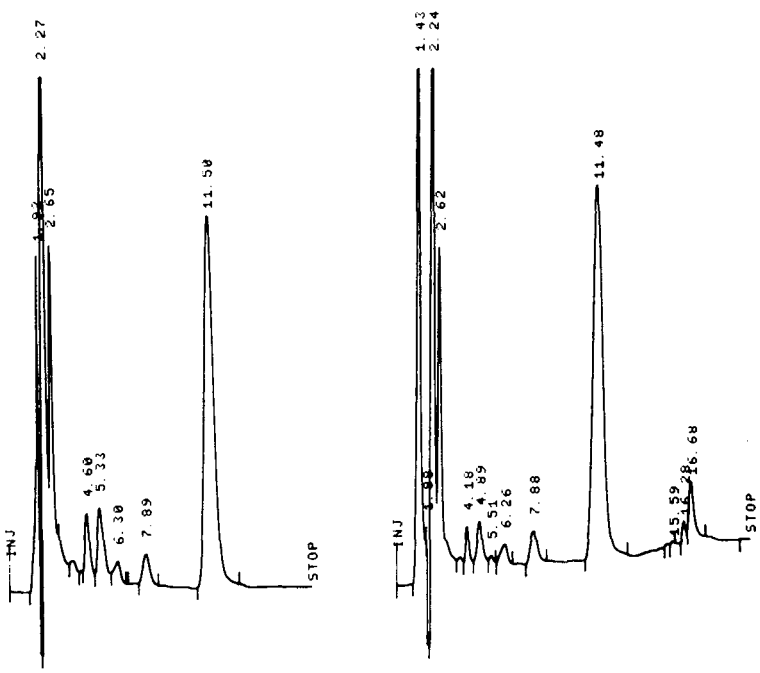

Fig. 4. Chromatograms from a steady-state patient representing 88 ng/ml of mebendazole (left, isocratic elution; right, gradient elution). Retention times: mebendazole, 7.89 min and 7.88 min; internal standard, 11.50 min and 11.48 min; sensitivity, 0.005 a.u.f.s. and attenuation × 8.

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REFERENCES

1 R. Marsboom, Toxicol. Appl. Pharmacol., 24 (1973) 371.
2 D.D. Heath and R.A.F. Chevis, Lancet, 2 (1974) 218.
3 W.S. Kammerer and D.M. Judge, Amer. J. Trop. Med. Hyg., 25 (1976) 714.
4 W.C. Campbell, R.O. McCracken and L.S. Blair, J. Amer. Med. Assoc., 230 (1974) 825.
5 A. Bekhti, J.-P. Schaaps, M. Capron, J.-P. Dessaint, F. Santoro and A. Capron, Brit. Med. J., 2 (1977) 1047.
6 P. Kern, M. Dietrich and K.-J. Volkmer, Tropenmed. Parasitol., 30 (1979) 65.
7 M.I. Al-Moslih, A.Y. Al-Rawas, S. Rassam, M.A. Al-Dabagh, T.A. Al-Janabi, M.A. Shafik and M.S. Al-Ani, Brit. Med. J., 2 (1978) 1435.
8 H.T. Goodman, Med. J. Aust., 2 (1976) 662.
9 W.E.G. Meuldermans, R.M.A. Hurkmans, W.F.J. Lauwers and J.J.P. Heykants, Eur. J. Drug Metab. Pharmacokinet., 1 (1976) 35.
10 J.P. Brugmans, D.C. Thienpont, I. van Wijngaarden, O.F. Vanparijs, V.L. Schuermans and H.L. Lauwers, J. Amer. Med. Assoc., 217 (1971) 313.
11 K.B. Alton, J.E. Patrick and J.L. McGuire, J. Pharm. Sci., 68 (1979) 880.
12 G. Karlaganis, G.J. Münst and J. Bircher, J. High Resol. Chromatogr. Chromatogr. Commun., 2 (1979) 141.

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CHROMBIO. 610

DETERMINATION OF AMITRIPTYLINE-N-OXIDE, AMITRIPTYLINE AND NORTRIPTYLINE IN SERUM AND PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KAREN MØLLER JENSEN

Biological Department, A/S DUMEX, 37, Prags Boulevard, DK-2300 Copenhagen S (Denmark)

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SUMMARY

A method for the determination of amitriptyline-N-oxide, amitriptyline and nortriptyline in serum and plasma has been developed. After extraction from serum or plasma the drugs were analysed by high-performance liquid chromatography.

The detection limit was 10 ng/ml (2 ml serum or plasma actually used). The coefficient of variation for all three compounds was below 10%.

Amitriptyline-N-oxide was found in rat plasma after an oral dose (10 mg/kg) of amitriptyline-N-oxide.

INTRODUCTION

During recent years a number of methods for the determination of tricyclic antidepressants in biological fluids have been published. Amitriptyline and nortriptyline (Fig. 1) have been measured by gas—liquid chromatography using flame-ionization detection [1—6] or alkali flame-ionization detection [7—13], and gas chromatography with electron-capture detection has also been applied [1, 14—16].

Mass fragmentographic methods are specific and often more sensitive than gas chromatographic methods, and this technique has been used for the determination of amitriptyline and nortriptyline [17—19]. Owing to the expensive equipment, however, many investigators prefer other methods, such as thin-layer chromatography.

Thin-layer chromatography may be used for the determination of amitriptyline and nortriptyline either by scanning the plates in situ [20—23], or by cutting off the spots followed by detection by fluorimetry [24]. Furthermore,

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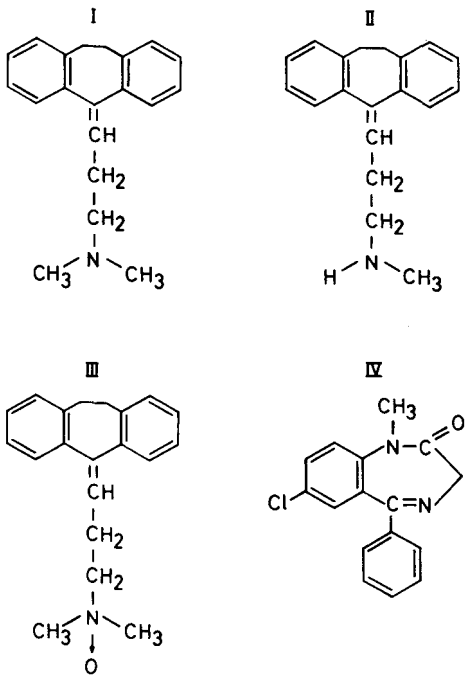

Fig. 1. Formulae of amitriptyline (I), nortriptyline (II), amitriptyline-N-oxide (III), and diazepam (IV).

it is possible to measure amitriptyline-N-oxide (Fig. 1) by thin-layer chromatography [25, 26].

Amitriptyline-N-oxide can also be determined by radioactivity measurements. Investigators have determined the drug in human plasma [27] and in plasma of rats and dogs [28] after a dose of ^{14}C-labelled amitriptyline-N-oxide. In other experiments amitriptyline-N-oxide was found as a metabolite in the urine of dogs [29] or of rats [30] after a dose of ^{14}C-labelled amitriptyline.

This paper describes a high-performance liquid chromatographic (HPLC) method for amitriptyline-N-oxide, amitriptyline and nortriptyline in serum and plasma. Several authors [31—39] have described HPLC methods for the determination of amitriptyline and nortriptyline in biological fluids. However, no such method for amitriptyline-N-oxide has been published so far.

EXPERIMENTAL

Materials and reagents

Hexane and dichloromethane were specially purified (nanograde) from Mallinckrodt (St. Louis, MO, U.S.A.). Acetonitrile (E. Merck, Darmstadt, G.F.R.) and methanol (Rathburn Chemicals, Walkerburn, Great Britain) were special HPLC grade. All other chemicals were analytical grade.

The tubes used for evaporation of hexane (tube I) and dichloromethane (tube II) were treated with a solution of 0.1% triethylamine in hexane and dried, and only used once.

The mobile phase for HPLC was membrane filtered (pore size 0.45 μm) and kept in an ultrasonic bath for 15 min immediately before use.

Amitriptyline and nortriptyline were available as hydrochlorides and amitriptyline-N-oxide as the base. All three compounds were synthesised by Synthesis Laboratories of A/S Dumex.

HPLC conditions

The apparatus consisted of a solvent delivery system, Model 6000 A (Waters Assoc.), a loop injection system U 6 K (Waters Assoc.), and a UV detector, Model 440, fixed wavelength of 254 nm (Waters Assoc.). The column was μBondapak C_{18} (30 cm × 3.9 mm, particle size 10 μm). Generally, the mobile phase was a mixture of acetonitrile and potassium dihydrogen phosphate (0.6% w/v) adjusted to pH 3 with phosphoric acid.

The mobile phase for the amitriptyline-N-oxide analysis was a mixture of 60% acetonitrile in 0.6% potassium dihydrogen phosphate. The flow-rate was 1.0 ml/min.

For the amitriptyline—nortriptyline analysis the mobile phase consisted of 50% acetonitrile in 0.6% potassium dihydrogen phosphate. The flow-rate was 0.9 ml/min.

Printing of the chromatograms and calculation of the peak areas were performed by an electronic integrator from Hewlett-Packard, Avondale, PA, U.S.A. (No. 3080). The peak heights of amitriptyline-N-oxide were measured and the peak areas for amitriptyline and nortriptyline were used.

Procedure

The extraction procedure is illustrated in Fig. 2. The internal standard (diazepam) was dissolved in dichloromethane before use.

The residue was dissolved in the mobile phase by shaking it in a whirlimixer immediately before injection into the chromatograph.

On the basis of calibration graphs constructed from control serum (plasma) containing known amounts of amitriptyline-N-oxide, amitriptyline and nortriptyline, the concentration of the three drugs in unknown samples was calculated.

Sampling

Male rats (Sprague—Dawley, weighing about 275 g) were fasted from the day before, but had free access to drinking water during the experiment.

A suspension of amitriptyline-N-oxide was given orally to the rats. The dose was 10 mg/kg. The rats were killed 0.5, 1, 2, 3, 4, 7, or 24 h after administration and heparinized blood was collected. The plasma was centrifuged off and kept at −18°C until analysed.

RESULTS

The calibration graph of amitriptyline-N-oxide in serum (serum concentration 50—250 ng/ml) was found to be satisfactory, but the best result was obtained using an internal standard (diazepam). The correlation coefficient was $r = 0.9559$ when the internal standard was not used and $r = 0.9828$ when the internal standard was used.

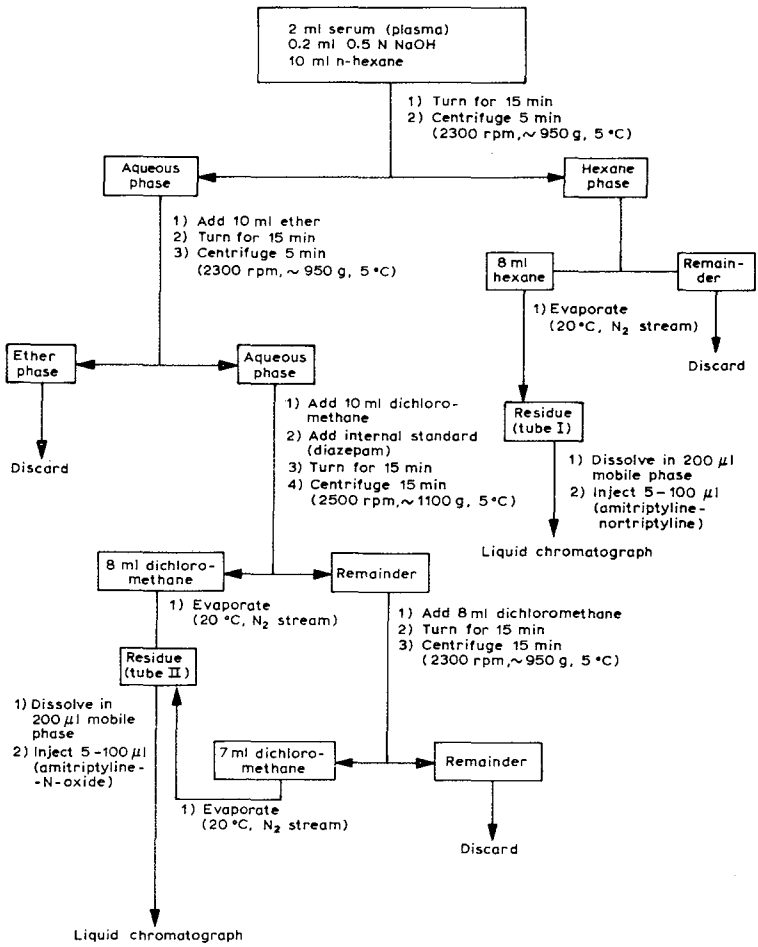

Fig. 2. Extraction scheme for amitriptyline-N-oxide, amitriptyline and nortriptyline.

Plotting the calibration graph of nortriptyline (50—400 ng/ml of serum) produced a correlation coefficient of $r = 0.9960$. Similarly, the correlation coefficient of amitriptyline was found to be $r = 0.9970$ (50—400 ng/ml of serum).

Fig. 3 shows a chromatogram of control serum (A) and of control serum with amitriptyline-N-oxide (200 ng in 2 ml) added (B). The retention time for amitriptyline-N-oxide was 5.2 min, and for diazepam (internal standard) 7.6 min.

Chromatograms of control serum (A) and of control serum with amitriptyline and nortriptyline (50 ng/ml) added (B) are shown in Fig. 4. The retention times were 6.1 min (amitriptyline) and 5.6 min (nortriptyline).

The reproducibility of the methods was tested for all three drugs (Table I). The coefficient of variation was found to be below 10%.

Extracted samples and standard solutions of amitriptyline-N-oxide were injected into the chromatograph, and the recovery of amitriptyline-N-oxide was found to be 75—80%.

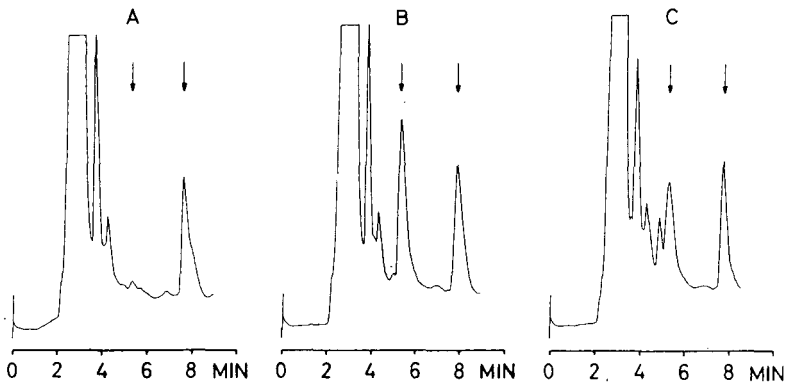

Fig. 3. Chromatograms of: (A) control serum; (B) control serum with amitriptyline-N-oxide (retention time 5.2 min) (100 ng/ml) added; and (C) rat plasma collected 30 min after an oral dose of amitriptyline-N-oxide (10 mg/kg). Internal standard: diazepam (retention time 7.6 min).

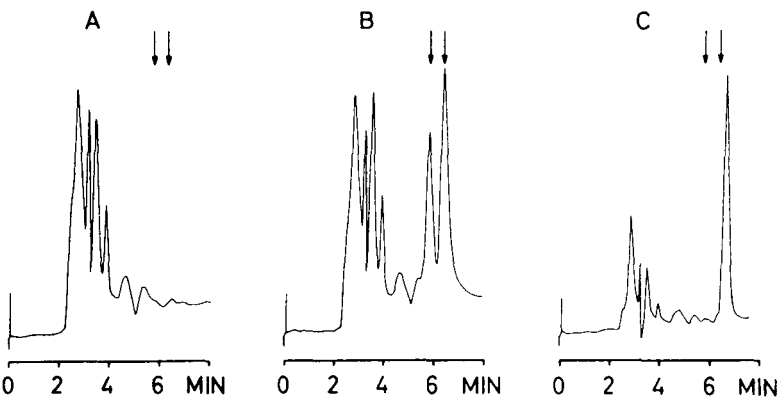

Fig. 4. Chromatograms of: (A) control serum; (B) control serum with amitriptyline (retention time 6.1 min) and nortriptyline (retention time 5.6 min) (50 ng/ml) added; and (C) rat plasma collected 30 min after an oral dose of amitriptyline-N-oxide (10 mg/kg). Peak with retention time 6.4 min: unknown.

The minimal concentration that can be determined is 10 ng/ml (2 ml actually used) for amitriptyline-N-oxide, amitriptyline and nortriptyline.

Various drugs which might interfere with the analysis were injected into the chromatograph; the retention times relative to amitriptyline-N-oxide are given in Table II.

Rat plasma

Fig. 3C shows a chromatogram of a rat plasma sample taken 30 min after an oral dose of amitriptyline-N-oxide, 10 mg/kg. The content of amitriptyline-N-oxide was calculated to be 270 ng/ml (500 μl of plasma were used).

The mean concentrations of amitriptyline-N-oxide in the rat plasma samples are illustrated in Fig. 5.

Fig. 4C shows a chromatogram of rat plasma, extracted and chromatographed as described for amitriptyline and nortriptyline. The plasma sample was drawn 30 min after an oral dose of amitriptyline-N-oxide, 10 mg/kg. No amitriptyline or nortriptyline was detectable in this sample.

TABLE I

REPRODUCIBILITY OF THE DETERMINATIONS OF AMITRIPTYLINE-N-OXIDE, AMITRIPTYLINE AND NORTRIPTYLINE

	No. of determinations	Added to 2 ml of serum (ng)	Found Mean (ng)	Found C.V.* (%)
Amitriptyline-N-oxide	6	90	96	8
	6	100	97	7
	6	150	153	6
	5	200	188	9
Amitriptyline	6	100	98	7
	5	150	146	8
	6	200	204	3
Nortriptyline	6	100	98	6
	5	150	146	8
	6	200	202	4

* C.V., coefficient of variation.

TABLE II

RELATIVE RETENTION TIMES FOR SOME COMPOUNDS

The chromatographic system for amitriptyline-N-oxide was used.

Compound	Relative retention time
Pindolole	0.52
Nortriptyline	0.77
Carbamazepine	0.78
Imipramine	0.81
Cyproheptadine	0.82
Amitriptyline	0.85
Nitrazepam	0.86
Imipramine-N-oxide	0.91
Amitriptyline-N-oxide	1.00
Desmethyldiazepam	1.00
Diazepam	1.32
2-Amino-5-nitrobenzophenone	1.42

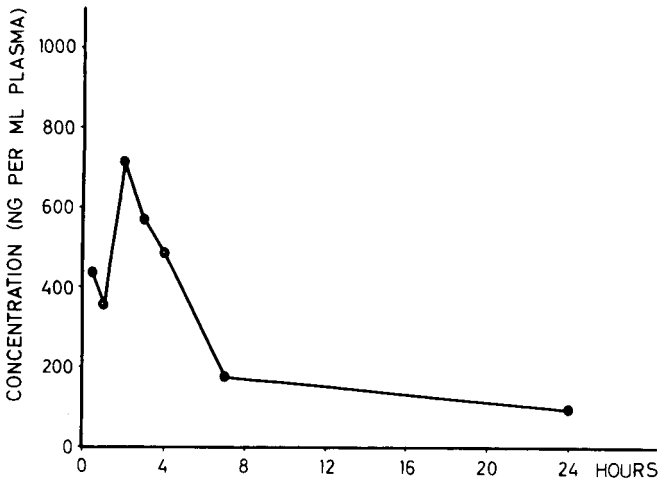

Fig. 5. Mean plasma concentration (n=3) of amitriptyline-N-oxide after an oral dose of amitriptyline-N-oxide (10 mg/kg) to rats.

DISCUSSION

In the present study amitriptyline, nortriptyline and amitriptyline-N-oxide were determined by HPLC without previous derivatization of the compounds.

The recovery of amitriptyline-N-oxide was found to be approximately 80% when the extracted drug was compared with standard solutions injected directly. About 10% was not extracted from the serum (checked by ^{14}C-labelled amitriptyline-N-oxide). The remainder (10%) may be lost by adsorption to glassware used during the analysis. Pretreatment with triethylamine to prevent this phenomenon is described by Jørgensen [10], who found a recovery of 83% (amitriptyline and nortriptyline). In the present study only the tubes containing the last residue were treated with triethylamine. Breyer-Pfaff et al. [26] found recoveries of 86% and 84% for amitriptyline-N-oxide and amitriptyline, respectively.

The reproducibility of the method for amitriptyline-N-oxide, amitriptyline and nortriptyline expressed as the coefficient of variation was found to be below 10%, which is comparable with the values reported by Breyer-Pfaff et al. [26], namely 4% (amitriptyline-N-oxide) and 9% (amitriptyline). The coefficient of variation was calculated by Jørgensen [10] to be 7.6% (amitriptyline and nortriptyline) and by Mellström and Braithwaite [37] to be 10% (amitriptyline) and 5% (nortriptyline).

In the present study the detection limit was 10 ng/ml of serum for all three compounds.

Santagostino et al. [25] 500 ng/ml the detection limit of amitriptyline-N-oxide in the urine to be reported. Breyer-Pfaff et al. [26] obtained a higher sensitivity (amitriptyline-N-oxide 15 ng/ml, amitriptyline 12 ng/ml), but they used 4 ml of plasma for the analysis. Jørgensen [10] stated that the lowest detectable concentration of amitriptyline was 5 ng/ml and of nortriptyline 10—15 ng/ml (2 ml plasma used), and Brodie et al. [32] reported a sensitivity of 5 ng/ml (amitriptyline and nortriptyline) using 4 ml of plasma.

For the determination of amitriptyline-N-oxide, diazepam was chosen as internal standard. It is separated from amitriptyline-N-oxide and suffers no interference from peaks of control serum. The internal standard was added immediately before the extraction with dichloromethane, and any possible diazepam content in the serum of patients was removed by hexane and ether before adding the internal standard. Desmethyldiazepam, if present, is also removed at this stage of the extraction procedure.

In the present study amitriptyline-N-oxide was found in rat plasma after an oral dose (10 mg/kg) of amitriptyline-N-oxide.

Amitriptyline-N-oxide is reported to be metabolised extensively in the rat [28], but neither amitriptyline nor nortriptyline were found in any of the plasma samples. An unknown peak, having a longer retention time than that of amitriptyline, may be a metabolite.

REFERENCES

1 S. Cooper, J.-M. Albert, R. Dugal, M. Bertrand and R. Elie, Arzneim.-Forsch., 29 (1979) 158.
2 G. Nyberg and E. Mårtensson, J. Chromatogr., 143 (1977) 491.
3 G.L. Corona and B. Bonferoni, J. Chromatogr., 124 (1976) 401.
4 P.C.N. Eichholtz, J. Chromatogr., 111 (1975) 456.
5 H.B. Hucker and S.C. Stauffer, J. Pharm. Sci., 63 (1974) 296.
6 J.E. Burch, M.A. Raddats and S.G. Thompson, J. Chromatogr., 162 (1979) 351.
7 J.N. Connor, G.F. Johnson and H.M. Solomon, J. Chromatogr., 143 (1977) 415.
8 H.B. Hucker and S.C. Stauffer, J. Chromatogr., 138 (1977) 437.
9 J. Vasiliades and K.C. Busch, Anal. Chem., 48 (1976) 1708.
10 A. Jørgensen, Acta Pharmacol. Toxicol., 36 (1975) 79.
11 L.A. Gifford, P. Turner and C.M.B. Pare, J. Chromatogr., 105 (1975) 107.
12 S. Reite, B. Salvesen and O. Skaug, Medd. Nor. Farm. Selsk., 37 (1975) 55.
13 S. Dawling and R.A. Braithwaite, J. Chromatogr., 146 (1978) 449.
14 P. Hartvig, S. Strandberg and B. Näslund, J. Chromatogr., 118 (1976) 65.
15 J.E. Wallace, H.E. Hamilton, L.K. Goggin and K. Blum, Anal. Chem., 47 (1975) 1516.
16 O. Borgå and M. Garle, J. Chromatogr., 68 (1972) 77.
17 R.G. Jenkins and R.O. Friedel, J. Pharm. Sci., 67 (1978) 17.
18 W.A. Garland, J. Pharm. Sci., 66 (1977) 77.
19 C.-G. Hammar, B. Alexanderson, B. Holmstedt and F. Sjöqvist, Clin. Pharmacol. Ther., 12 (1971) 496.
20 P. Haefelfinger, J. Chromatogr., 145 (1978) 445.
21 D.C. Fenimore, C.J. Meyer, C.M. Davis, F. Hsu and A. Zlatkis, J. Chromatogr., 142 (1977) 399.
22 U. Breyer and K. Villumsen, Eur. J. Clin. Pharmacol., 9 (1976) 457.
23 D.B. Faber, C. Mulder and W.A. Man in 't Veld, J. Chromatogr., 100 (1974) 55.
24 P.N. Kaul, L.R. Whitfield and M.L. Clark, J. Pharm. Sci., 67 (1978) 60.
25 G. Santagostino, R.M. Facino and D. Pirillo, J. Pharm. Sci., 63 (1974) 1690.
26 U. Breyer-Pfaff, M. Ewert and R. Wiatr, Arzneim.-Forsch., 28 (1978) 1916.
27 I. Midgley, D.R. Hawkins and L.F. Chasseaud, Arzneim.-Forsch., 28 (1978) 1916.
28 R.R. Brodie, L.F. Chasseaud, D.R. Hawkins and I. Midgley, Arzneim.-Forsch., 28 (1978) 1907.
29 H.B. Hucker, A.J. Balletto, J. Demetriades, B.H. Arison and A.G. Zacchei, Drug. Metab. Dispos., 5 (1977) 132.
30 E. Eschenhof and J. Rieder, Arzneim.-Forsch., 19 (1969) 957.
31 H.-J. Kuss and M. Nathmann, Arzneim.-Forsch., 28 (1978) 1301.

32 R.R. Brodie, L.F. Chasseaud and D.R. Hawkins, J. Chromatogr., 143 (1977) 535.
33 I.D. Watson and M.J. Stewart, J. Chromatogr., 132 (1977) 155.
34 J.C. Kraak and P. Bijster, J. Chromatogr., 143 (1977) 499.
35 J.H.M. van den Berg, H.J.J.M. de Ruwe, R.S. Deelder and Th.A. Plomp, J. Chromatogr., 138 (1977) 431.
36 I.M. Johansson, K.-G. Wahlund and G. Schill, J. Chromatogr., 149 (1978) 281.
37 B. Mellström and R. Braithwaite, J. Chromatogr., 157 (1978) 379.
38 J.H. Knox and J. Jurand, J. Chromatogr., 103 (1975) 311.
39 R.A. de Zeeuw and H.G.M. Westenberg, J. Anal. Toxicol., 2 (1978) 229.

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CHROMBIO. 618

SIMULTANEOUS DETERMINATION OF PHENYLBUTAZONE AND ITS METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TERUYOSHI MARUNAKA*, TOSHIAKI SHIBATA, YOSHINORI MINAMI and YUKIHIKO UMENO

Research Laboratory, Taiho Pharmaceutical Co. Ltd., Kawauchi-cho, Tokushima 771-01 (Japan)

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SUMMARY

A high-performance liquid chromatographic method was developed for the simultaneous determination of phenylbutazone and its metabolites, oxyphenbutazone and γ-hydroxyphenylbutazone, in plasma and urine. Samples were acidified with hydrochloric acid and extracted with benzene—cyclohexane (1:1, v/v). The extract was redissolved in methanol and chromatographed on a μBondapak C_{18} column using a mobile phase of methanol—0.01 *M* sodium acetate buffer (pH 4.0) in a linear gradient (50 to 100% methanol at 5%/min; flow-rate 2.0 ml/min) in a high-performance liquid chromatograph equipped with an ultraviolet absorbance detector (254 nm). The detection limit for phenylbutazone, oxyphenbutazone and for γ-hydroxyphenylbutazone was 0.05 μg/ml.

A precise and sensitive assay for the determination of phenylbutazone and its metabolites was established.

INTRODUCTION

Burns et al. [1] reported an ultraviolet (UV) method for the determination in plasma of phenylbutazone, which is widely used as an antihistaminic agent. Many studies using this UV method were then reported [2—9]. However, the method had many problems in sensitivity and in specificity of determination.

Recently, several methods using gas—liquid chromatography (GLC) [10—15] and high-performance liquid chromatography (HPLC) [16, 17] have been reported as having improved sensitivity and precision for the determination of phenylbutazone and its metabolites.

Midha et al. [12] determined phenylbutazone and oxyphenbutazone as methylated derivatives by GLC, but each compound gave two peaks on the chromatograph. Bruce et al. [13] determined simultaneously phenylbutazone

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and a heptaflurobutyrated derivative of oxyphenbutazone by GLC. Tanimura et al. [14] determined phenylbutazone and trimethylsilylated derivatives of oxyphenbutazone and γ-hydroxyphenylbutazone by GLC. This method was a two-step analysis, phenylbutazone being determined first and then the trimethylsilylated derivatives of two metabolites.

Pound and co-workers [16, 17], on the other hand, determined phenylbutazone and oxyphenbutazone by HPLC using an adsorption chromatography column.

We examined the simultaneous determination of phenylbutazone and its metabolites, oxyphenbutazone and γ-hydroxyphenylbutazone, in plasma and urine by HPLC using a reversed-phase chromatographic column. This report describes the precise and sensitive assay of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone in plasma and urine.

EXPERIMENTAL

Materials and reagents

Phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone were obtained from Esteve Products (Barcelona, Spain). Other chemicals used were all purchased from Wako Pure Chemicals (Osaka, Japan). Benzene, cyclohexane, chloroform and methanol were of liquid-chromatography grade.

A 0.01 *M* sodium acetate buffer (pH 4.0) used as a mobile phase for HPLC was prepared by dissolving 1.36 g of sodium acetate trihydrate and 2.36 ml of acetic acid in 1 l of ion-exchanged water.

Instruments

A Shimadzu Model LC-2 high-performance liquid chromatograph (Kyoto, Japan) equipped with a Model SIL-1A high-pressure injection valve, a Model UVD-2 detector (UV, 254 nm) and a Model GRE-2 gradient elution equipment was used.

A μBondapak C_{18} chromatographic column (30 cm × 4 mm I.D., 8—10 μm particle size) (Waters Assoc., Milford, MA, U.S.A.) was used for the separation and a pre-column (Permaphase ODS, 5 cm × 2.1 mm I.D., Shimadzu) was connected between the separation column and the injector. The mobile phase was a linear gradient (5%/min) of 50% methanol in 0.01 *M* sodium acetate buffer (pH 4.0) as the initial concentration and 100% methanol as the final concentration; the flow-rate was 2.0 ml/min. The column was maintained at room temperature, and compounds thus eluted were recorded by the detector at a constant wavelength of 254 nm; the attenuator was set at 0.04 a.u.f.s. Peak areas were determined by a Shimadzu Chromatopac Model 1A apparatus.

A JEOL Model JMS-01SG-2 mass spectrometer with an electron impact ion source (Tokyo, Japan) was used for identification of phenylbutazone and its metabolites. The mass spectrometric analysis was carried out under the following conditions: ionization energy 75 eV, ionization current 200 μA, and accelerating voltage 10 kV.

Extraction procedure

Blood samples were collected in heparinized containers and centrifuged to separate the plasma. The plasma (1.0 ml) was diluted with 1.0 ml of physiological saline, adjusted to pH 2.0 with 5 *N* HCl, and then shaken vigorously with 20 ml of benzene—cyclohexane (1:1, v/v) at room temperature for 20 min. After centrifugation at 2000 *g* for 5 min, the organic layer was separated. This extraction was repeated once more. The organic layer containing phenylbutazone and its metabolites was evaporated to a suitable volume under nitrogen gas at 30°C, then transferred to a test-tube (10 ml capacity) and dried under nitrogen gas. The residue was dissolved in 100 μl of methanol, and 20 μl of this solution were injected into the liquid chromatograph.

Urine was treated by the same procedure except that a 2.0-ml sample was used and then was extracted with 30 ml of benzene—cyclohexane.

Calibration curves

Calibration curves for the determination of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone by HPLC were prepared by adding known amounts of these compounds to plasma and urine, and assaying the mixture by the same extraction procedure; the peak areas were plotted against the concentrations of these compounds. As shown in Fig. 1, all these calibration curves were linear.

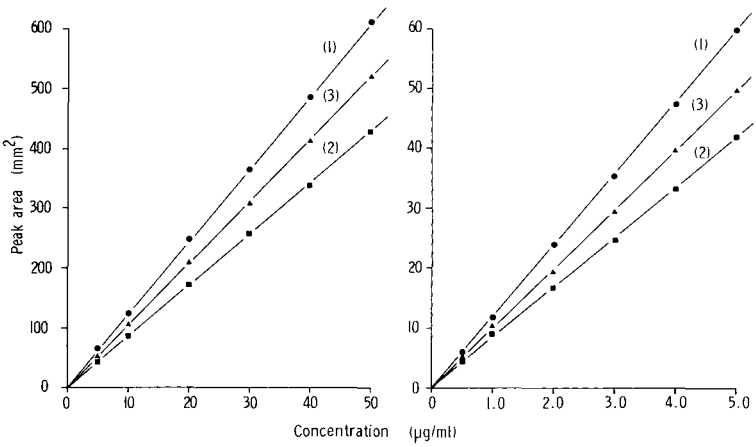

Fig. 1. Calibration curves of phenylbutazone (1), oxyphenbutazone (2) and γ-hydroxyphenylbutazone (3) extracted from plasma and urine. Calibration curves for the ranges 5—50 μg/ml and 0.5—5 μg/ml are presented.

RESULTS AND DISCUSSION

A reversed-phase chromatographic column, μBondapak C_{18}, was used for the separation. A mobile phase consisting of a linear gradient (0 to 100% methanol at 8%/min, flow-rate 2.0 ml/min) of methanol—water (mobile phase A), methanol—0.05 *M* KH_2PO_4 (mobile phase B) or methanol—0.01 *M* sodium acetate buffer (pH 4.0) (mobile phase C) was found to be suitable for the

separation of standard phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone. Furthermore, the separation patterns using a linear gradient (5%/min, flow-rate 2.0 ml/min) of 50% methanol as the initial concentration and 100% methanol as the final concentration in mobile phases A, B and C were investigated to obtain a satisfactory analysis time. As a result, the mobile phase C system showed good separation and analysis time.

Next, a suitable solvent for the extraction of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone from plasma and urine was investigated. Chloroform extracts under the acidic conditions of hydrochloric acid gave no interfering peaks from biological components on analysis using mobile phase A, but the same extract gave peaks due to biological components overlapping in elution time with phenylbutazone and oxyphenbutazone on analysis with mobile phase B or C. Benzene—cyclohexane (1:1, v/v) extracts under the acidic conditions of hydrochloric acid gave no interfering peaks on analysis with mobile phase A, B or C.

On the basis of the above results, benzene—cyclohexane (1:1, v/v) was used as extraction solvent for phenylbutazone and its metabolites from plasma and urine, and a linear gradient (50 to 100% methanol at 5%/min, flow-rate 2.0 ml/min) of methanol—0.01 *M* sodium acetate buffer (pH 4.0) was used as the mobile phase for reversed-phase HPLC for the subsequent experiments.

The chromatograms showing the separation of benzene—cyclohexane extract from rat plasma control and from rat plasma at 4 h after oral administration of phenylbutazone (100 mg/kg) are shown in Fig. 2; those from rat urine control and from rat urine at 6—12 h are shown in Fig. 3. Analysis time was 15 min. The retention times for phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone under the present HPLC conditions were 7.0, 6.1 and 4.9 min, respectively. Each fraction eluting from the HPLC column was separately

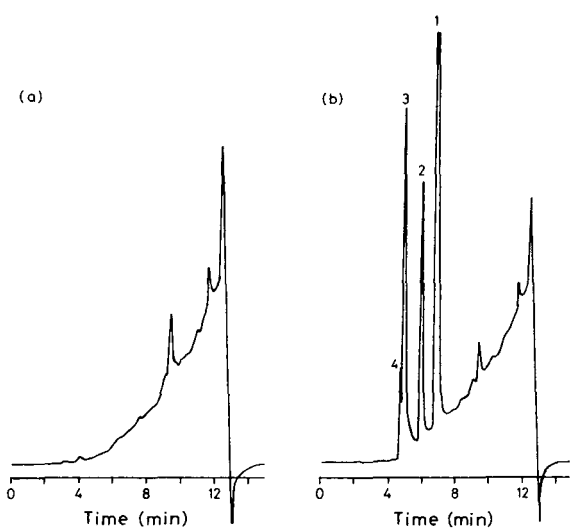

Fig. 2. Liquid chromatograms showing the separation of (a) control, and (b) phenylbutazone (1), oxyphenbutazone (2), γ-hydroxyphenylbutazone (3) and unknown metabolite (4) extracted from rat plasma. Details are described in the text.

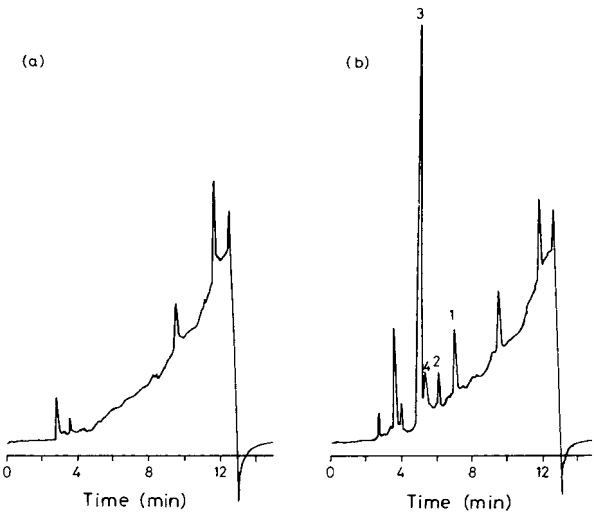

Fig. 3. Liquid chromatograms showing the separation of (a) control, and (b) phenylbutazone (1), oxyphenbutazone (2), γ-hydroxyphenylbutazone (3) and *p*,γ-dihydroxyphenylbutazone (4) extracted from rat urine. Details are described in the text.

collected and identified by mass spectrometry: phenylbutazone, *m/e* 308 (M^+), 252, 183, 105 and 77; oxyphenbutazone, *m/e* 324 (M^+), 268, 199, 93 and 77; γ-hydroxyphenylbutazone, *m/e* 324 (M^+), 309, 280, 266, 183, 83 and 77.

The detection limits under the present method were 0.05 μg/ml of plasma or urine for all compounds. The present method had an accuracy of ± 1.5—3.1% and a very good reproducibility.

Methanol was used as a sample solvent since phenylbutazone and its metabolites are all readily soluble in methanol and no decomposition at all was observed. Similar results were also obtained when water was used as a sample solvent.

Known amounts of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone were added to the plasma and urine, and then the recovery for each compound was examined. As shown in Table I, the overall recoveries of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone were 96.7 ± 1.7%, 93.1 ± 3.7% and 81.7 ± 4.2%, respectively.

In addition, *p*,γ-dihydroxyphenylbutazone has been found as a metabolite of phenylbutazone [18]. This metabolite showed a retention time of 5.4 min, and did not affect the separation of phenylbutazone and other metabolites. This corresponding peak was confirmed by gas chromatography—mass spectrometry as its trimethylsilylated derivative: *m/e* 556 (M^+), 541 ($M-CH_3$), 426, 334, 246, 181 and 73. This metabolite, however, could not be determined because of recovery problems under the present method.

The concentrations of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone in plasma after oral administration of 100 mg/kg phenylbutazone to male Wister rats (180 g body weight) determined by the present method were compared with those determined by GLC [14]. The results obtained are shown in Fig. 4. The overall difference between the HPLC and GLC methods was 4.8%.

TABLE I

RECOVERIES ON EXTRACTION OF PHENYLBUTAZONE, OXYPHENBUTAZONE AND γ-HYDROXYPHENYLBUTAZONE FROM PLASMA AND URINE

Each value is the mean of five determinations.

Compound	Added (μg/ml)	Recovery from plasma (%)	Recovery from urine (%)
Phenylbutazone	50.00	96.8	97.1
	25.00	96.3	96.5
	5.00	96.9	96.1
	0.50	96.7	96.8
	0.10	97.3	96.9
Mean ± S.D.		96.7 ± 1.7	
Oxyphenbutazone	50.00	92.8	92.7
	25.00	93.1	93.0
	5.00	93.3	93.0
	0.50	92.9	93.2
	0.10	93.7	93.5
Mean ± S.D.		93.1 ± 3.7	
γ-Hydroxyphenylbutazone	50.00	79.9	80.7
	25.00	81.9	81.1
	5.00	82.4	81.9
	0.50	81.9	82.6
	0.10	82.1	82.9
Mean ± S.D.		81.7 ± 4.2	

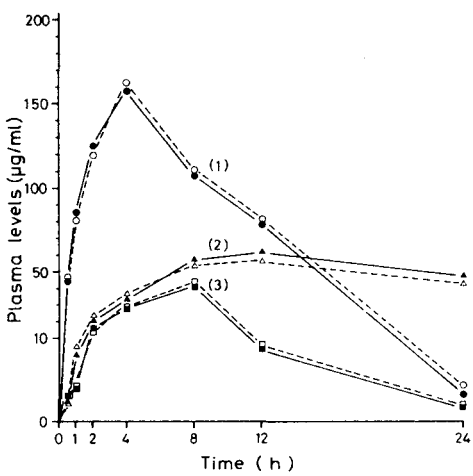

Fig. 4. Comparison of plasma levels of phenylbutazone (1), oxyphenbutazone (2) and γ-hydroxyphenylbutazone (3) determined by HPLC (●, ▲, ■) and GLC (○, △, □). The single oral dose given to male Wistar rats was 100 mg (0.3 mmol) phenylbutazone per kg.

The present assay method was then applied to the plasma and urine of other animals and man to which phenylbutazone and its metabolites were added. The results obtained for the chromatographic separation, recoveries, precision and sensitivities were in good agreement with those obtained with rat plasma and urine.

Furthermore, the conjugates of phenylbutazone and its metabolites [18] in urine could be determined using the same extraction procedure and under the same HPLC conditions as non-conjugated compounds after the following treatment. Sodium chloride was added to saturation to the aqueous layer after extraction of non-conjugated compounds with benzene—cyclohexane (1:1, v/v). The solution was extracted twice with fifteen volumes of ethyl acetate, and the organic layer containing the conjugates was evaporated to dryness under nitrogen gas. The residue was dissolved in 1/15 *M* sodium acetate buffer (pH 5.5), then 3000 units of β-glucuronidase and 10 units of sulfatase were added, and the mixture was incubated at 37°C for 3 h to convert the conjugates into non-conjugated compounds.

Phenylbutazone (100 mg/kg) was given orally to a beagle dog (9 kg body weight) and the concentrations of phenylbutazone and its metabolites, and their conjugates in urine, were determined using the present method. These results obtained are shown in Table II.

TABLE II

CUMULATIVE URINARY EXCRETION OF PHENYLBUTAZONE AND ITS METABOLITES, AND THEIR CONJUGATES AFTER ORAL ADMINISTRATION OF PHENYLBUTAZONE

Each value is the mean (± S.D.) of three determinations of molar per cent of dose. Dose administered was 100 mg/kg.

Compound	Time (h)			
	0—12	12—14	24—48	48—72
Phenylbutazone	0.42 ± 0.29	0.74 ± 0.16	1.35 ± 0.54	1.41 ± 0.52
Phenylbutazone conjugate	0.54 ± 0.32	1.01 ± 0.21	1.09 ± 0.18	1.09 ± 0.18
Oxyphenbutazone	0.00	0.05 ± 0.01	0.29 ± 0.10	0.29 ± 0.10
Oxyphenbutazone conjugate	0.39 ± 0.15	2.04 ± 0.44	3.39 ± 0.58	3.55 ± 0.62
γ-Hydroxyphenylbutazone	2.40 ± 1.60	3.78 ± 0.68	19.47 ± 0.60	20.70 ± 0.51
γ-Hydroxyphenylbutazone conjugate	0.13 ± 0.09	0.47 ± 0.16	1.01 ± 0.35	1.01 ± 0.35

The precision and sensitivity of the assay described appear to be satisfactory for the determination of the plasma and urine levels of phenylbutazone and its metabolites.

REFERENCES

1 J.J. Burns, R.K. Rose, T. Chenkin, A. Goldman, A. Schulert and B.B. Brodie, J. Pharmacol. Exp. Ther., 109 (1953) 346.
2 B. Hermann, Med. Exp., 1 (1960) 170.
3 G.R. Van Petten, H. Feng, R.J. Withey and H.F. Letten, J. Clin. Pharmacol., 11 (1971) 117.
4 B.G. Lukas, C.B. Borman and S.B. Zak, J. Pharm. Sci., 65 (1976) 86.
5 J.E. Wallace, J. Pharm. Sci., 57 (1968) 2053.
6 E. Jänchen and G. Levy, Clin. Chem., 18 (1972) 984.
7 M.W. Fuchs, Rheumatology, 16 (1964) 489.
8 H.M. Stevens, Clin. Chem., 16 (1970) 437.
9 R. Pulver, Schweiz. Med. Wochenschr., 80 (1957) 308.

10 R. Perego, E. Martinelli and P.C. Vanoni, J. Chromatogr., 54 (1971) 280.
11 I.J. McGilveray, K.K. Midha, R. Brien and L. Wilson, J. Chromatogr., 89 (1974) 17.
12 K.K. Midha, I.J. McGilveray and C. Charette, J. Pharm. Sci., 63 (1974) 1234.
13 P.B. Bruce, W.R. Maynard and L.K. Dunning, J. Pharm. Sci., 63 (1974) 446.
14 Y. Tanimura, Y. Saitoh, F. Nakagawa and T. Suzuki, Chem. Pharm. Bull., 23 (1975) 651.
15 A. Sioufi, F. Caudal and F. Marfill, J. Pharm. Sci., 67 (1978) 243.
16 N.J. Pound, I.J. McGilveray and R.W. Sears, J. Chromatogr., 89 (1974) 23.
17 N.J. Pound and R.W. Sears, J. Pharm. Sci., 64 (1975) 284.
18 W. Dieterle, J.W. Faigle, F. Früh, H. Mory, W. Theobald, K.O. Alt and W.J. Richter, Arzneim.-Forsch., 26 (1976) 572.

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CHROMBIO. 621

ANALYSIS OF ISOXAZOLYL PENICILLINS AND THEIR METABOLITES IN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H.H.W. THIJSSEN

Department of Pharmacology, University of Maastricht, Biomedical Centre, P.O. Box 616, 6200 MD Maastricht (The Netherlands)

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SUMMARY

A high-performance liquid chromatographic assay method to quantitate the isoxazolyl penicillins, their active metabolites, and their penicilloic acids in serum or urine is described. Separation and analysis is performed using reversed-phase chromatography. Urine samples, after the appropriate dilution, can be assayed directly. Serum samples (0.1 ml) are either extracted with methylene chloride or treated with perchloric acid—methanol. Serum levels as low as 0.4 μg/ml (extraction procedure) can be assayed accurately.

INTRODUCTION

In studying the disposition of antibiotics, an accurate and rapid assay procedure that differentiates between the different compounds or metabolites is preferred over the well-known bioassay, especially when it is recognized that active metabolites are formed. High-performance liquid chromatography (HPLC), particularly the reversed-phase mode, has found wide application as an analytical tool to quantitate, among others, antibiotic drugs in biological fluids [1—5].

The formation of active metabolites of the isoxazolyl penicillins (the active metabolites are the 5-hydroxymethyl derivatives of the parent compounds [6]) has been described in some detail and it was shown that in anephric patients the active metabolite may represent 40—50% of the total penicillin activity [7]. The parent compound and the metabolite were assayed by bioassay after separation by reversed-phase thin-layer chromatography. A further investigation of the clinical relevance of the formation of active metabolites of the iso-

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xazolyl penicillins in anephric patients [8] prompted the development of a rapid and reliable assay technique. This paper describes a reversed-phase HPLC method to determine the isoxazolyl penicillins, their 5-hydroxymethyl metabolites and their penicilloic acids in plasma, serum or urine.

EXPERIMENTAL

HPLC instrumentation and conditions

The HPLC system consisted of a Waters M-6000A pump equipped with a U6K injector. UV absorbance at 220 nm was monitored with a Perkin-Elmer LC-55 spectrophotometer. A Lichrosorb RP-8 column (particle size 5 μm; 250 mm × 4.6 mm I.D.; Chrompack, Middelburg, The Netherlands) was used in combination with a guard column (ODS pellicular material; 50 mm × 2.1 mm I.D.) to extent life time. The mobile phase was either a buffer—methanol or a buffer—acetonitrile mixture.

Materials

Sodium oxacillin and sodium dicloxacillin were gifts of Bristol Labs. (The Netherlands); sodium cloxacillin and sodium flucloxacillin were gifts of Beecham Research Labs. (The Netherlands). The active metabolites were isolated from rat urine as described previously [6]. The corresponding penicilloic acids were prepared by treating solutions of the isoxazolyl penicillins with alkaline (50 μl 1 *N* sodium hydroxide to 1 mg of the penicillin dissolved in 1 ml of water) for 30 min at room temperature whereafter the reaction mixture was neutralized with hydrochloric acid.

Sample preparation

Samples were analyzed with the aid of an internal standard. One of the isoxazolyl penicillins was added to the sample before the work-up procedure. Plasma (serum) samples were either treated with methanol—perchloric acid or extracted with methylene chloride. In the former case, to 100 μl sample spiked with the internal standard, 300 μl of ice cold 0.05 *N* perchloric acid in methanol was added. The mixture was allowed to stand for 10 min in ice. Aliquots of the mixture, clarified by centrifugation at 0°C, were injected. In the extraction procedure, to 100 μl sample spiked with the internal standard, 100 μl citric acid buffer (0.5 *M*, pH 2.2) and 20 μl 0.5 *N* hydrochloric acid were added. Extraction was performed with 2.5 ml methylene chloride. The organic phase was evaporated to dryness at 35°C by a stream of nitrogen. The residue was redissolved in an appropriate volume of mobile phase. Urine samples were diluted accordingly with water.

RESULTS AND DISCUSSION

Good resolution of the isoxazolyl penicillins and their 5-hydroxymethyl metabolites could be achieved on LiChrosorb RP-8 with either buffer—methanol mixtures [for instance: 0.02 *M* sodium acetate (pH 5.5)—methanol (10:8, v/v)] or buffer—acetonitrile mixtures [for instance: 0.02 *M* ammonium acetate (pH 6.6)—acetonitrile (100:34, v/v)]. The former mobile phase system only was

TABLE I

CAPACITY FACTORS, k', OF THE ISOXAZOLYL PENICILLINS, THE 5-HYDROXYMETHYL METABOLITES (M) AND THE PENICILLOIC ACIDS (PA)

Compound	Mobile phase*	
	I	II
Oxacillin	2.70	1.92
M	1.43	0.96
PA	0.86	–
Cloxacillin	3.55	2.95
M	1.80	1.46
PA	1.20	–
Flucloxacillin	4.00	4.19
M	2.10	2.19
PA	1.53	–
Dicloxacillin	6.00	6.23
M	2.70	2.96
PA	2.30	–

* I, 0.02 *M* sodium acetate (pH 5.5)—methanol (10:8, v/v); II, 0.02 *M* ammonium acetate (pH 6.6)—acetonitrile (100:34, v/v).

suitable for analysing the penicilloic acids. In the buffer—acetonitrile mobile phase the penicilloic acids eluted with the unretained solute (Table I). At first these results made it obvious to select the buffer—methanol system. Once in use, however, some disadvantages were discovered. Besides a higher working pressure, the main problem appeared to be the instability of the penicillins. Fig. 1 shows the degradation of cloxacillin with time. The reaction product, which probably was the monomethyl ester of the α-penicilloate [9], was less

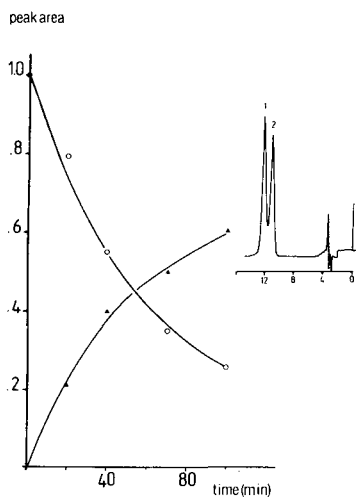

Fig. 1. Degradation of cloxacillin in methanol. Cloxacillin solution (100 μl) (1 mg cloxacillin in 1 ml 0.9% sodium chloride in water) was diluted with methanol to 1 ml. At different time intervals aliquots were injected for analysis. Cloxacillin, O; product, ▲, Insertion: chromatogram of the reaction mixture after 40 min at room temperature. Cloxacillin, 1; product, 2. Chromatographic conditions: mobile phase, 0.02 *M* sodium acetate (pH 5.5)—methanol (100:75, v/v); flow-rate, 1.2 ml/min; timescale in minutes.

retained on the column. The observed methanolic degradation was slowed down considerably in the cold.

The extent of the detector response per absolute amount (expressed in peak area) at 220 nm appeared to be the same for the isoxazolyl penicillins (Table II). UV absorption per mass equalled the parent compounds for the 5-hydroxymethyl metabolites and the penicilloic acids. It was therefore obvious to use one of the isoxazolyl penicillins as the internal standard for the sample analysis.

TABLE II

DETECTOR RESPONSE (PEAK AREA, mm^2) AT 220 nm

Chromatographic conditions: mobile phase, 0.02 *M* ammonium acetate (pH 6.6)—acetonitrile (100:34, v/v); flow-rate, 1 ml/min; detector sensitivity, 0.01 a.u.f.s.; chart speed, 5 mm/min.

Amount injected (ng)	Penicillin*			
	O	Cl	FCl	Cl_2
40	144	140	144	146
100	358	361	362	361
500	1785	1838	1822	1800

* Oxacillin, O; cloxacillin, Cl; flucloxacillin, FCl; dicloxacillin, Cl_2.

Plasma or serum samples in which the amount of penicilloic acid was also to be analyzed were handled by the methanol—perchloric acid treatment (see Experimental). The extraction procedure, which resulted in much cleaner samples, could not be applied because the recovery of penicilloic acids was shown to be poor and most often degradation was observed due to the acid treatment [9]. When the serum penicilloic acid levels were not important, the methylene chloride extraction procedure was used. By preference, the buffer—acetonitrile system was chosen as the mobile phase.

Examples of chromatograms obtained under different chromatographic conditions from biological specimens containing isoxazolyl penicillin and its metabolic products are shown in Fig. 2.

Using the internal standard procedure, the accuracies for the different sample preparation methods were 100 ± 16 (S.D., n = 12, concentrations $\geqslant$ 1 μg/ml) and 100 ± 6 (S.D., n = 11, concentrations $\geqslant$ 0.4 μg/ml) for the methanol—perchloric acid method and the extraction method, respectively. Serum concentration—time profiles and the cumulative excretions of cloxacillin and flucloxacillin and their respective metabolites after their oral administration to a healthy fasting individual (1.5 g in capsules) are shown in Fig. 3. The same volunteer participated in these experiments. The time between the first and second experiment was two weeks. As can be seen, the formation of the 5-hydroxymethyl metabolites was low. Serum levels were low (peak ratios for metabolite and parent compound 1/16 and 1/25 for cloxacillin and flucloxacillin, respectively) and small amounts were excreted in urine (10% or less of the total penicillin amount).

The amount of the penicilloic acids excreted in urine over the time period of collection was even less. The serum levels, however, appeared to be higher and, most striking, their half-lives were at least twice as long as the half-lives of the

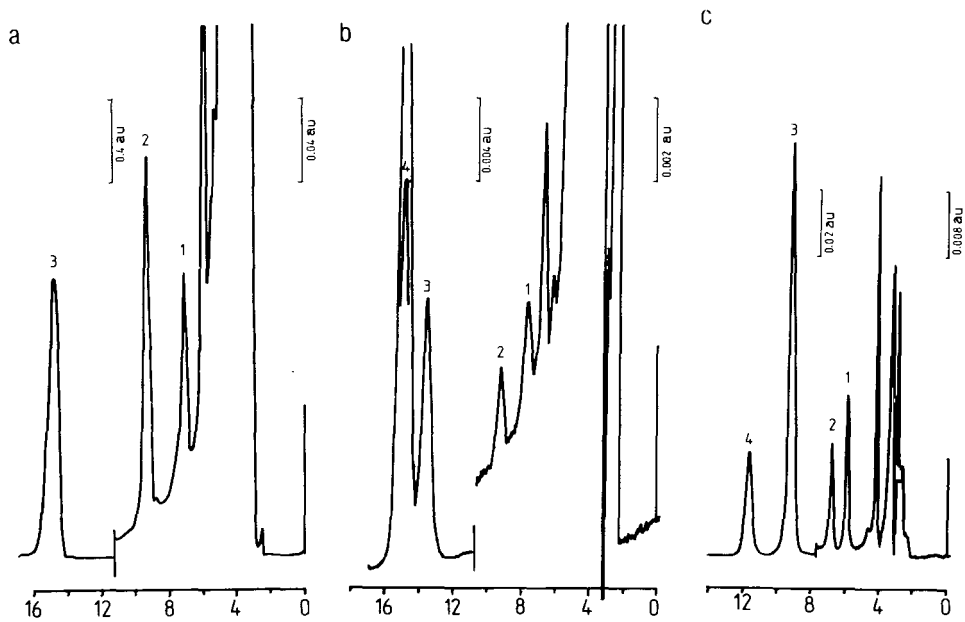

Fig. 2. Chromatograms of (a) urine sample after the oral administration of flucloxacillin. The urine sample was diluted twenty fold with water, 20 μl were injected; peaks: 3, flucloxacillin, 1320 μg/ml; 2, 5-hydroxymethyl metabolite, 122 μg/ml; 1, penicilloic acid, 54 μg/ml. Chromatographic conditions: mobile phase, 0.02 *M* sodium acetate (pH 5.5)—methanol (10:8, v/v), flow-rate 0.8 ml/min; timescale in minutes. (b) Serum sample after the oral administration of flucloxacillin. 100 μl serum, spiked with cloxacillin, 10 μg/ml, was treated with 300 μl 0.05 *N* perchloric acid in methanol (see Experimental); peaks: 4, flucloxacillin, 35.2 μg/ml; 3, cloxacillin, 10 μg/ml; 2, 5-hydroxymethyl metabolite, 1.3 μg/ml; 1, penicilloic acid, 1.5 μg/ml. Chromatographic conditions as in (a). (c) Serum sample after the oral administration of cloxacillin. 100 μl serum, spiked with flucloxacillin, and oxacillin, (5 μg/ml and 1 μg/ml respectively), were extracted with methylene chloride (see Experimental); peaks: 4, flucloxacillin, 5 μg/ml; 3, cloxacillin, 14.6 μg/ml; 2, oxacillin, 1 μg/ml; 1, 5-hydroxymethyl metabolite, 1.3 μg/ml. Chromatographic conditions: mobile phase, 0.02 *M* ammonium acetate (pH 6.6)—acetonitrile (100:34, v/v); flow-rate, 0.8 ml/min; timescale in minutes.

parent compounds (for instance, 170 and 69 min for the penicilloic acid and cloxacillin, respectively). A clear picture of the fate of the penicilloic acids in plasma (serum) after the administration of a penicillin is not yet known. Cole et al. [10] assumed on the basis of the prolonged urinary excretion of the penicilloic acids that their plasma half-lives should be longer than the half-lives of the corresponding penicillins.

Hellström et al. [11] observed a plasma radioactivity half-life of about 150 min after the administration of [^{35}S]cloxacillin which is compatible with the serum half-life of the cloxacillin penicilloic acid found in the present study.

Some relevant pharmacokinetic data, describing the experiments from Fig. 3, are summarized in Table III. Although the data are from one subject only, the kinetic parameters show that one of the factors determining the higher serum levels after an oral dosage of flucloxacillin as compared with cloxacillin is the slower elimination of the former (compare renal clearances of the penicillins and the area under the curve values of the penicilloic acids). These findings are in agreement with earlier data [12, 13].

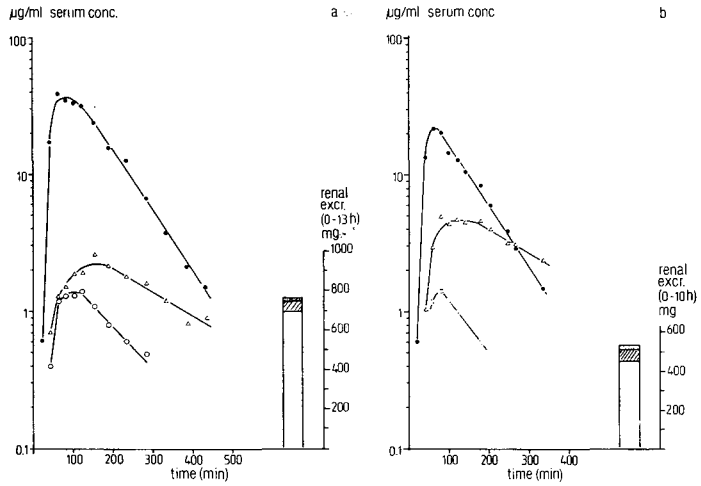

Fig. 3. Serum concentration—time profile and cumulative renal excretion of (a) flucloxacillin and its metabolites after an oral dose of flucloxacillin (1.5 g, capsules); serum: flucloxacillin, •; 5-hydroxymethyl metabolite, O; penicilloic acid, △ and urine: flucloxacillin, □; 5-hydroxymethyl metabolite, ▨; penicilloic acid, ▣. (b) Cloxacillin and its metabolites after an oral dose of cloxacillin (1.5 g, capsules); serum: cloxacillin, •; 5-hydroxymethyl metabolite, ○; penicilloic acid, △ and urine: cloxacillin, □; 5-hydroxymethyl metabolite, ▨; penicilloic acid, ▣.

TABLE III

PHARMACOKINETIC PARAMETERS OF CLOXACILLIN AND FLUCLOXACILLIN AFTER ORAL ADMINISTRATION (FIG. 3)

M = 5-hydroxymethyl metabolite, PA = penicilloic acid. The serum half-life ($t_{½}$) was estimated from the elimination phase by the least-squares method. The area under the curve (AUC) was estimated by the trapezoidal rule. The area from the last serum data to infinity was calculated by $C_t \times t_{½} \times (\ln 2)^{-1}$. Renal clearance was estimated by dividing the amount excreted in urine by AUC.

Compound	$t_{½}$ (min)	AUC (µg/ml·min)	Renal clearance (ml/min)	fr*
Cloxacillin	69	2913	160	31.0
M	78	217	220	3.2
PA	170	1675	17	2.3
Flucloxacillin	68	7300	96	46.5
M	76	285	166	3.2
PA	170	819	26	1.4

* The percentage of the dose cumulatively excreted in the urine.

CONCLUSIONS

The HPLC analysis for the determination of the isoxazolyl penicillins, their 5-hydroxymethyl metabolites and their penicilloic acids is a simple, sensitive and reliable method. Its advantages over microbiological assays is evident because of its ability to differentiate between the various antibacterial active species in a sample.

REFERENCES

1 B.B. Wheals and I. Jane, Analyst (London), 102 (1977) 625.
2 I. Nilsson-Ehle, Acta Pathol. Microbiol. Scand. Sect. B Suppl., 259 (1977) 61.
3 R.P. Bush, T.E. Maxim, N. Allen, T.A. Jacob and F.J. Wolf, J. Chromatogr., 99 (1974) 609.
4 T.B. Vree, Y.A. Hekster, A.M. Baars and E. van der Kleyn, J. Chromatogr., 145 (1978) 496.
5 I. Nilsson-Ehle and P. Nilsson-Ehle, J. Infect. Dis., 139 (1979) 712.
6 H.H.W. Thijssen, J. Antibiot., 32 (1979) 1033.
7 H.H.W. Thijssen and H. Mattie, Antimicrob. Ag. Chemother., 10 (1976) 441.
8 In preparation.
9 J.P. Hou and J.W. Poole, J. Pharm. Sci., 60 (1971) 503.
10 M. Cole, M.D. Kenig and V.A. Hewitt, Antimicrob. Ag. Chemother., 3 (1973) 463.
11 K. Hellström, A. Rosén and A. Swahn, Eur. J. Clin. Pharmacol., 7 (1974) 125.
12 J.E. Rosenblatt, A.C. Kind, J.L. Brodie and W.M.M. Kirby, Arch. Int. Med., 121 (1968) 345.
13 E.H. Nauta and H. Mattie, Brit. J. Clin. Pharmacol., 2 (1975) 111.

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Note

High-performance liquid chromatography of 13-*cis*-retinoic acid and of endogenous retinol in human plasma

JEAN-GUY BESNER* and ROBERT LECLAIRE

Faculty of Pharmacy, University of Montreal, P.O. Box 6128, Montreal, Quebec H3C 3J7 (Canada)

and

PIERRE R. BAND

Institut du Cancer de Montréal, Montreal (Canada)

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Retinoids (vitamin A and analogues) are essential for normal growth and differentiation of epithelial tissues [1], and possess prophylactic and therapeutic activity in a variety of chemically induced epithelial cancers in experimental animal tumor systems [2—4]. To circumvent the toxicity of

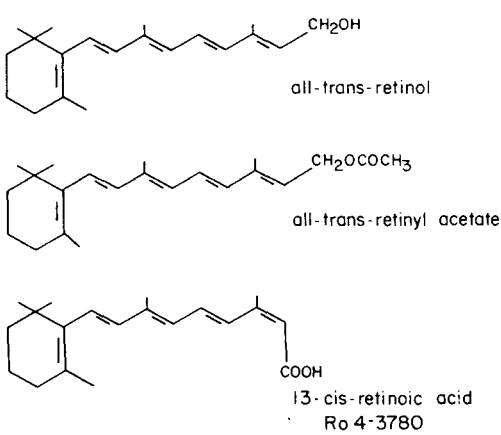

Fig. 1. Chemical structures of assayed retinoids.

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pharmacologic doses of natural retinoids, less toxic derivatives have been synthesized [5]. The synthetic structural analogue of all-*trans*-retinoic acid (RA), 13-*cis*-retinoic acid (13-*cis*-RA), has been shown to be less toxic than the parent compound [6], to be effective in the prevention of lung and bladder carcinogenesis in rodents [7, 8], and has been used successfully in the treatment of various dermatoses in man [9—11]. In view of the potential chemopreventive effect of 13-*cis*-RA in human epithelial cancer [12], we have undertaken a clinical Phase I pharmacologic and toxicologic study of 13-*cis*-RA, and have modified the method of Frolik et al. [13] to assay the drug. This report describes a sensitive and specific high-performance liquid chromatographic (HPLC) assay for the simultaneous determination of 13-*cis*-RA and of endogenous plasma retinol (R-OL), using all-*trans*-retinyl acetate as the internal standard (Fig. 1).

MATERIALS AND METHODS

All manipulations were carried out in the dark allowing only slight indirect bulb light. Fluorescent light was totally avoided to prevent photoisomerisation reactions.

Reagents

All solid chemicals used were of reagent grade; solvents used for extraction and chromatography were of chromatoquality (99%) grade and were purchased from Canlab Ltd., Montreal, Canada.

Columns

The columns were 250 mm × 3.2 mm I.D. stainless steel containing 10 μm Altex LiChrosorb C_{18} reversed-phase packing.

Instruments

A Perkin-Elmer Model 601 dual-pump high-performance liquid chromatograph was used, equipped with a Rheodyne injector permitting injection of volumes between 1 and 150 μl.

The detector was a Perkin-Elmer Model LC-55 set at 350 nm, and the recorder a Perkin-Elmer Model 56 set at 1 or 2 mV. Peak areas were calculated with a Varian integrator Model 485 equipped with filtering and baseline tracking devices.

Mobile phase

Throughout the procedures, the mobile phase was constituted of a mixture of acetonitrile and water containing 1% of ammonium acetate (80:20). The flow-rate was 1.5 ml/min, keeping the pressure at 70—105 bars, well below the critical pressure of 205 bars.

Analytical standards

All-*trans*-R-OL, all-*trans*-RA, and all-*trans*-retinyl acetate were purchased from Eastman Kodak, Rochester, NY, U.S.A.; 13-*cis*-RA (RO-4-3780) was

obtained from Hoffmann-LaRoche, Nutley, NJ, U.S.A. All these substances have a purity greater than 99%.

Preparation of standards

Stock solutions of all retinoids were made by diluting 10 mg of pure substance in 100 ml of methanol; these solutions were stable for over four months. Working standard solutions of 1 mg% were made by dilution with methanol and discarded after single use.

Buffer

An acetate buffer of 0.2 *M* was made by adding 12 ml of 0.2 *M* to 700 ml of water; the pH was adjusted to 3.00 with 0.2 *M* sodium hydroxide, and the volume completed to 1 liter.

Extraction procedure

To 15-ml Teflon screw-cap tubes containing 1 ml of plasma, 0.5 μg of retinyl acetate as internal standard was added to 1 ml of methanol. The plasma was vortexed for 15 sec to ensure good protein precipitation; 1 ml of buffer was then added to 2 ml of extracting solvent containing hexane—methylene chloride—isopropanol (80:19:1, v/v), and the tubes were vortexed for 1 min and centrifuged. The organic layer was removed and the extraction procedure repeated on the residual plasma fraction. The two organic fractions were then combined and evaporated under dry nitrogen at 45° C in the dark. The residue was dissolved in 100 μl of acetonitrile and a 10-μl aliquot was used for the assay.

Calibration curve

To aliquots of 1 ml of plasma, previously tested for the absence of interfering substances, were added 0, 50, 100, 150, 200, 300, 500, and 700 ng of R-OL and of 13-*cis*-RA; to each tube 500 ng of all-*trans*-retinyl acetate were also added. Analysis at each of these concentrations was repeated five times.

RESULTS

The individual values obtained for the construction of standard curves are shown in Table I. Standard deviations at each of the concentrations studied were less than 10% of the mean for both 13-*cis*-RA and R-OL. Routine analyses performed on different days of plasma spiked with standards were also within the S.D. indicated in Table I. The coefficients of regression were 0.99 for both 13-*cis*-RA and R-OL calibration curves. To allow for a more quantitative determination of the tested compounds and to circumvent biases induced by possible variations of peak shapes over time, surface areas instead of peak heights were evaluated. Comparisons of peak areas of spiked plasma extracts of R-OL, 13-*cis*-RA, and all-*trans*-retinyl acetate with standards injected under the same conditions indicated a recovery from plasma of 85 ± 10% for each substance.

Typical chromatograms obtained with blank plasma, and with plasma from a patient who received a single oral dose of 60 mg (35 mg/m^2) of 13-*cis*-RA are shown in Fig. 2. The peaks are well defined with no extraneous substance

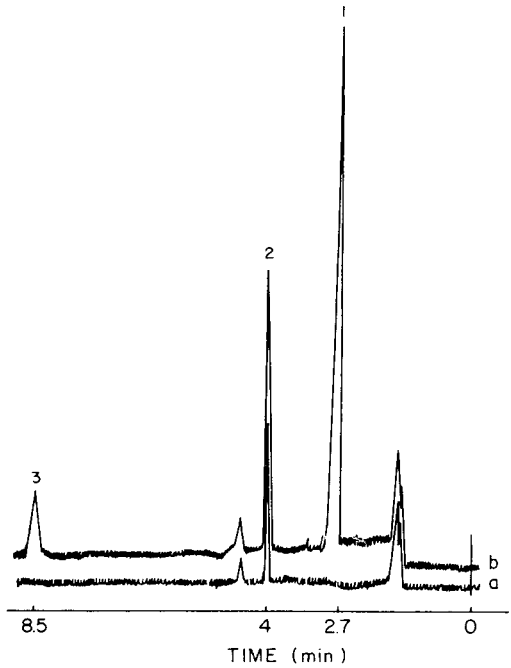

Fig. 2. Chromatograms of the HPLC analysis of (a) control plasma containing endogenous all-*trans*-retinol (peak 2); and (b) plasma of a patient treated with a single oral dose of 60 mg of 13-*cis*-retinoic acid (peak 1); peak 3 represents the internal standard (all-*trans*-retinyl acetate).

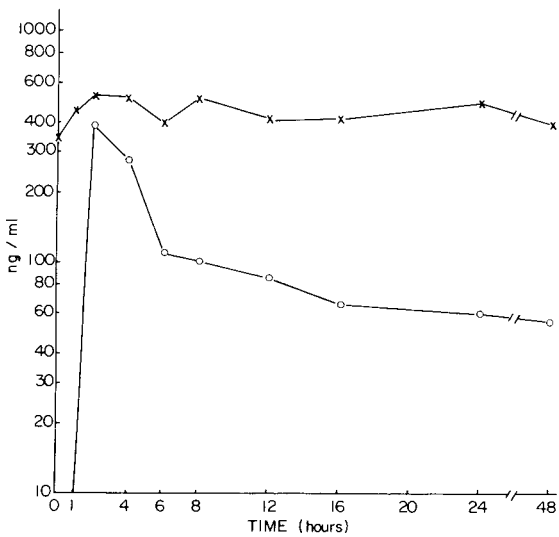

Fig. 3. Plasma levels of endogenous retinol (x) and of 13-*cis*-retinoic acid (o) in a patient receiving a single dose of 60 mg of 13-*cis*-retinoic acid.

TABLE I

SURFACE RATIOS OF PLASMA 13-*CIS*-RETINOIC ACID AND OF ALL-*TRANS*-RETINOL OVER INTERNAL STANDARD OBTAINED FOR THE CALIBRATION CURVES

Amount of retinoids added to plasma (ng/ml)	Surface ratios			
	All-*trans*-retinol / Internal standard		13-*cis*-retinoic acid / Internal standard	
	Values	Mean ± S.D.	Values	Mean ± S.D.
0	1.26, 1.21, 1.17 1.12, 1.25*	1.20* ± 0.06		
50	1.52, 1.38, 1.64 1.72, 1.61	1.57 ± 0.13	0.35, 0.38, 0.32 0.31, 0.30	0.33 ± 0.03
100	1.95, 1.82, 2.20 2.30, 2.28	2.11 ± 0.21	0.56, 0.57, 0.54 0.67, 0.56	0.58 ± 0.05
150	2.35, 2.30, 2.48	2.44 ± 0.15	0.76, 1.04, 0.82 0.69, 0.80	0.83 ± 0.13
200	2.80, 2.70, 2.64 3.02, 2.88	2.81 ± 0.15	1.16, 1.20, 1.12	1.14 ± 0.05
300	3.24, 3.34, 3.42 2.98, 3.18	3.23 ± 0.17	1.72, 1.75, 1.73 1.60, 1.53	1.67 ± 0.10
400	3.72, 3.89, 4.02 3.81, 3.59	3.81 ± 0.17	2.33, 2.34, 2.25 2.17, 2.21	2.26 ± 0.07
500	4.83, 4.79, 4.62 4.55, 4.52	4.66 ± 0.14	2.72, 2.72, 2.50 2.83, 3.03	2.76 ± 0.19
700	6.08, 5.98, 5.65 5.96, 5.87	5.91 ± 0.16	4.10, 4.10, 3.29 4.08, 3.84	3.88 ± 0.35

*In the standard curves these values, corresponding to endogenous plasma all-*trans*-retinol, were subtracted from all other ratios.

interfering with the assay. Fig. 3 illustrates the pharmacokinetics of 13-*cis*-RA in the same patient. The drug is absorbed rapidly and follows a bicompartmental model with a distribution and an elimination phase. Endogenous plasma R-OL concentrations are also shown.

DISCUSSION

All-*trans*-retinyl acetate, a closely related structural analogue of R-OL and 13-*cis*-RA, was chosen as the internal standard because its elution time is long and occurs in a region of the chromatogram free from interference. Although the maximum UV absorbance of retinyl acetate is at 326 nm, sensitivity at 350 nm is sufficient to obtain good surface ratios with the retinoids studied.

The method outlined is rapid and accurate with a limit of sensitivity of about 25 ng/ml for both R-OL and 13-*cis*-RA. All technical procedures, however, must be performed in the dark as degradation occurs when plasma extracts are submitted to light. Also, one must ensure that the patients avoid the intake of exogenous vitamin A preparations, particularly those containing retinyl acetate.

The HPLC assay reported in this paper differs from the method originally described by Frolik et al. [13]. Firstly, the extraction procedure is directly performed on plasma without prior lyophilization. The use of a solvent—solvent partition system gives a clear separation of the retinoids assayed from interfering substances which are rapidly eluted in the chromatogram, and the analytical separation procedure is completed within 10 min (Fig. 2). Secondly, to avoid variations in peak shapes and elution times, the column pressure was kept below 105 bars. This enabled us to inject about 100 samples before changing the inlet frit to ensure quality and reproducibility of results. All-*trans*-RA has a retention time of 3.5 min (not shown in Fig. 2) just between those of R-OL and of 13-*cis*-RA. Due to the possibility of photoisomerisation between 13-*cis*-RA and all-*trans*-RA, an endogenous metabolite of R-OL present in the plasma [14], the separation of both isomers is of importance. Further, Keilson et al. [15] have noted a decrease in endogenous plasma R-OL in rats deficient in vitamin A and given all-*trans*-RA. In our clinical study [16] we have observed a similar decrease in plasma R-OL in some patients chronically treated with 13-*cis*-RA. Our analytical method provides the means to study the kinetic parameters of 13-*cis*-RA, to assay R-OL and all-*trans*-RA, and to evaluate the possible interactions between these retinoids. The procedure, rapid and practical, is particularly suitable for multiple sample analysis.

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REFERENCES

1 T. Moore, in W.H. Sebrell and R.S. Harris (Editors), The Vitamins, Vol. 1, Academic Press, New York, 2nd ed., 1967, p. 245.
2 E.W. Chu and R.A. Malmgren, Cancer Res., 25 (1965) 884.
3 U. Saffiotti, R. Montesano, A.R. Sellakumar and S.A. Borg, Cancer, 20 (1967) 857.
4 R.C. Moon, C.J. Grubbs and M.B. Sporn, Cancer Res., 36 (1976) 2626.
5 M.B. Sporn, N.M. Dunlop, D.L. Newton and J.M. Smith, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 35 (1976) 1332.
6 E.J. Hixson and E.P. Denine, Toxicol. Appl. Pharmacol., 44 (1978) 29.
7 C.D. Port, M.B. Sporn and D.G. Kaufman, Proc. Amer. Ass. Cancer Res., 16 (1975) 21.
8 P.J. Becci, H.J. Thompson, C.J. Grubbs, R.A. Squire, C.C. Brown, M.B. Sporn and R.C. Moon, Cancer Res., 38 (1978) 4463.
9 G.L. Peck and F.W. Yoder, Lancet, ii (1976) 1172.
10 G.L. Peck, T.G. Olsen, F.W. Yoder, J.S. Strauss, D.T. Downing, M. Pandya, D. Butkus and J. Armand-Battandier, N. Engl. J. Med., 300 (1979) 329.
11 M.L. Reed, J. Stanley, F. Stengel, J.L. Shupack and D.M. Benjamin, Arch. Dermatol., 115 (1979) 605.
12 Editorial Comment. J. Amer. Med. Ass., 235 (1976) 1409.
13 C.A. Frolik, T.E. Tavela, G.L. Peck and M.B. Sporn, Anal. Biochem., 86 (1978) 743.
14 M.G. De Ruyter, W.E. Lambert and A.P. De Leenheer, Anal. Biochem., 98 (1979) 402.
15 B. Keilson, B.A. Underwood and J.D. Loerch, J. Nutr., 109 (1979) 787.
16 J.-G. Besner, R. Leclaire and P.R. Band, in preparation.

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Note

Gas chromatographic analysis of naloxone in biological fluids

PETER J. MEFFIN* and KERRY J. SMITH

Department of Clinical Pharmacology, Flinders University of South Australia, Bedford Park, South Australia 5042 (Australia)

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Several methods have been described for the analysis of naloxone in biological fluids [1—5]. However, only the radio-immunoassay of Berkowitz et al. [3] has sufficient sensitivity to enable the characterisation of naloxone disposition in man after usual doses. Sams and Malspeis [5] have described a gas chromatographic method with electron capture detection, based on the formation of a perfluoroalkylester derivative of naloxone which has comparable sensitivity to the method of Berkowitz et al., but this technique has not been applied to biological samples. We describe a method which has equivalent sensitivity and reproducibility to the radio-immunoassay of Berkowitz et al., but offers a chromatographic approach to the analysis of naloxone in biological fluids.

EXPERIMENTAL

Method

The sample preparation is illustrated in Fig. 1. Naltrexone, a close structural analogue of naloxone (Fig. 2), was used as an internal standard. The internal standard solution contained 100 ng of naltrexone per 100 μl of 0.1 *M* sulphuric acid. Each sample was assayed as follows. Whole blood or plasma (1 ml) and 100 ng of internal standard were added to a 100 × 13 mm culture tube with a PTFE-lined screw-cap, which contained 250 μl of carbonate buffer (pH 9, 1.0 *M*), together with 5 ml of toluene. Each tube was mixed by tilting for 30 min on a mechanical mixer and centrifuged at 1000 *g* for 5 min. The organic and aqueous layers were separated by freezing each tube in a dry-ice—acetone bath and pouring the unfrozen organic layer into a second tube containing 250 μl of 0.1 *M* sulphuric acid. This tube was mixed for 5 min, frozen in dry-ice—acetone and the toluene discarded. To the remaining aqueous phase in each

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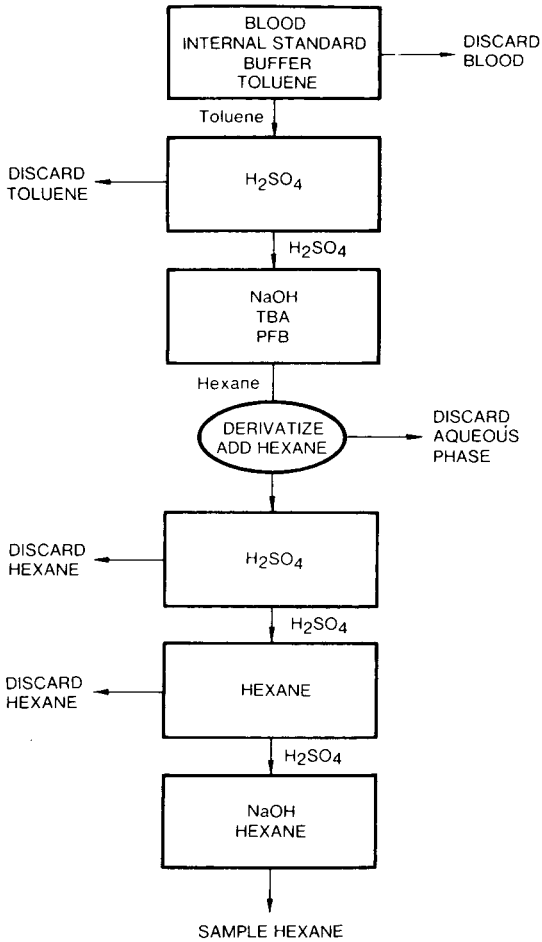


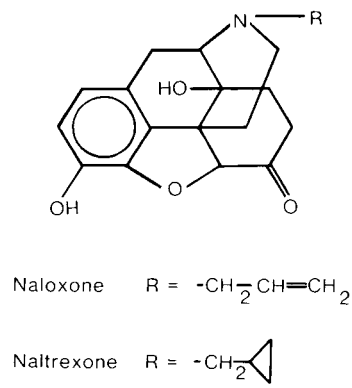


Fig. 1. Flow diagram of sample preparation procedure.

Fig. 2. Structures of naloxone and the internal standard (naltrexone).

tube were added 500 μl of aqueous 0.1 *M* tetrabutylammonium hydroxide (TBA) and 200 μl of a 0.4% solution of pentafluorobenzyl bromide (PFB) in dichloromethane. This mixture was shaken at room temperature for 30 min in order to carry out the derivatisation reaction. A 2-ml volume of hexane was added to each tube, which was then vortexed for 10 sec, centrifuged for 5 min and frozen in dry-ice—acetone.

The organic layer was poured into a third 100 × 13 mm culture tube, containing 0.5 ml of 0.5 *M* sulphuric acid. This mixture was vortexed for 1 min, centrifuged for 1 min and the organic phase discarded. This procedure was repeated with an additional 2 ml of hexane. Finally, 100 μl of 5 *N* sodium hydroxide solution and 100 μl of hexane were added to the tube, which was mixed for 5 min and centrifuged for 5 min. Approximately 5 μl of the hexane layer was sampled from the tube and injected into the gas chromatograph.

Apparatus

A 1.8 m × 3.2 mm glass column packed with 3% OV-17 on Gas-Chrom Q, 100—120 mesh (Applied Science Labs., PA, U.S.A.) was used in a Hewlett-Packard Model 5710A gas chromatograph fitted with a ^{63}Ni electron-capture detector. The column was conditioned at 300°C for 48 h before being connected to the detector. The oven temperature was 280°C, the injector port 250°C and the detector 350°C. The flow-rate of the carrier gas (5% methane in argon) was 60 ml/min. The output from the detector was recorded with a dual-pen recorder with voltage spans set at 1 and 5 mV.

Calibration

The assay was calibrated by analysing samples containing, 1, 5, 10, 25, 50, 100, 150 and 200 ng of naloxone. The peak height ratio of naloxone to naltrexone was plotted versus the amount of naloxone in each sample. Linear and power functions were fitted to these data using least-squares regression analysis. The reproducibility of the assay was measured by determining the coefficient of variation for ten samples, each containing the same amount of naloxone. The reproducibility was determined in this way for 5 and 50 ng of naloxone.

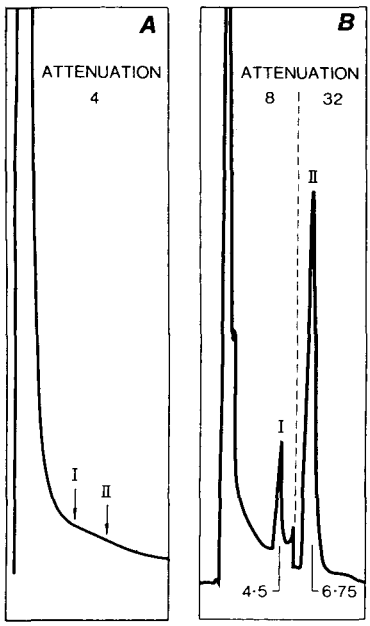
RESULTS AND DISCUSSION

Under the conditions described the retention times were 4.5 min for naloxone and 6.75 min for naltrexone. Fig. 3 shows a chromatogram from a 1-ml control blood sample (A) and from a 1-ml blood sample containing 5 ng of naloxone and 100 ng of internal standard (B). There were no significant peaks which interfered with the peaks of interest in control blood samples from six subjects.

Fig. 4 shows a typical calibration curve for naloxone from whole blood. Although the data shown in Fig. 4 could be reasonably well fitted by the equation for a straight line [$y = 0.103 + 0.0184x$, $r^2 = 0.9091$, where y is the peak height ratio of naloxone to naltrexone, x is the amount of naloxone in the sample and r is the correlation coefficient], close examination shows data have an upward trend. This visual impression was confirmed by dividing the peak height ratio by the amount of naloxone in the sample to give a normalised peak height ratio. When analysed in this manner, the normalised peak height ratio for the 1-ng sample was 1.04 and this value increased progressively with increasing amounts of naloxone, to reach a value of 1.62 for the 200-ng sample. For this reason data in Fig. 3 were fitted to a power function ($y = ax^b$), $y = 0.00856x^{1.11447}$, $r^2 = 0.998$. The mean r^2 value for 12 calibration curves prepared over a period of several months was 0.989 + 0.02 (S.D.).

Attempts to produce linear calibration curves, by reducing on-column loss of the naloxone derivative with the injection of desmethylimipramine as described by Brotell et al. [6] and Swezey et al.[7] for a similar analysis of pentazocine, were not successful.

The coefficient of variation in the peak height ratio of ten blood samples containing the same concentration of naloxone was 2.68% for 50 ng and 4.1% for 5 ng. Calibration curves from plasma were similar to those obtained for whole blood.



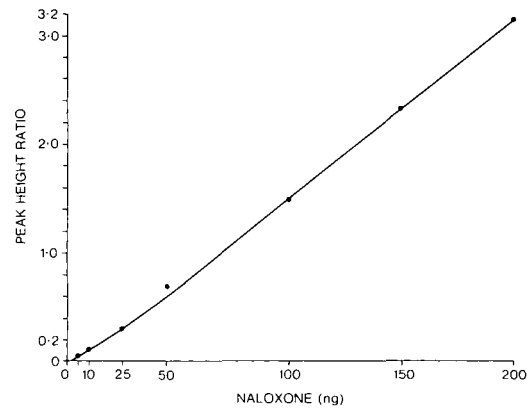


Fig. 3. Chromatograms of (A) control blood sample and (B) sample containing 5 ng of naloxone (I) and 100 ng of naltrexone (internal standard) (II). I and II in A indicate the retention times of naloxone (4.5 min) and internal standard (6.75 min).

Fig. 4. Calibration curve for naloxone. Data were fitted to the expression $y = 0.00856x^{1.11447}$, $r^2 = 0.998$.

It is sometimes convenient when analysing large numbers of samples to prepare samples on one day and chromatograph them the following day. The stability of the derivatised samples was investigated by preparing calibration samples on one day and immediately chromatographing them and then re-chromatographing them 24 h later, during which time the samples had been stored at 4°C. When a power function was fitted to these data, values of a = 0.0209, b = 1.212, r^2 = 0.999, were obtained on the first chromatographic analysis and a = 0.0233, b = 1.182, r^2 = 0.998, were obtained 24 h later. This analysis indicates that during this period storage at 4°C did not affect the accuracy of the method.

Of the previously available methods for the analysis of naloxone in biological fluids, only the radio-immunoassay of Berkowitz et al. for which the lowest point on the calibration curve is 5 ng, has sufficient sensitivity to enable the disposition of naloxone to be determined after usual doses. The analysis of naloxone reported by Sams and Malspeis [5] which utilises gas chromatography with electron capture detection has good sensitivity (2 ng) and reproducibility (coefficient of variation 1—8.5%) but has not been applied to biological samples. A further disadvantage of this method is the instability of the perfluoroalkylester derivatives of naloxone and the internal standard naltrexone, which gives rise to changes in peak height ratio with time. The present analysis was designed to retain the sensitivity of electron capture detection while elim-

inating the disadvantages of the ester derivatives of naloxone. Our approach is based on that described by Swezey et al. [7] for pentazocine, in which a more stable ether derivative is formed by ion-paired extractive alkylation of the phenolic anion using tetrabutylammonium hydroxide and pentafluorobenzyl bromide.

Ether derivates of naloxone and naltrexone formed in this way are stable to strong acid and alkali and thus show no tendency to hydrolyse prior to chromatography as do the ester derivatives described by Sams and Malspeis [5]. The stability of the ether derivatives of naloxone and naltrexone also facilitates the removal of excess derivatising agent prior to chromatography (see Fig. 1).

Although ethyl acetate [2] and 1% isopropanol in chloroform [4] have been used to extract naloxone from biological fluids, preliminary experiments with [^{3}H]naloxone in which 1 ml of blood, adjusted to pH 9, was extracted with 5 ml of organic solvent, showed that, under these conditions, toluene extracted 78% of the radioactivity. Corresponding values for other solvents were hexane 25%, ethyl acetate 78% and diethyl ether 88%. For reasons of convenience, safety and to enhance specificity, toluene was selected as being the most suitable solvent [7].

In summary, the approach described provides a chromatographic method with equivalent sensitivity and reproducibility to the radio-immunoassay method of Berkowitz et al. [3]. Only these two methods offer sufficient sensitivity to enable the characterisation of the disposition of naloxone after usual doses.

ACKNOWLEDGEMENT

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REFERENCES

1 S.H. Weinstein, M. Pfeffer, J.M. Schor, L. Indindoli and M. Mintz, J. Pharm. Sci., 60 (1971) 1567.
2 J. Fishman, H. Roffwarg and L. Hellman, J. Pharm. Exp. Ther., 187 (1973) 575.
3 B.A. Berkowitz, S.H. Ngai, J. Hempstead and S. Spector, J. Pharm. Exp. Ther., 195 (1975) 499.
4 S.H. Weinstein, M. Pfeffer and J.M. Schor, Advan. Biochem. Psychopharm., 8 (1974) 525.
5 R.A. Sams and L. Malspeis, J. Chromatogr., 125 (1976) 409.
6 H. Brotell, H. Ehrsson and O. Gyllenhaal, J. Chromatogr., 78 (1973) 293.
7 S.E. Swezey, T.F. Blaschke and P.J. Meffin, J. Chromatogr., 154 (1978) 256.

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CHROMBIO. 607

Note

High-performance liquid chromatographic assay for measurement of cefoxitin in serum

LARRY A. WHEELER*,*, MICHEL DE MEO, BARBARA D. KIRBY, RICHARD S. JERAULD and SYDNEY M. FINEGOLD

Research and Medical Services, Veterans Administration Wadsworth Medical Center, Los Angeles 90073 and Departments of Pharmacology and Medicine, U.C.L.A. School of Medicine, Los Angeles, CA 90024 (U.S.A.)

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Cefoxitin is a new semi-synthetic cephamycin that is highly resistant to hydrolysis by β-lactamase [1]. This property makes cefoxitin highly active against many bacterial species which are resistant, in vitro and in vivo, to the available cephalosporin antibiotics. Such bacterial pathogens include indole-positive *Proteus*, *Serratia marcescens*, *Bacteroides fragilis* and *Providencia* spp. [2]. Recent clinical trials have shown that cefoxitin is effective in treating a variety of serious infections [3].

Present methods for the analysis of cefoxitin in plasma are bioassay techniques using either a cup-plate technique [1, 4] or disk-agar diffusion [5]. Bioassay procedures lack chemical specificity and require incubation for 12 to 15 h. Buhs et al. [6] have reported on the use of anion-exchange resins with high-performance liquid chromatography (HPLC) to quantitate cefoxitin in the urine. This method is not ideally suited to rapid clinical analysis because of the long retention time of cefoxitin (19 min).

The present study was undertaken to develop a rapid, simple and sensitive assay for cefoxitin in serum using reversed-phase HPLC. The HPLC assay was used to monitor drug levels in adult patients with serious infections and the results compared with a microbiological assay.

*Address for correspondence: Anaerobic Bacteriology Research Laboratory, VA Wadsworth Medical Center, Building 114, Room 317, Los Angeles, CA 90073, U.S.A.

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MATERIALS AND METHODS

Antibiotic

Sodium cefoxitin was supplied by Merck, Sharp and Dohme Labs. (West Point, PA, U.S.A.).

Patients

Serum cefoxitin concentrations were determined for patients in a clinical study evaluating the efficacy of intravenous cefoxitin in aerobic and anaerobic infections. Fourteen patients with bacterial infections including endocarditis, pneumonia, pyogenic cellulitis, abscess and pyogenic arthritis were treated with cefoxitin. Cefoxitin was administered as 1 or 2-g doses, diluted in 100 ml of either normal saline or 5% dextrose in water, given over 30 min. Doses were given at 4- or 6-h intervals.

Sample preparation

Clotted blood was centrifuged and serum pipetted into a sterile tube. The serum samples were stored at −20°C if they were not analyzed immediately.

After thawing, 0.5 ml of serum was mixed with 0.5 ml of 5% trichloracetic acid (TCA) dissolved in methanol. The sample was then mixed by vortexing and kept on ice for 30 min. The TCA solution was made fresh every 3 to 5 h. As the TCA solution ages a new peak appears on the chromatogram with a retention time of 10 min. The peak is first observed approximately 3 h after the methanolic TCA is prepared. As the TCA solution ages the peak will increase in size. This peak does not interfere with the cefoxitin analysis but prolongs the assay time since the peak must come off the column before injection of the next sample. The sample was centrifuged for 5 min at 2000 *g* in a Sorvall SP centrifuge to remove the precipitated serum proteins. The supernatant was collected and filtered through a 0.22-μm filter (GSWP; Millipore, Bedford, MA, U.S.A.), and 25 μl were then injected into the chromatograph.

Standard curves were prepared by adding known amounts of an aqueous solution of sodium cefoxitin to water or blank serum. Concentrations of cefoxitin in serum were calculated by the peak-height ratio method. Cephalothin (25 μg/ml final concentration in the serum) can be used as an internal standard.

HPLC procedure

Chromatography was carried out on an ALC/GPC 204 liquid chromatographic system (Waters Assoc., Milford, MA, U.S.A.). A Pye Unicam LC3 variable-wavelength detector (Cole Scientific, Calabasis, CA, U.S.A.) set at 235 nm monitored the effluent from the column. A reversed-phase column of μBondapak C_{18}, 10-μm particle size (30 cm × 3.9 mm; Waters Assoc.) was used to separate cefoxitin from other UV absorbing compounds in the serum. The solvent system was a mixture of acetonitrile—acetic acid—0.005 *M* potassium dihydrogen phosphate (25:0.5:74.5, v/v/v). The flow-rate was 2 ml/min.

The microbiological assay for measuring cefoxitin in sera was performed at Merck, Sharp and Dohme using the cup-plate technique of Sonneville [7] with *Bacillus subtilis* MB 36.

RESULTS

The ultraviolet absorption of cefoxitin in 0.005 *M* potassium dihydrogen phosphate was characterized by maxima at 235 nm and 263 nm. The extinction coefficients at each wavelength were 16,400 and 9500 respectively. Therefore, 235 nm was chosen as the most sensitive wavelength to use for the cefoxitin assay.

The retention time of cefoxitin was 3.2 min. No interfering peaks were observed in serum containing no cefoxitin. Fig. 1 shows a chromatogram of a cefoxitin standard and a typical chromatogram of serum from a patient on cefoxitin therapy.

The method was linear for concentrations of cefoxitin from 2 μg/ml to 150 μg/ml. Standard curves in both distilled water and blank serum gave similar results. The coefficients of variation for replicate samples obtained on the same day were 2.3% (n = 3); 2.4 (n = 3); 2.6 (n = 6); 3 (n = 5) and 10 (n = 3) for 3, 6, 10, 30 and 50 μg/ml respectively. Blank serum samples with 10 or 30 μg/ml cefoxitin were frozen and thawed on four different days to determine day-to-day variation of the method. The coefficients of variation were 4 and 5% (n = 4) respectively.

The practical limit for the detection of cefoxitin is 1 μg/ml. Levels below 1 μg/ml of cefoxitin can be measured if desired by increasing the injection

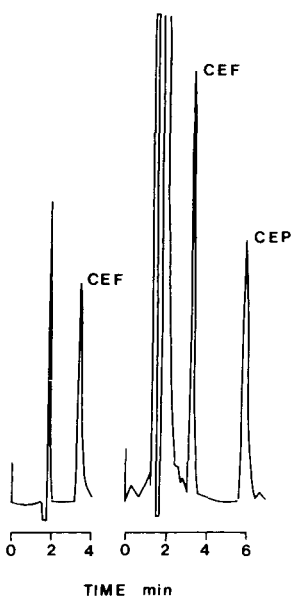

Fig. 1. On the left is a representative chromatogram of cefoxitin (CEF) in blank serum (30 μg/ml). Attenuation was set at 0.08. The peak at 2.1 min is present when blank serum with no cefoxitin is injected. On the right is a typical chromatogram from a patient receiving cefoxitin therapy with cephalothin added as an internal standard (25 μg/ml final concentration in the serum). Attenuation was set at 0.04. The retention time of cefoxitin (CEF) is 3.2 min and 5.8 min for cephalothin (CEP).

volume and/or lowering the attenuation. These manipulations can cause the solvent front to mask the cefoxitin peak.

Sera from patients receiving cefoxitin intravenously were assayed first by the HPLC method. Table I shows the concentrations (using HPLC) in the sera of each patient at the end of the cefoxitin infusion (time 0) and at 30, 120, and 240 min post-infusion. The mean values after a 2-g infusion were 106, 38, 13 and 7 μg/ml, respectively.

A duplicate serum sample was frozen and sent to Merck, Sharp and Dohme Labs. to determine the cefoxitin present using the microbiological assay. The data in Table II show the concentrations of cefoxitin in the sera of patients when measured microbiologically.

The correlation between serum cefoxitin concentrations obtained by HPLC and the microbiological assay was determined by linear regression analysis. The equation for the line was $y = 0.77\,x + 3.9$ (y = microbiological assay, x = HPLC data). The correlation coefficient was 0.93. These results indicate the HPLC assay tends to give higher values than the microbiological assay but that there is still an acceptable correlation between the two methods.

TABLE I

SERUM CONCENTRATIONS OF CEFOXITIN DETERMINED BY HPLC AT VARIOUS TIMES AFTER INTRAVENOUS ADMINISTRATION*

Patient number	Minutes post-infusion			
	0**	30	120	240
2 g every 4 h				
55	122	38.4	15.2	43.5
56	91.4	18.8	6.1	3
57	130	93	37.1	15
58	n.d.§§	21	5.3	3.2
60***	n.d.	21.3	8.9	3.7
62	n.d.	15.1	3.3	18.9
63	138	54.3	28.2	13.8
64	114	41	10.7	101
66	83	32	6.1	1.4
67	60.4	40	12.7	6.7
$X \pm$ S.D.	106±24	38±22	13±10	7±5§
1.5 g every 4 h				
61	n.d.	31.4	5.5	4.8
1.0 g				
59 every 6 h	n.d.	10.5	–	–
65 every 4 h	38.2	18.9	2.4	2.2

*Cefoxitin concentrations are reported as μg/ml serum.

**Infusion time for cefoxitin was 30 min. Time 0 is at the end of the 30 min-infusion.

***Patient 60 received 2 g every 6 h.

§This mean excludes patients 55, 62 and 64. In this group of patients the blood sample was accidently taken just after the 30-min infusion of cefoxitin instead of just before.

§§n.d. = Not determined.

TABLE II

SERUM CONCENTRATIONS OF CEFOXITIN DETERMINED BY THE MICROBIOLOGICAL ASSAY AT VARIOUS TIMES AFTER INTRAVENOUS ADMINISTRATION*

Patient number	Minutes post-infusion			
	0**	30	120	240
2 g every 4 h				
55	119.7	40.6	16.3	77.7
56	34	17.0	7.9	4.0
57	117.2	52.4	24.2	13.7
58	n.d.§§	27.3	7.7	4.3
60***	n.d.	23.8	11.0	3.7
62	n.d.	10.4	1.8	13.0
63	82.4	36.9	17.5	16.3
64	114.2	34.7	10.8	83.2
66	78.2	33.1	7.1	1.6
67	53.9	31.1	15.3	14.3
$X \pm$ S.D.	86±31	31±11	12±6	8±6§
1.5 g every 4 h				
61	n.d.	49.5	8.0	3.6
1.0 g				
59 every 6 h	n.d.	12.7	5.0	2.1
65 every 4 h	32.9	13.4	6.0	2.3

*Cefoxitin concentrations are reported in μg/ml serum.

**Infusion time for cefoxitin was 30 min. Time 0 is at the end of the 30 min-infusion.

***Patient 60 received 2 g every 6 h.

§ The mean excludes patients 55, 62 and 64. In this group of patients the blood sample was accidently taken just after the 30-min infusion of cefoxitin instead of just before.

§§ n.d. = Not determined.

DISCUSSION

A rapid, reliable and sensitive procedure was developed for the analysis of cefoxitin in serum. The mean cefoxitin concentrations as determined by either HPLC or the microbiological assay are comparable to those determined by Heseltine et al. [3] using a microbiological assay technique. Their means were: 108, 72, 12 and 10 μg/ml for the end of infusion (time 0) and 30 min, 2 h and 4 h post-infusion respectively. In the current study using HPLC the means were 106, 38, 13 and 7 respectively.

The possible use of cefoxitin in selected dental infections prompted us to measure cefoxitin in the saliva of three patients. Saliva was diluted in half with the HPLC solvent and passed through a 0.22-μm filter before injection. Saliva samples were obtained at the end of the 30-min infusion of 2 g of cefoxitin. In one patient additional samples were collected at 30 and 60 min post-infusion. No cefoxitin (<1 μg/ml) was found in any of the samples.

Monitoring the serum level is important in adjusting the dosage of cefoxitin in renal insufficiency since greater than 90% of the drug is eliminated into the urine [1]. Cefoxitin concentrations can be determined by HPLC in approximately 40 min compared to 12—15 h using a conventional microbiological assay.

HPLC is an easy and versatile method of determining cefoxitin in serum and saliva.

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REFERENCES

1 H. Onishi, D. Daoust, S. Zimmerman, D. Hendlin and E. Stapley, Antimicrob. Ag. Chemother., 5 (1974) 38.
2 W. Brumfitt, J. Kosmidis, J. Hamilton-Miller and N.G. Gilchrist, Antimicrob. Ag. Chemother., 6 (1974) 290.
3 P. Heseltine, D. Busch, R. Meyer and S. Finegold, Antimicrob. Ag. Chemother., 11 (1977) 427.
4 S. Goodwin, E. Raftery, A. Goldberg, H. Skeggs, A. Tiu and C. Martin, Antimicrob. Ag. Chemother., 6 (1974) 338.
5 K. Miller, E. Celozzi, Y. Kong, B. Pelak, D. Hendlin and E. Stapley, Antimicrob. Ag. Chemother., 5 (1974) 33.
6 R.P. Buhs, T.E. Maxim, N. Allen, T.A. Jacob and F.J. Wolf, J. Chromatogr., 99 (1974) 609.
7 P.F. Sonneville, K. Albert, H. Skeggs, H. Gentner, K. Kwan and C. Martin, Eur. J. Clin. Pharmacol., 12 (1977) 273.

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CHROMBIO. 608

Note

High-performance liquid chromatographic analysis of the anticancer agent methylglyoxal bis(guanylhydrazone) (MGBG, NSC-32946) in biological fluids

MICHAEL G. ROSENBLUM* and TI LI LOO

Pharmacology Branch, Department of Developmental Therapeutics, The University of Texas System Cancer Center M.D. Anderson Hospital and Tumor Institute, Houston, TX 77030 (U.S.A.)

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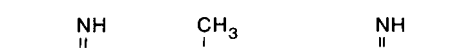
In 1958, Freedlander and French demonstrated that methylglyoxal bis-(guanylhydrazone) (MGBG, Fig. 1) was an effective agent against murine L1210 and P388 leukemias [1]. In the ensuing phase I and phase II clinical trials, MGBG was shown to have significant antileukemic activity [2—5]. In one study, 72% of patients with acute myeloblastic leukemia whose blast cells had granules or were Auer body positive obtained a remission [3]. However, efficacious therapy was often accompanied by toxicities that were not significantly attenuated by dose scheduling. As a result, further therapeutic trials with MGBG were discontinued.

Recently, Knight et al. [6] reported that the dose-limiting toxicity of MGBG may be largely diminished utilizing a weekly i.v. dose schedule. As a result, MGBG is being intensively re-evaluated for remission induction in patients with oligoleukemia.

To facilitate the renewed clinical trial of MGBG, it is advantageous to have knowledge of certain pharmacokinetic properties of this agent. Accordingly, a fast, sensitive, specific, and reproducible assay for MGBG is desirable. This paper describes a high-performance liquid chromatographic (HPLC) method to quantify MGBG in biological fluids. It is readily applicable to the monitoring of MGBG in cancer patients during therapy so that toxicity may be avoided.



METHYLGLYOXAL bis(GUANYLHYDRAZONE)
(MGBG, METHYL-GAG, NSC-32946)

Fig. 1. Structure of MGBG.

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EXPERIMENTAL

Chromatography

All analyses were performed with a Waters Assoc. (Milford, MA, U.S.A.) Model 204 liquid chromatograph equipped with a Model M 6000A pump, a variable-wavelength UV detector (Varian Vari Chrom) set at 283 nm and a Varian recorder (Model 9176). Peak areas were determined by electronic integration (Varian Model CDS-111). An analytical reversed-phase μBondapak C_{18} column (Waters, 4 mm × 30 cm, 10 μm particle size) equipped with a reversed-phase μBondapak C_{18} guard column (Waters 1 mm × 6 cm, 10 μm particle size) was used for separation. Only reagents of analytical grade were used and all solvents were filtered and vacuum-degassed before use. The mobile phase was 0.03 *M* sodium acetate adjusted to pH 4.3 with glacial acetic acid and contained 5% methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). The flow-rate was 2 ml/min.

Preparation of biological fluids

Plasma. Aliquots (1 ml) of plasma containing MGBG (supplied by the Drug Development Branch, National Cancer Institute) were placed in 10-ml plastic centrifuge tubes. Plasma proteins were precipitated with 100 μl of cold perchloric acid (PCA). The tubes were centrifuged at 8,000 *g* (Sorvall centrifuge model RC2B) for 20 min and the supernatants were decanted into centrifuge tubes containing 100 μl of cold 10 *N* potassium hydroxide. The potassium perchlorate precipitate was removed by centrifugation at 5,000 *g* for 10 min and the supernatants were chromatographed as described above.

Urine. Urine samples of 1 ml containing various amounts of MGBG were acidified to pH 1 with 12 *N* hydrochloric acid and added to centrifuge tubes in which was placed 100 mg of XAD-2 resin (Supelco, Bellefonte, PA, U.S.A.) previously equilibrated in 1 *N* hydrochloric acid. The samples were vigorously stirred for 5 min and the supernatants were decanted, neutralized with 10 *N* potassium hydroxide and chromatographed as described previously.

Cerebrospinal fluid. Cerebrospinal fluid (CSF) was assayed for MGBG by the precipitation of contaminating proteins with PCA. Excess PCA was removed by addition of 10 *N* potassium hydroxide.

Leukocytes. A sample of MGBG was added to one ml of phosphate-buffered saline (PBS) containing $1 \cdot 10^8$ leukocytes. The cells were disrupted by sonication (Fisher Sonic Dismenbrator) and the proteins were precipitated with PCA. The PCA soluble fractions were neutralized and chromatographed.

RESULTS AND DISCUSSION

Its intense absorbance in the ultraviolet region (λ_{max} = 28 nm, ϵ = 33,800 at pH 4), permits MGBG to be readily assayed in leukocytes and biological fluids by means of HPLC. Under the conditions described in Experimental, MGBG was eluted from the reversed-phase column 6 min after injection (Fig. 2). In complex mixtures such as urine (Fig. 2E), the separation of MGBG from other components may have been slightly enhanced by a 40% reduction in the methanol content of the mobile phase. However, this slight advantage was offset by exaggerated tailing of the MGBG peak.

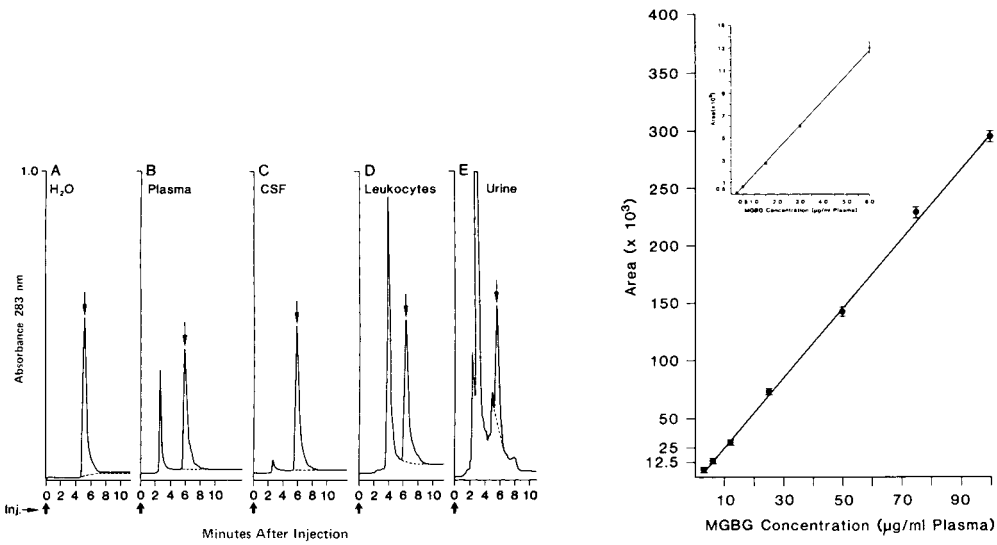


Fig. 2. Chromatograms of MGBG (peaks indicated by arrows) in various media and biological fluids. Broken line under the MGBG peak is the chromatographic pattern of the same biological fluid without MGBG. In all cases, MGBG concentration was 50 μg/ml.

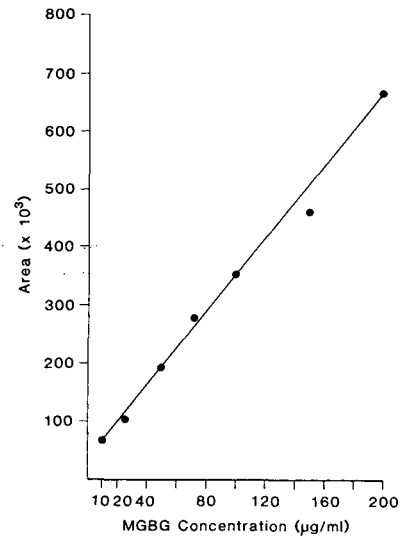
Fig. 3. Standard curve for MGBG in plasma. Values shown are means ± S.E.M. for duplicates of three separate determinations. Insert shows the linearity of the assay over the concentration range 0.25—6 pg MGBG per ml plasma.

Perchloric acid extraction of MGBG from plasma resulted in the loss of between 6% and 22% of the drug at various concentrations. The mean recovery rate was 84 ± 2% for duplicate determinations on nine drug concentrations between 0.25 and 100 μg MGBG/ml plasma. No change in recovery rate was noted when plasma and MGBG were incubated at 37°C for periods of up to 4 h (Table I). Slightly higher recovery rates were found for CSF and for a suspension of leukocytes (88% and 84% respectively).

TABLE I

RECOVERY OF MGBG FROM VARIOUS BIOLOGICAL FLUIDS

Medium	MGBG concentration (μg/ml)	Peak area (× 10^3) (Mean ± S.E.M.)	Recovery (%)
Water	50	181 ± 0.4	100
Plasma	50	138 ± 1.2	76
Plasma, incubated 2 h at 37°C	50	138 ± 0.7	76
Plasma, incubated 4 h at 37°C	50	138 ± 1.5	77
CSF	50	159 ± 0.9	88
Phosphate buffered saline containing 1 · 10^8 leukocytes	50	152 ± 0.7	84



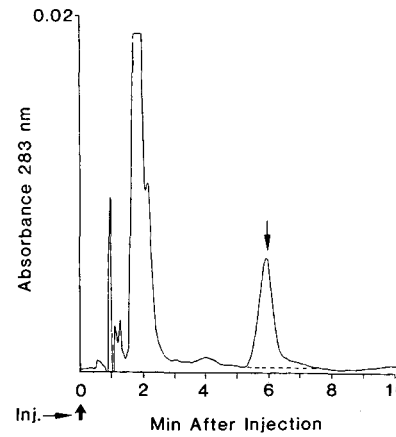


Fig. 4. Standard curve for MGBG in urine. Values are averages for duplicate determinations.

Fig. 5. Chromatogram of plasma from a cancer patient 3 h after intravenous MGBG (200 mg/m^2). Broken line indicates the chromatographic pattern of plasma from the same patient prior to MGBG therapy.

A standard curve for MGBG in plasma (Fig. 3) shows that this HPLC assay has a lower limit of detection of approximately 250 ng and is linear over the concentration range 0.25 to 100 μg/ml. In urine (Fig. 4), this assay has a detection limit of 10 μg/ml due to interfering substances in urine.

MGBG was readily detected in the plasma of a patient who received MGBG therapy (Fig. 5). This assay method will therefore be useful for monitoring patients who receive MGBG therapy and for determining the pharmacokinetics of MGBG in patients with cancer.

ACKNOWLEDGEMENT

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REFERENCES

1 B.L. Freedlander and F.A. French, Cancer Res., 18 (1958) 360.
2 W. Regelson and J.F. Holland, Cancer Chemother. Rep., 27 (1963) 15.
3 E.J. Freireich, E. Frei, III and M. Karon, Cancer Chemother. Rep., 16 (1962) 183.
4 R.H. Levin, E. Henderson, M. Karon and E.J. Freireich, Clin. Pharmacol. Ther., 6 (1965) 31.
5 M. Boiron, C. Jacquillat, M. Weil and J. Bernard, Cancer Chemother. Rep., 45 (1965) 69.
6 W.A. Knight, C. Livingston, C. Fabian and J. Costanzi, Proc. Amer. Soc. Clin. Oncol., (1979) 319.

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CHROMBIO. 614

Note

High-performance liquid chromatographic determination of prazosin in human plasma, whole blood and urine

EMIL T. LIN*, ROBERT A. BAUGHMAN, Jr. and LESLIE Z. BENET

Department of Pharmacy and The Drug Studies Unit, School of Pharmacy, University of California, San Francisco, CA 94143 (U.S.A.)

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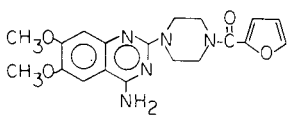
Prazosin hydrochloride (Minipress^{R}, Pfizer) is a new, effective antihypertensive agent that is currently being studied in patients with congestive heart failure for preload and afterload reduction. Previous assays of prazosin [1—4] involve spectrofluorometry after extraction of the alkaline sample with ethyl acetate and then back extraction with hydrochloric acid [4]. This technique is lengthy and requires 3—4 ml of plasma. Most recently Twomey and Hobbs [5] have reported a high-performance liquid chromatographic fluorescence detector method which also involves double extraction similar to that described above.



PRAZOSIN

A new method for the determination of prazosin in human plasma, whole blood and urine is reported here. The method is simple, rapid, sensitive, involves no extraction steps and requires only 0.2 ml of biological sample.

EXPERIMENTAL

Reagents

Prazosin hydrochloride was supplied as prazosin standard 7866-271-A from Pfizer (Groton, CT, U.S.A.). Carbamazepine (Tegretol^{R}) was obtained from Geigy Pharmaceuticals, Division of Ciba-Geigy (Ardsley, NY, U.S.A.). All other reagents were from Fischer Scientific (Fair Lawn, NJ, U.S.A.) and certified

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HPLC grade. The mobile phase was a solution of 43% methanol with 0.6 ml glacial acetic acid, pH 5.0, which was filtered through a Millipore filter and degassed prior to use. Stock solutions of prazosin were prepared by dissolving the drug in methanol and diluting with distilled water.

Chromatographic system

A Perkin-Elmer (PE) Series 2 liquid chromatograph equipped with a rotary valve injector, a PE fluorescence spectrophotometer (Model 204A), a PE fixed-wavelength (254 nm) UV detector (Model 250) and a Linear Model 300 Series dual-pen recorder was used with a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm ID, 10-μm particle size) from Waters Assoc. (Milford, MA, U.S.A.). The fluorescence detector was operated at an excitation wavelength of 340 nm and an emission wavelength of 384 nm. Due to the narrow bandwidth of the fluorometer, only prazosin and metabolite concentrations are detectable under the conditions described and as such the internal standard, carbamazepine, is measured using UV detection.

Procedures

All samples (0.2 ml biological fluid) were deproteinated by adding 0.4 ml of acetonitrile which contained the internal standard (IS) carbamazepine (2.55 μg/ml). After vortexing for 1 min and centrifuging for 10 min at 1500 *g* in an IEC HN-S centrifuge, the supernatant was transferred to a clean test tube and evaporated to 0.1 ml with nitrogen. A 40—60-μl sample is then injected onto the column

The assay limitation for prazosin is 0.1 ng/ml in plasma, urine and whole blood. In actual pharmacokinetic studies only concentrations as low as 1 ng/ml were observed in 12-h samples following 5-mg doses. Calibration graphs were constructed from spiked plasma and urine samples using the sample procedure described above. Prazosin was added to prove a standard curve concentration range of 2—76 ng/ml in actual studies, although calibration curves were shown to be linear from 1—164 ng/ml. The peak height ratios (prazosin:IS) were plotted versus drug concentration in ng/ml, and the calibration graph was used for the calculation of the plasma, whole blood or urine concentrations in human subjects. All calibration graphs were linear over the concentration ranges measured and a mean correlation coefficient for eleven calibration curves was 0.993 ± 0.004, with a coefficient of variation of 0.39%.

An acetonitrile precipitation method was used to determine the extent of prazosin recovery from plasma proteins. Three sets of six samples (sets designated A through C in Table I), with each set consisting of two samples containing 0.2 ml water (Water 1 and 2), and four samples containing 0.2 ml plasma (Plasma 1—4), were prepared at three different concentrations (17, 54, 118 ng/ml). The absolute peak height of prazosin in water and plasma was compared after injecting identical volumes (50 μl) of supernatant obtained by the procedure described above.

The within-day precision of this method was assessed by conducting replicate analyses (n = 10) of the same spiked plasma samples. Four different concentrations were used.

TABLE I

RECOVERY OF PRAZOSIN FROM PLASMA PROTEINS

Set	Concentration (ng/ml)	Sample	Prazosin peak height (cm)	Carbamazepine peak height (cm)	Peak height ratio
A	17.0	Water-1	3.7	10.5	0.35
		Water-2	3.7	10.45	0.35
		Plasma-1	3.9	10.8	0.36
		Plasma-2	3.9	10.65	0.36
		Plasma-3	3.6	10.5	0.34
		Plasma-4	3.7	11.2	0.33
B	54.0	Water-1	11.0	10.9	1.01
		Water-2	10.9	10.3	1.06
		Plasma-1	10.6	10.8	0.98
		Plasma-2	10.9	10.8	1.01
		Plasma-3	11.0	10.8	1.01
		Plasma-4	10.8	10.2	1.06
C	118	Water-1	24.0	10.9	2.20
		Water-2	24.0	9.7	2.47
		Plasma-1	24.8	10.6	2.32
		Plasma-2	23.8	10.1	2.36
		Plasma-3	23.6	10.8	2.18
		Plasma-4	25.0	10.6	2.36

RESULTS AND DISCUSSION

Representative chromatograms of spiked plasma and plasma from a human volunteer are shown in Fig. 1. Control samples of plasma, urine and whole blood show no interfering peaks. The use of the narrow bandwidth PE spectrofluorometer allows prazosin to be detected in biological fluids without interfering peaks even though no extractions are involved. However, the use of the narrow bandwidth required the IS to be determined independently using a UV detector. Although the above method requires two detectors there is an advantage in this arrangement as relatively high concentrations of IS to drug may be used and compensated for by the different sensitivity settings on the individual detectors. Under the above conditions the retention time for prazosin is 7 min and 10 min for the IS.

The results of the acetonitrile precipitation method (Table I) show that virtually all of the compound is removed from protein, that is, no difference is noted between water and plasma samples. The within-day precision (Table II) was assessed by conducting replicate analyses. The coefficient of variation (C.V.) for the four different concentrations ranged from 0.82 to 8.5%. Frozen plasma samples at various concentrations were found to be stable through the four weeks tested. Although the data suggest that the day-to-day reproducibility of this method is acceptable for analytical measurement, when patient plasma samples were being analyzed two known concentrations were included with the standard curve to insure the day-to-day reproducibility.

The method described here has been utilized in pharmacokinetic studies of prazosin in nine congestive heart failure patients and in five normal volunteers,

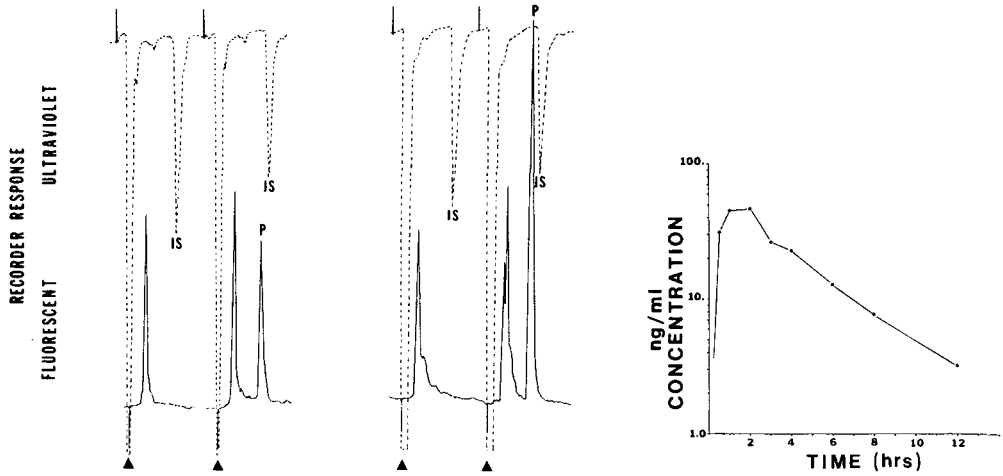

Fig. 1. (Left) dual-pen recording of chromatograms for blank plasma and plasma spiked with prazosin. In the dual-pen chromatograms the UV response is shifted to the left of the fluorescent response, such that the solid line on the UV tracing corresponds to the inject symbol on the fluorescent response. Symbols (retention times in parentheses): ▲, inject; P, prazosin (7 min); IS, internal standard, (10 min). (Right) dual-pen recording of chromatograms for blank plasma and plasma sample from a normal subject. Samples obtained 2 h after a 5-mg oral dose of prazosin. Flow-rate, 2 ml/min; UV detector sensitivity, 1.0 a.u.f.s.; input, 20 mV; fluorescent detector sensitivity, 10; PM gain, 4; input, 20 mV.

Fig. 2. Representative plasma concentration—time curve for prazosin after oral administration of 5 mg to a congestive heart failure patient.

TABLE II

PRECISION OF PLASMA PRAZOSIN ASSAY

$n = 10$.

Mean + S.D. (ng/ml)	C.V. (%)
6.5 ± 0.09	1.35
13.0 ± 0.27	1.85
19.5 ± 1.67	8.50
46.0 ± 0.37	0.82

all of whom received a 5-mg oral dose of prazosin hydrochloride (Fig. 2). When the plasma and blood samples from the subjects were analyzed for prazosin, two time-dependent chromatograph peaks were noted. To date the identities of these peaks have not been determined as no authentic samples of prazosin metabolites are available. The isolation and identification of these peaks are currently being carried out in our laboratory.

CONCLUSION

The technique described here should be useful in future pharmacokinetic studies with prazosin, as the method is sensitive, simple, rapid and requires the

use of only 0.2 ml of sample. The total time for a sample chromatogram is 12 min and no prior extraction steps are required. We are currently using this method in prazosin pharmacokinetic studies in man and animals.

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REFERENCES

1 A.J. Wood, P. Bolli and F.O. Simpson, Brit. J. Clin. Pharmacol., 3 (1976) 199.
2 I.S. Collins and P. Pek, Clin. Exp. Pharmacol. Physiol., 2 (1976) 445.
3 F.O. Simpson, P. Bolli and A.J. Wood, Med. J. Aust. (Suppl.), 2 (1977) 17.
4 R. Verbesselt, A. Mullie, T.B. Tjandramaga, P.J. de Schepper and P. Dessian, Acta Therapeutica, 2 (1976) 27.
5 T.M. Twomey and D.C. Hobbs, J. Pharm. Sci., 67 (1978) 1468.

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CHROMBIO. 615

Note

Plasma and tissue concentrations of hycanthone in the mouse determined by reversed-phase high-performance liquid chromatography

EDWIN D.W. MOORE

Department of Pharmacology, Mayo Clinic and Foundation, Rochester, MN 55901 (U.S.A.)

and

GARTH POWIS*

Division of Developmental Oncology Research, Department of Oncology, Mayo Clinic and Foundation, Rochester, MN 55901 (U.S.A.)

(Received February 4th, 1980)

Hycanthone, 1-{[2-(diethylamino)ethyl]amino}4-(hydroxymethyl)-thioxanthen-9-one (Fig. 1) is widely used in the treatment of schistosomiasis [1]. Hycanthone has activity against murine leukemias L-1210 and P-388 and rat Walker 256 carcinosarcoma [2, 3] and has been studied as a potential anticancer drug in man [4, 5]. Hycanthone is a major metabolite of lucanthone, 1-{[2-(diethylamino)ethyl]amino}4-(methyl)-thioxanthen-9-one [1, 6], a drug with radiosensitizing properties in vitro [7, 8] and in vivo [9]. The radiosensitizing effects of lucanthone are rapidly reversed when it is removed and exposure to the drug for several hours is necessary to produce the optimal effect [8]. Hycanthone has been reported to enhance the effects of radiation on mammary tumors in mice [10] and it is currently being evaluated as a radiosensitizing agent in man. Metabolic studies have been conducted using radioactively labelled hycanthone [11] and plasma and tissue levels of solvent extractable radioactivity reported following the administration of radioactively

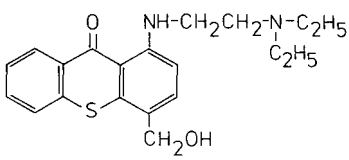

Fig. 1. Hycanthone structure.

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labelled hycanthone to monkeys and rats [12]. Plasma levels of non-labelled hycanthone determined by a normal-phase high-performance liquid chromatographic (HPLC) procedure have been reported in man [5] but the method was not adapted for use with an internal standard and does not resolve hycanthone metabolites. We report a simple reversed-phase HPLC assay for hycanthone and its lipophilic metabolites. This assay has been used to measure the time course of the disappearance of hycanthone from plasma and tissues in the mouse.

EXPERIMENTAL

Animal studies

Male BDF_1 mice weighing between 25 and 30 g were injected intraperitoneally (i.p.) with an aqueous solution of hycanthone methanesulfonate at a dose of 80 mg hycanthone base per kg body weight (343 mg/m^2). Groups of six mice were killed at different times by decapitation and blood collected into heparinized tubes. The different organs were removed and immediately frozen with crushed dry ice. Tissues were homogenized in 4 volumes water at 4°C using a Polytron homogenizer (Brinkman Instruments, Westbury, NY, U.S.A.).

Preparation of samples

Portions (0.5 ml) of plasma or of tissue homogenate (4 ml) were extracted with 8 ml chloroform. The efficiency of extraction of hycanthone was 93%. The chloroform extract was dried with sodium sulfate and taken to dryness under nitrogen. The residue was dissolved in 100 μl acetonitrile and 50 μl injected into the HPLC system. With plasma and most tissues an internal standard of 10 μg hycanthone acid was employed. Analysis was, however, always conducted with and without an internal standard. Standard curves were prepared for each tissue.

High-performance liquid chromatography

Hycanthone and non-polar metabolites were separated by reversed-phase HPLC on a 25-cm C_2-bonded LiChrosorb RP-2 column, 10-μm particle size (Merck, Darmstadt, G.F.R.), under isocratic conditions with 50% 0.01 *N* sodium acetate buffer (pH 4.0) in acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) as the mobile phase at a flow-rate of 2 ml/min. More reproducible results were obtained if, after each separation, the column was flushed with 100% acetonitrile for 10 min. The eluting compounds were detected at 257 nm with a reference wavelength of 520 nm. A Hewlett-Packard 1084B liquid chromatograph and variable-wavelength detector were employed. The output from the detector was fed into a Hewlett-Packard 79850B liquid chromatograph terminal, and peak areas integrated.

Drugs

Hycanthone monomethanesulfonate, lucanthone, hycanthone acid (1-{[2-(diethylamino)ethyl]amino}-4-carboxy-thioxanthen-9-one) and hycanthone N-oxide (1-{[2-(diethylamino-N-oxide)ethyl]amino}-4-hydroxymethyl-thioxanthen-9-one) were supplied by Sterling Winthrop Research Institute (Rensselaer, NY, U.S.A.).

RESULTS AND DISCUSSION

The normal-phase HPLC separation of hycanthone previously reported from this laboratory [5] could not be adapted to an assay with the internal standards available since it failed to resolve hycanthone and lucanthone while the more polar hycanthone N-oxide and hycanthone acid gave broad unresolved peaks. All of these compounds were well separated by reversed-phase HPLC on a C_2-bonded support (Fig. 2). Hycanthone bound so tightly to a C_{18}-bonded support that it could not be eluted with 100% acetonitrile. The procedure for the extraction of hycanthone from plasma is simpler than that previously reported [5]. All of the compounds had an absorption maximum at 257 nm which was chosen as the wavelength for detection. Hycanthone acid was chosen, for convenience, as the internal standard rather than the slowly eluting lucanthone. Although hycanthone acid was a possible metabolite it was not present in the plasma or in tissues other than the liver and gastrointestinal tract.

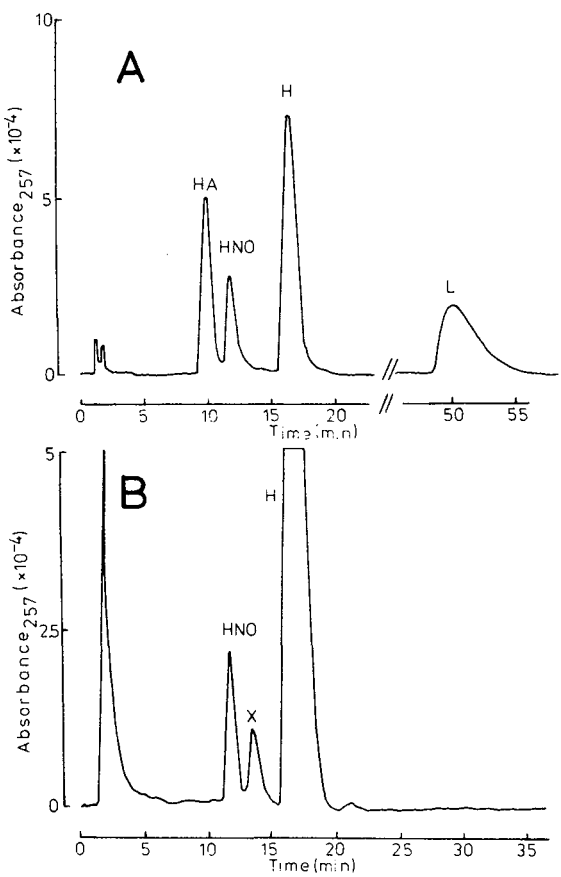

Fig. 2. Chromatograms of A, mouse plasma to which had been added 5 μg/ml each of hycanthone (H); hycanthone acid (HA); hycanthone N-oxide (HNO); and lucanthone (L); B, mouse kidney homogenate 100 min after the i.p. administration of hycanthone 80 mg/kg; X, unidentified metabolite.

All samples were routinely assayed with and without added hycanthone acid. The acid was used as an internal standard only when it was not detected in the tissue under study. Using hycanthone acid as an internal standard in plasma, the assay was linear up to 100 μg hycanthone per ml and had a lower limit of detectability of 50 ng/ml. The coefficient of variation of the assay was 6.4%.

Following i.p. administration, hycanthone was rapidly absorbed and disappeared from the plasma in a biphasic manner (Fig. 3) with a distributive half-life ($t\frac{1}{2}^{\alpha}$) of 19 min, a postdistributive half-life ($t\frac{1}{2}^{\beta}$) of 56 min, and a steady state volume of distribution (Vss) of 43,888 mg per kg body weight. The concentrations of hycanthone in the tissues studied were always higher than in plasma but fell with about the same half-life as the plasma hycanthone (Table I). This suggests a reversible binding of hycanthone to sites within the tissues.

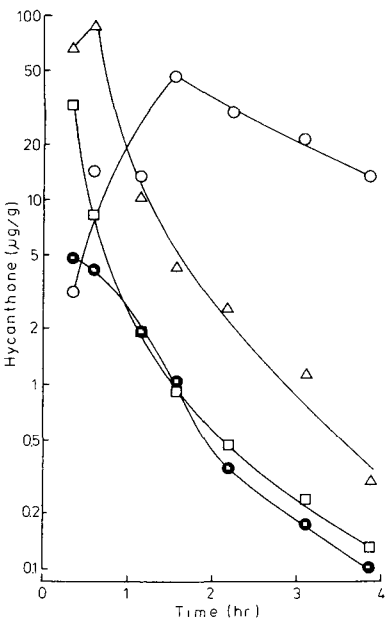

Fig. 3. Plasma and tissue levels of hycanthone •, plasma; □, liver; △, lung; and ○, gastrointestinal tract. Each point represents a pooled sample from 6 mice administered hycanthone i.p. at a dose of 80 mg per kg body weight.

Hycanthone binds reversibly with high affinity to double stranded DNA by intercalation [13] but it may also bind to other macromolecules. Hycanthone has been reported to be an alkylating agent and to bind covalently to tissue macromolecules [14], although this has not been found by all workers [15]. Hycanthone bound covalently to tissue marcromolecules would not be detected by solvent extraction.

Tissues can be divided into three types depending on the time course of the appearance and disappearance of hycanthone. Examples are shown in Fig. 3. In liver, epididimal fat pad and brain the peak levels of hycanthone occur within 15 min of administration. In lung, kidney and heart muscle the peak levels of hycanthone are not reached until 30 min. The ratio of peak tissue concentra-

TABLE I

TISSUE HYCANTHONE CONCENTRATIONS

Tissue	Peak concentration (μg/g)*	Tissue: plasma concentration ratio**	Biologic half-life (min)***
Plasma	4.9	–	56
Kidney	89.4	21.1	42
Lung	89.2	21.0	38
Fat pad	47.1	9.7	34
Brain	42.8	8.8	35
Liver	32.4	6.7	47
Heart	11.2	2.6	51
Gastrointestinal tract §	47.0	44.3	80

*Values are the mean of 3 determinations on pooled samples from 6 mice.

**The plasma concentration employed was that at the time of the peak tissue concentration.

***This represents the postdistributive half-life ($t_{1/2}^{\beta}$).

§ Including contents. The peak concentration is much delayed (see Fig. 3).

tions of hycanthone to the plasma hycanthone concentrations are shown in Table I. In the gastrointestinal tract, which includes the intestines, caecum and colon together with their contents, there was a delayed rise in the level of hycanthone. This can be attributed to a biliary excretion, which in most species is the major route of elimination for hycanthone and its metabolites [11].

Hycanthone metabolites were present in several tissues. This is in contrast to previous animal studies conducted with radioactively labelled hycanthone in which only liver and bile were found to contain metabolites [9]. Hycanthone N-oxide was detected in kidney (Fig. 2), liver, lung, and gastrointestinal tract, and at low levels in plasma. Hycanthone acid was present in liver and gastrointestinal tract. An unidentified metabolite with a retention time of 14 min was present in liver and in smaller quantities in kidney (Fig. 2), lung, and heart. A second unidentified metabolite with a retention time of 20 min was found only in the gastrointestinal tract. These unidentified peaks may represent either the sulfoxide or deethylated metabolites which have been found in rat bile and liver [11, 12] or the sulfone which has been found in mouse bile [16].

Clinical studies with lucanthone suggest that the gastrointestinal tract is particularly sensitive to the enhancing effects of the thioxanthenones on radiation [17]. This could be due to high levels of hycanthone or its metabolites in the gastrointestinal tract. It could also reflect a dependence of intestinal stem cells on an ability to repair sublethal radiation damage [18]. A major effect of hycanthone is to prevent the accumulation and repair of sublethal radiation damage [8]. The effect of different concentrations of hycanthone and its metabolites in various tissues on the radiosensitizing properties of hycanthone remain to be determined.

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REFERENCES

1 P.E. Hartman and P.B. Hulbert, J. Toxicol. Environ. Health, 1 (1975) 243.
2 S.M. Sieber, J. Whang-Peng and J.G. Johns, Biochem. Pharmacol., 22 (1973) 1253.
3 S. Legha, Hycanthone NSC 142982. Brochure, Investigational Drug Branch, Cancer Therapy Evaluation, National Cancer Institute, Bethesda, MD, 1975.
4 S. Legha, W.E. Gorse and G.P. Bodey, Cancer Treat. Rep., 62 (1978) 1173.
5 J.S. Kovach, C.G. Moertel, A.J. Schutt and R.T. Eagan, Cancer Treat. Rep., 63 (1979) 1965.
6 E. Hirschberg, I.B. Weinstein, R. Carchman and S. Archer, Proc. Amer. Ass. Cancer Res., 9 (1968) 30.
7 R. Bases, Cancer Res., 30 (1970) 2007.
8 D.B. Leeper, J.E. Karamuz and J.D. Blannet, Int. J. Rad. Oncol. Biol. Phys., 4 (1978) 219.
9 A.J. Milligan, H.R. Katz and D.B. Leeper, J. Nat. Cancer Inst., 60 (1978) 1023.
10 J. Pittcock, D.Q. Brown and T. Schulz, Radiat. Res., 70 (1977) 674.
11 C. Davison, M. Scrime and J. Edelson, Arch. Int. Pharmacodyn., 230 (1977) 4.
12 P. Hernandez, E.W. Dennis and A. Farah, Bull. World Health Org., 45 (1971) 27.
13 M. Jahangir, P.B. Hulbert and J.A. Grundy, J. Pharm. Pharmacol., 29 (1977) 78P.
14 J.L. Miller and P.G. Hulbert, J. Pharm. Pharmacol., 28 (1978) Suppl. 18.
15 R. Bases, F. Mendez, F. Elequin, D. Liebeskind, A. Kozin and S. Neubort, Cancer Res., 38 (1978) 781.
16 R. Strufe, Med. Chem., 7 (1963) 337.
17 M.P. Nobler and A.J. Scher, Int. J. Rad. Oncol. Biol. Phys., 4 (1978) 1039.
18 A.J. Milligan and D.B. Leeper, Radiology, 130 (1979) 499.

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Note

Determination of cinnarizine in plasma by high-performance liquid chromatography

H.K.L. HUNDT*, L.W. BROWN and E.C. CLARK

Department of Pharmacology, University of the O.F.S., Bloemfontein 9301 (South Africa)

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To be able to carry out a bioavailability comparison between a generic and an innovator's brand of cinnarizine (Stugeron®, Janssen Pharmaceuticals, Beerse, Belgium), an analytical method for its determination had to be devised since no assay method could be traced despite a thorough literature survey. A gas—liquid chromatographic method has since been published [1]. This paper describes a high-performance liquid chromatographic method for the determination of cinnarizine in plasma. The assay is sufficiently sensitive to follow reliably plasma levels of cinnarizine for a period of four half-lives after a therapeutic dose of 50 mg.

EXPERIMENTAL

Reagents

The chemical structures of cinnarizine and the internal standard, chlorbenoxamine, are shown in Fig. 1. Cinnarizine was obtained from Labethica (Bethlehem, South Africa) and chlorbenoxamine from Lennon (Port Elizabeth, South Africa).

Stock solutions of cinnarizine and chlorbenoxamine were made up in 0.05 *M* sulphuric acid and methanol, respectively, and stored at 4°C. Methanol and ammonium dihydrogen phosphate used for the mobile phase were guaranteed reagent grade (Merck, Darmstadt, G.F.R.) and were used as received. Diethyl ether used for the extractions was of anaesthetic grade (Natal Cane By-products, Merebank, South Africa) and was freshly distilled over sodium hydroxide pellets before use.

Apparatus

An M6000 pump and a WISP autosampler (Waters Assoc., Milford, MA, U.S.A.) were coupled to a 30 cm × 4 mm I.D. stainless-steel column packed

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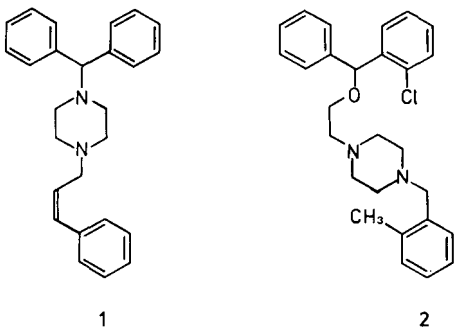

1 2

Fig. 1. Chemical structures of cinnarizine (1) and chlorbenoxamine (2).

with Spherisorb 5 ODS (Phase Separations, Queensferry, Great Britain). A Waters variable-wavelength detector Model 450 was used to measure absorbance of the eluate. The results were processed on a Waters 730 data module.

Other apparatus used consisted of glass centrifuge tubes with B24 and B19 ground-glass joints and stoppers, a variable-speed reciprocating shaker (Kötterman, Hänigsen, G.F.R.), a Damon CRU-5000 refrigerated centrifuge (Needham Heights, MA, U.S.A.), a Reactitherm heating module (Pierce Chemical Company, Rockford, IL, U.S.A.), 5-ml glass ampoules, high-purity nitrogen and a 100-μl Hamilton syringe.

Stock solutions

A cinnarizine stock solution was prepared by making 20 mg (accurately weighed) of cinnarizine up to 1 l with 0.05 *M* sulphuric acid. The internal standard solution was 40 mg of chlorbenoxamine made up to 100 ml with methanol.

Extraction procedure

To 5 ml of plasma, in a B24 ground-glass centrifuge tube, were added 100 μl of internal standard solution, 1 ml of 0.25 *M* NaOH and 10 ml of diethyl ether. The stoppered tubes were shaken for 10 min on a reciprocating shaker at the rate of 180 strokes/min. The tubes were centrifuged for 5 min at 900 *g* at 5°C. The supernatant ether layer was then transferred to 20-ml B19 ground-glass centrifuge tubes containing 2 ml of 0.05 *M* sulphuric acid. After shaking and centrifuging as before, the ether layer was aspirated off and discarded. The aqueous acidic solution was alkalinised by adding 0.5 ml of 0.5 *M* NaOH and extracted with 5 ml of diethyl ether as before and centrifuged. As much of the supernatant ether layer as possible was transferred to 5-ml ampoules in which the ether was evaporated at 40°C under a gentle stream of high-purity nitrogen.

The residue in the ampoules was redissolved in 120 μl of the mobile phase used for chromatography and 100 μl of this solution were injected for analysis.

Chromatography

The mobile phase consisted of 850 ml of methanol plus 150 ml of aqueous 0.05 *M* ammonium dihydrogen phosphate. A constant flow-rate of 1.0 ml/min was maintained with a pressure of 1500 p.s.i. at ambient temperature through

a 30 cm × 4 mm I.D. stainless-steel column containing Spherisorb ODS (5 μm). The absorbance of the eluate was monitored at 285 nm and 0.04 a.u.f.s.

Retention times of cinnarizine and chlorbenoxamine were 6.2 and 8.7 min, respectively.

RESULTS AND DISCUSSION

Fig. 2 shows representative chromatograms obtained and demonstrates the lack of interfering endogenous compounds.

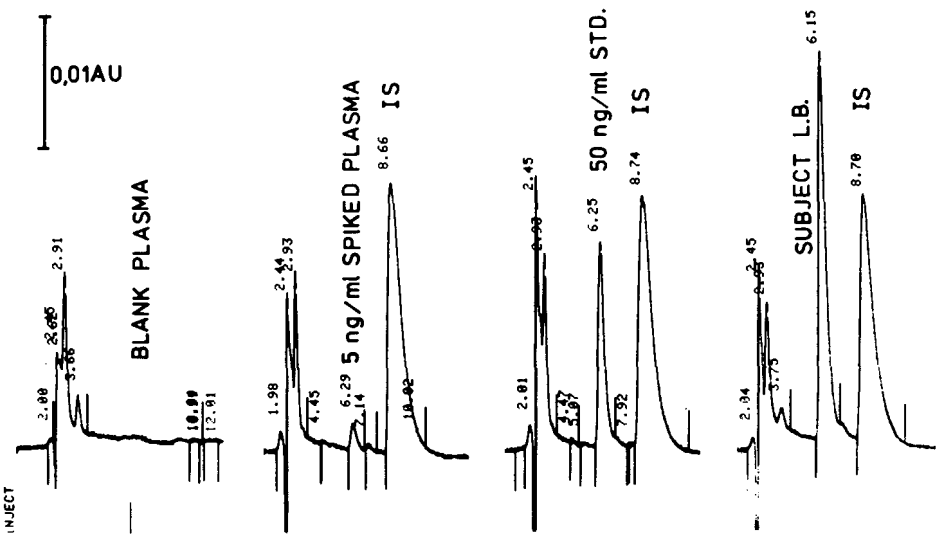

Fig. 2. Typical chromatograms of plasma samples.

Linear calibration curves of cinnarizine peak heights/internal standard peak heights vs. plasma concentrations were obtained with plasma standards containing 20—100 ng/ml. The lines passed very close to the origin and the slopes remained relatively constant as can be seen from the following equation

$$y = (47.00 \pm 1.90)x + (0.44 \pm 1.47)$$

This equation represents the average equation of ten calibration curves (obtained by linear regression analysis) constructed during a period of two weeks while the assays of cinnarizine in actual plasma samples of the bioavailability trial were being carried out. Correlation coefficients for these linear regressions were consistently greater than 0.999 (n=4) making one-point calibration feasible.

A summary of the results obtained with spiked samples during the trial using a single plasma standard concentration of cinnarizine to calibrate the data module is presented in Table I.

The limit of sensitivity for this method, defined as 2 × S.D. obtained at zero concentration from the intercept of a straight line plot of S.D. of the mean vs. plasma concentration of replicate spiked samples, was found to be 2 ng/ml.

TABLE I

RECOVERY OF CINNARIZINE IN SPIKED PLASMA SAMPLES

Concentration spiked (ng/ml)	Mean concentration found (ng/ml)	C.V. (%)	*N*
26.0	25.7	8.4	6
52.0	52.7	4.3	6
78.0	76.2	6.3	5
104.0	107.8	5.7	5

Only chloroform and diethyl ether were evaluated for their extraction efficiencies and ability to separate cinnarizine from impurities in the plasma. Direct chloroform extraction of cinnarizine from ten buffer solutions ranging from pH 1 to pH 10 and with cinnarizine concentrations of 20 μg/ml revealed a remarkable consistency in the extraction efficiency over the whole pH range investigated. This was not the case with diethyl ether as can be seen from Fig. 3.

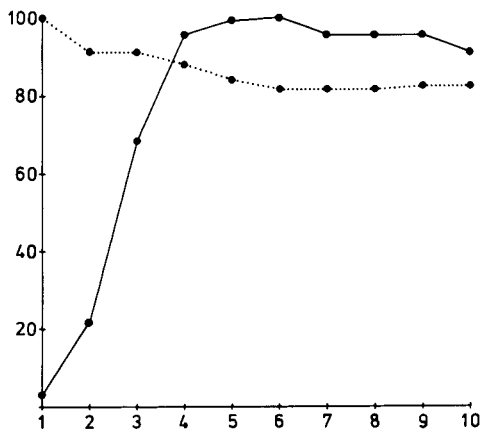

Fig. 3. Effect of pH on extraction of cinnarizine from buffer solutions with chloroform (•· · · · ·•) and diethyl ether (•———•). *Y* axis: relative extraction efficiency. *X* axis: pH of solution.

Even more remarkable is the fact that cinnarizine, being a basic compound, extracts best into chloroform at the lowest pH values; a circumstance which ncessitated the addition of 1 ml of 1 *N* sulphuric acid by Morrison et al. [1] in order to obtain good recoveries for extractions from 1 ml of plasma.

Since further purification of the extract from plasma was required before injection into the liquid chromatograph, diethyl ether was chosen as extraction solvent because cinnarizine could be back-extracted from it into acid medium.

The absolute extraction yield of cinnarizine carried through the whole extraction procedure with spiked plasma samples containing 100 ng/ml cinnarizine and using chlorbenoxamine as an external standard was 67%. That of chlorbenoxamine at 8 μg/ml, using cinnarizine as external standard, was 55%.

The low absolute extraction yield of cinnarizine was compensated for by the purity of the extract allowing the injection of the entire extract from 5 ml of plasma into the chromatography column.

TABLE II

PHARMACOKINETIC PARAMETERS FOR ORAL ABSORPTION OF 50 mg OF CINNARIZINE AS TABLETS IN TWO DIFFERENT FORMULATIONS

Values are means (± S.D.) in six normal trial subjects.

	Mean peak plasma conc. (ng/ml)	Mean time to peak plasma conc. (h)	Mean AUC* (0—24 h) (ng·h/ml)	Mean plasma half-life (h)
A	73.7 ± 34.5	2.3 ± 0.4	583 ± 180	4.4 ± 1.0
B	88.6 ± 42.3	2.4 ± 1.1	721 ± 268	5.3 ± 1.6

* AUC, Area under the curve.

Since this method was used only during a bioavailability trial during which time the participating volunteers were instructed to refrain from the ingestion of medicines, no drugs were tested for interference with this assay.

The results obtained in the bioavailability trial are summarised in Table II.

REFERENCE

1 P.J. Morrison, I.D. Bradbrook and H.J. Rogers, Brit. J. Clin. Pharmacol., 7 (1979) 349.

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CHROMBIO. 619

Note

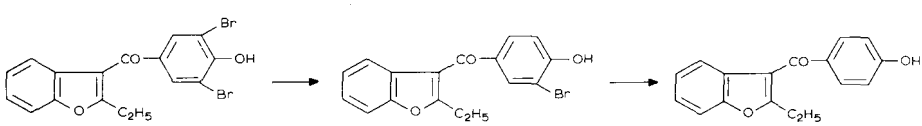
High-performance liquid chromatographic determination of benzbromarone and the main metabolite benzarone in serum

HARTMUT VERGIN* and GILLIAN BISHOP

Heumann-Pharma, Biochemical Department, P.O. Box 2260, D-85 Nürnberg (G.F.R.)

(First received February 25th, 1980; revised manuscript received April 21st, 1980)

Benzbromarone, 2-ethyl-3-(4-hydroxy-3,5-dibromobenzoyl)-benzofuran, a well-known uricosuric agent, reduces serum uric acid concentrations by increasing renal clearance thereof in both healthy volunteers and gouty patients [1—3]. After absorption from the gastrointestinal tract benzbromarone is successively dehalogenated in the liver to its main metabolite, benzarone.



Bromobenzarone (II), the monohalogen metabolite, accounts for approximately 2% of the benzbromarone administered and is excreted exclusively in the faeces (radiochemical investigations) [4]; benzbromarone (I) and benzarone (III), on the other hand, are excreted almost entirely via the liver and the bile. Using radioactive-labelled material it has been shown that, for gouty patients, approximately 0.1% of the total dose appears as conjugated benzbromarone and 1% as conjugated benzarone in the urine [4]. In addition to the radiochemical methods, benzbromarone and benzarone serum concentrations can be determined using both spectrofluorimetry [5] and gas chromatography (GC) [6, 7].

This paper is aimed at describing a specific, sensitive and rapid procedure for simultaneously determining benzbromarone and benzarone serum concentrations using high-performance liquid chromatography (HPLC). This method is

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also suitable for measuring urine levels, but as renal excretion of the mother substance is negligible, urine level determination plays a subordinate role in benzbromarone therapy.

EXPERIMENTAL

Chemicals

All chemicals and solvents were p.a. standard, water bidistilled; all were prefiltered using a GV 100/1 glass filtration apparatus (ref. No. 392700) and filterdiscs, RC 58, 0.2 μm (ref. No. 371628) both from Schleicher and Schüll (Dassel, G.F.R.). Methanol, acetic acid, ethyl acetate were obtained from E. Merck (Darmstadt, G.F.R.) and acetonitrile HPLC Grade S from Rathburn Chemicals (Walkerburn, Great Britain). For calibrating the system benzbromarone charge No. Wi302782 and benzarone charge No. Wi30577 were used. Benzbromarone is manufactured as Narcaricin^{R} (Heumann-Pharma, Nürnberg, G.F.R.) and as Uricovac^{R} (Labaz, Erkrath, G.F.R.). Benzarone is manufactured as Fragivix^{R} (Sanol, Monheim, G.F.R.).

Instruments

All investigations described in this paper were carried out on an ALC/GPC-204 system (Waters, Königstein/Taunus, G.F.R.) which was equipped with an injector, Model U6K and UV absorbance detector (254 nm), Model 440. The peaks obtained were quantitatively integrated by a Waters Data Module, Model 730.

Sample preparation

Serum (3 ml) was mixed with methanol (4 ml) and shaken for 1 min (Vibro-Fix, Jahnke and Kunkel, Staufen, G.F.R.). After centrifuging for 15 min at 3270 *g* (Hettich EBA 3S) the aqeuous methanolic phase was extracted and filtered through 0.2-μm filterholders of the type FP 030/3 (Schleicher and Schüll). Finally 10—30 μl of the filtrate were injected into the HPLC system.

HPLC system

Stationary phase. A reversed-phase material, μBondapak C_{18}, 10 μm (Waters) was packed into a column of 30 cm × 4 mm I.D.

Mobile phase. A mixture was employed consisting of methanol—water—acetonitrile—ethyl acetate—acetic acid (72:23:3:1:1). The system was, in addition, flow programmed to rise linearly from 1 to 2 ml/min, 3 min after sample injection, and thereafter remain constant.

RESULTS AND DISCUSSION

Both benzbromarone and benzarone are eluted as well-defined peaks with slight tailing, when the mobile phase methanol—water—acetonitrile—ethyl acetate—acetic acid is employed and the flow-rate is programmed as previously described. Under these conditions all retention times are reproducible. Ten aliquots, each 10 μl, of a spiked aqueous—methanolic serum extract (2.84 μg/ml benzbromarone and 1.25 μg/ml benzarone) were injected successively

into the reversed-phase system. Even over a space of several days reproducible mean retention times of 4.88 (coefficient of variation 0.95%) and 9.47 min (coefficient of variation 0.55%) were obtained for benzarone and benzbromarone respectively (Table I).

TABLE I

RECOVERY VALUES FOR BENZBROMARONE AND BENZARONE FROM SPIKED SERUM SAMPLES

Benzbromarone		Benzarone	
Concentration (μg/ml)	Recovery (%), mean ± S.E.	Concentration (μg/ml)	Recovery (%), mean ± S.E.
0	—	0	—
1.29	78.4 ± 1.10	0.91	89.1 ± 1.1
2.58	79.0 ± 0.61	1.83	91.2 ± 0.8
5.16	81.4 ± 2.96	3.66	89.2 ± 0.7
10.32	80.9 ± 3.12	7.32	90.8 ± 1.3
$\bar{x}$ ± S.D.	80.0 ± 1.45		90.02 ± 1.04

The relationship peak area to concentration is linear in the range 0.25—22.5 μg/ml for benzbromarone and for benzarone in the range 0.15—15 μg/ml. The detection limit for this particular method and for benzbromarone lies in the region of 140 ng/ml; benzarone has a detection limit of about 90 ng/ml.

Table I shows extraction reproducibility for the mother substance and the main metabolite. Recoveries of 80 ± 1.45% were obtained for benzbromarone over a range of 1.29—10.30 μg/ml and of 90 ± 1% for benzarone over a range of 0.91—7.30 μg/ml. Due to the high degree of reproducibility for both substances, standardisation for routine analysis can be carried out externally. Fig. 1 shows typical chromatograms of spiked and patients' sera (Fig. 1B and C, respectively). Peaks from the blanks (Fig. 1A) can be seen to interfere in no way whatever with those of either benzbromarone or benzarone.

The HPLC method described in this paper allows simultaneous determination of the uricosuric agent benzbromarone and its main metabolite benzarone from both volunteer and patient sera. In comparison to the GC method already published [7] an extremely rapid extraction and quantification of the two substances can be achieved, without forgoing the high reproducibility. The method is thus suitable for bioavailability studies on preparations containing benzbromarone. After administration of a single oral dose (100 mg) to seven healthy volunteers, maximum serum levels of 1.84 ± 0.87 mg/l occur [8]. The elimination half-life is 2.77 ± 1.07 h. Benzarone, the major metabolite, can be detected in the serum 3 h after dosing of benzbromarone. The maximum benzarone serum levels of 0.79 ± 0.21 mg/l occur 6 h after dosing. Benzarone is eliminated from the volunteers serum with an elimination half-life of 13.52 ± 2.18 h.

Both substances are excreted mainly via the liver and bile. In urine, only benzarone can be detected (1.55% of the benzbromarone dose) conjugated as a β-glucuronide.

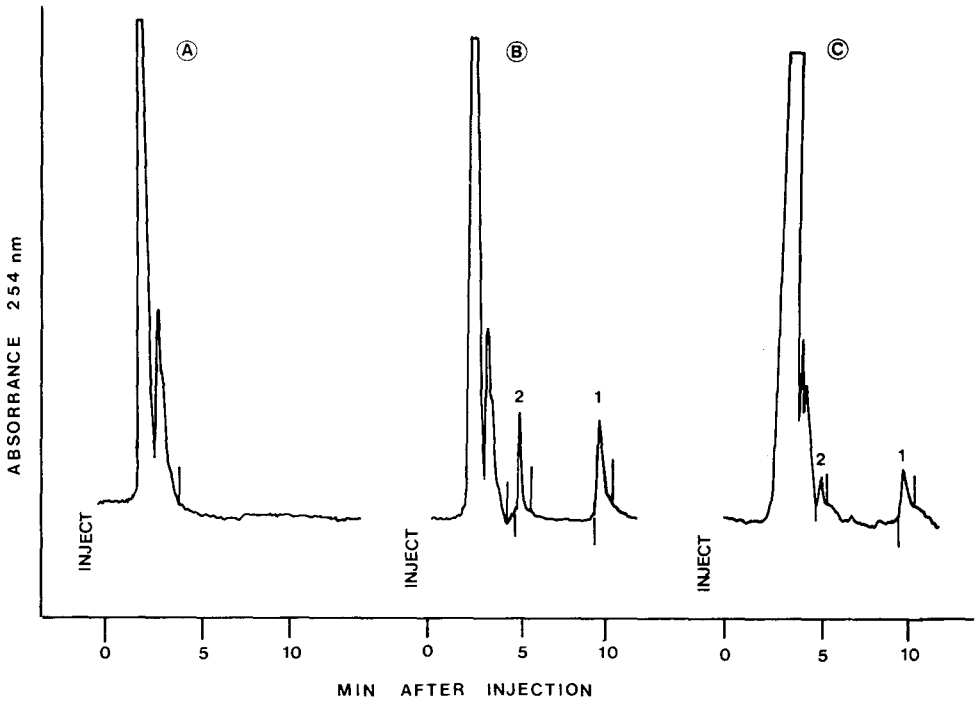

Fig. 1. Chromatograms of (A) serum blank; (B) serum spiked with 2.85 μg/ml benzbromarone and 1.83 μg/ml benzarone and (C) serum sample of a volunteer taken 6 h after administration of 100 mg benzbromarone where peaks 1 and 2 are benzbromarone and benzarone, respectively.

ACKNOWLEDGEMENT

We are indebted to Miss Heike Joch for her valuable technical assistance.

REFERENCES

1 J. Sternon, P. Kocheleff, E. Couturier, E. Balasse and P. van den Abeele, Acta Clin. Belg., 22 (1967) 285.
2 R. Kotzaurek and E.F. Hueber, Wien. Med. Wochenschr., 118 (1968) 1014.
3 D.P. Merzt, Münch. Med. Wochenschr., 111 (1969) 491.
4 J. Broekhuysen, M. Bacco, R. Sion, L. Demeulenaere and M. Van Hee, Eur. J. Clin. Pharmacol., 4 (1972) 125.
5 W. Rothe, Pharm. Ind., 39 (1977) 806.
6 T.-F. Yu, J. Rheumatol., 3 (1976) 305.
7 M.J. Bartek, Mead Johnson Report BART-MJ-05641, 1975.
8 H. Ferber, G. Hitzenberger and H. Vergin, Eur. J. Clin. Pharmacol., submitted for publication.

Journal of Chromatography, 183 (1980) 387—388
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CHROMBIO. 611

Letter to the Editor

A quantitative method for the simultaneous determination of carbamazepine, mephenytoin, phenylethylmalonamide, phenobarbital, phenytoin and primidone in serum by thin-layer chromatography. Additional comments to the analysis of carbamazepine-10,11-epoxide

Sir,

In a previous note [1] we described a quantitative method for measuring antiepileptic drugs in serum by thin-layer chromatography (TLC). While checking for substances interfering in the method, we found that phenylethylmalonamide (PEMA), a metabolite of primidone [2], has an R_F value similar to that of carbamazepine-10,11-epoxide. This means that the four patients in our carbamazepine study [1] who were given both carbamazepine and primidone, have to be excluded. The amended values will therefore be: mean carbamazepine concentration in 27 sera, 4.2 mg/l with a range 1.3—7.3; and for the

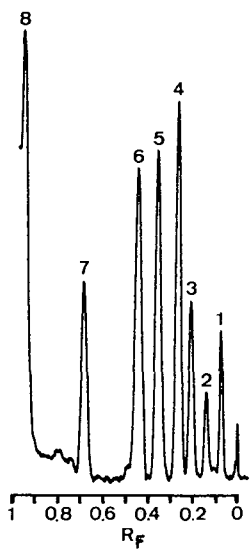

Fig. 1. TLC separation of a serum extract containing 2.5 μg of caffeine (3) and carbamazepine (4), and 5.0 μg of primidone (1), phenylethylmalonamide (2), phenytoin (5), phenobarbital (6) and mephenytoin (7). Peak 8 is the solvent front. Developing solvent: chloroform—acetone (87:13). In situ scan of reflectance at 215 nm.

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epoxide metabolite, 0.9 mg/l with a range 0.3—1.7, which is 21.4% of the carbamazepine concentration (previously determined, 26.2%).

PEMA was kindly donated by ICI-Pharma, Luzern, Switzerland. A working standard solution was prepared containing 12.5 mg each of caffeine and carbamazepine, together with 25 mg each of mephenytoin, PEMA, phenobarbital, phenytoin and primidone, per 100 ml of ethanol. This standard was added to drug-free serum, extracted and chromatographed as described previously [3, 4]. A scan of this thin-layer separation is shown in Fig. 1. PEMA does not exhibit any absorption maxima between 200 and 300 nm, only increasing absorption towards 200 nm. The recovery of PEMA added to serum was found to be 61%.

Department of Clinical Chemistry,
Universitätsspital Zürich, CH-8006
Zürich (Switzerland)

N. WAD
E. WEIDKUHN
H. ROSENMUND

1 N.T. Wad and H. Rosenmund, J. Chromatogr., 146 (1978) 167—168.
2 B.B. Gallagher and I.P. Baumel, in D.M. Woodbury, J.K. Penry and R.P. Schmidt (Editors), Antiepileptic Drugs, Raven Press, New York, 1972, pp. 361—366.
3 N. Wad, E. Hanifl and H. Rosenmund, J. Chromatogr., 143 (1977) 89—93.
4 N.T. Wad and H. Rosenmund, J. Chromatogr., 146 (1978) 361—362.

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CHROMBIO. 620

Book Review

Electrophoresis in the separation of biological macromolecules, by Ö. Gaál, G.A. Medgyesi and L. Vereczkey, Wiley, Chichester, New York, Brisbane, Toronto, 1980, 422 pp., price £21.00, ISBN 0-471-99602-5.

The domain of electrophoretic separation is, no doubt, in the field of biopolymers. A book devoted to this topic has been long expected and this one fills well the gap in the current world literature. After a brief theoretical survey, one can find here detailed descriptions of the strategy of the separation of proteins (and enzymes), nucleic acids and glycosidic components (glycoproteins). The categorization of proteins is done especially well, and includes specialized staining techniques for enzymes (pp. 261—285). The main emphasis on proteins is naturally on blood and membrane proteins. However, some other categories are omitted. I would not like to mention here connective tissue proteins as here I feel personally too involved and my judgement may be unjustified. However, such areas as muscle proteins, specific binding proteins, proteins of neoplastic tissues and, perhaps the field now called molecular anatomy, should be given some attention. The latter is partly covered in the section on isoelectric focusing followed by SDS—polyacrylamide gel electrophoresis (Fig. 81, p. 218). Another drawback could be considered the omission of mentioning such methods as rheophoresis or migration in the magnetic field. These techniques are not exploited much these days, but they do provide some perspectives.

Although the book is entitled Electrophoresis in the Separation of Biological Macromolecules, one wonders whether supramolecular structures should not be considered as well, at least for the benefit of the readers. The authors were wise enough to use an appropriate title to avoid any question of whether to include a chapter on the separation of cells and cellular particles.

The above comments are really minor ones and, in essence, represent suggestions for the possible second edition of the book, which I believe, will appear in due course. In my opinion the book is a good one and would be a valuable purchase for those involved in the application of electrophoretic methods. Unfortunately, the price is somewhat too high to expect many private buys, but this is common nowadays with almost any book.

Prague (Czechoslovakia) ZDENĚK DEYL

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PUBLICATION SCHEDULE FOR 1980

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	197/1 197/2 198/1	198/2 198/3 198/4 199	200/1 200/2	The publication schedule for further issues will be published later.
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Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2	183/3	183/4		

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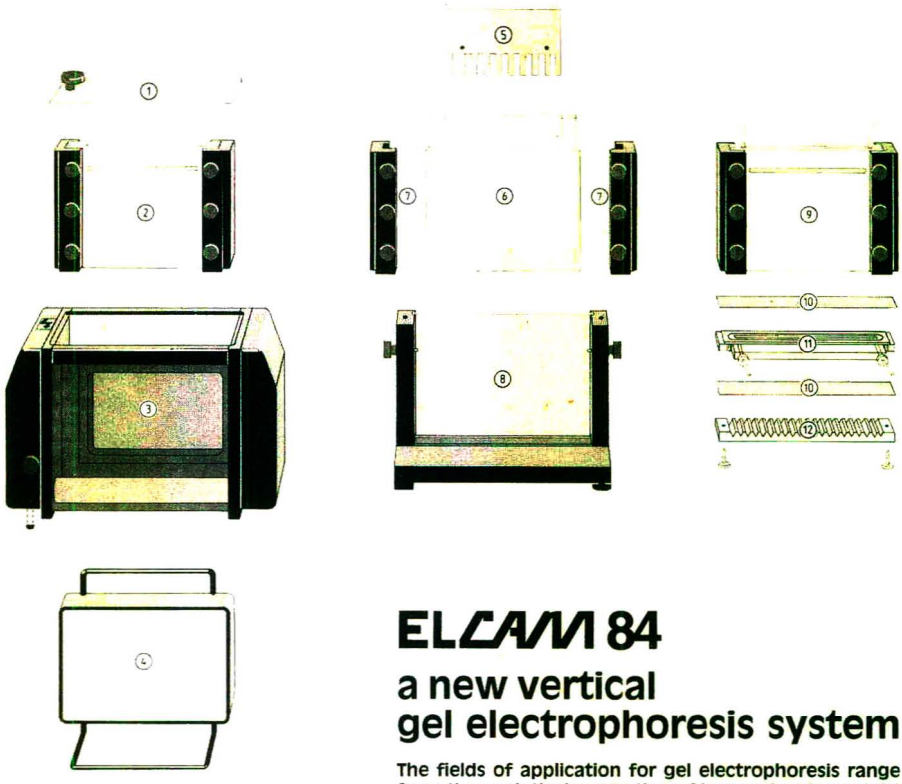

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