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VOL. 230, NO. 1 JOURNAL OF CHROMATOGRAPHY JUNE 11, 1982

(Biomedical Applications, Vol. 19, No. 1)

CONTENTS

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

(Continued overleaf)

Contents (continued)

Notes

JOURNAL OF CHROMATOGRAPHY

VOL. 230 (1982)

(Biomedical Applications, Vol. 19)

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CHROMBIO. 1245

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ANALYSIS OF ORGANIC ACIDS IN RENAL TISSUE BIOPSY

IDENTIFICATION OF 4-HYDROXYBUTYRIC ACID AND 4-HYDROXY-2-BUTENOIC ACID

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SUMMARY

The organic acids in renal tissue biopsy (0.5—1 mg) obtained from chronic glomerulonephritic patients were analyzed by capillary column gas chromatography—mass spectrometry. Some twenty compounds were identified in the renal tissue. The organic acid profile of renal tissue showed a marked difference from those of urine and serum. In particular, 4-hydroxybutyric acid and 4-hydroxy-2-butenoic acid, which are usually undetectable in urine and serum, were detected for the first time in renal tissue in considerably large amounts.

INTRODUCTION

So far the biopsy samples of renal tissue have been studied only by

0378-4347/82/0000—0000/$02.75

morphological methods such as light microscopy, fluorescence microscopy and electron microscopy. Recently Goodman et al. [1] described a method of analyzing the organic acids in various tissue specimens by gas chromatography—mass spectrometry (GC—MS). To determine the metabolism in renal tissue and, if possible, to diagnose renal disease from the viewpoint of metabolic profile, we have attempted to analyze the organic acids in renal tissue specimens by glass capillary column GC—MS. Organic acid profiles in renal tissue showed differences from those in urine or serum. 4-Hydroxybutyric acid and 4-hydroxy-2-butenoic acid have been detected in renal tissue for the first time.

EXPERIMENTAL

Materials

4-Hydroxybutyric acid was obtained from Tokyo Kasei Co. (Tokyo, Japan). 4-Hydroxy-2-butenoic acid was synthesized according to the method of McClure [2]. The synthesized compound was confirmed to be 4-hydroxy-2-butenoic acid by use of nuclear magnetic resonance, infrared spectroscopy and MS.

N,O-Bis(trimethylsilyl)trifluoroacetamide was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other reagents were commercially available products of analytical grade.

Sample preparation and GC—MS

Biopsied renal tissue samples were obtained from five patients with chronic glomerulonephritis. An amount of 0.5—1 mg of the tissue was homogenized with 0.2 ml of distilled water in a ground-glass homogenizer. After the addition of 0.8 ml of distilled water and sodium chloride, the homogenized solution was adjusted to pH 1.0 with 1 *N* hydrochloric acid, and the organic acids were extracted with 3 ml of ethyl acetate three times. After dehydration over anhydrous sodium sulfate, the extract was evaporated to dryness with a nitrogen stream. The extract was reacted with 1 mg of methoxylamine hydrochloride at 60°C for 1 h. After evaporation, the extract was trimethylsilylated with 20 μl of N,O-bis(trimethylsilyl)trifluoroacetamide at 90°C for 1 h. Three microlitres of the sample were subjected to GC—MS. The GC—MS instrument and the experimental conditions were the same as those reported previously [3].

RESULTS

Fig. 1 shows a gas chromatogram of organic acids in a renal tissue biopsy obtained from a patient with steroid-resistant nephrotic syndrome. The pathological finding was focal glomerulosclerosis. The peaks were identified by comparing their mass spectra with those of the trimethylsilylated authentic compounds or the mass spectra in the literature. The GC profile of renal tissue biopsy showed differences from those of urine and serum. In particular, the compound of peak 19 was detected in renal tissue in a comparatively large amount. In urine, however, the compound was barely detected or not detected at all. The electron-impact (EI) mass spectrum of peak 19 is shown in Fig. 2

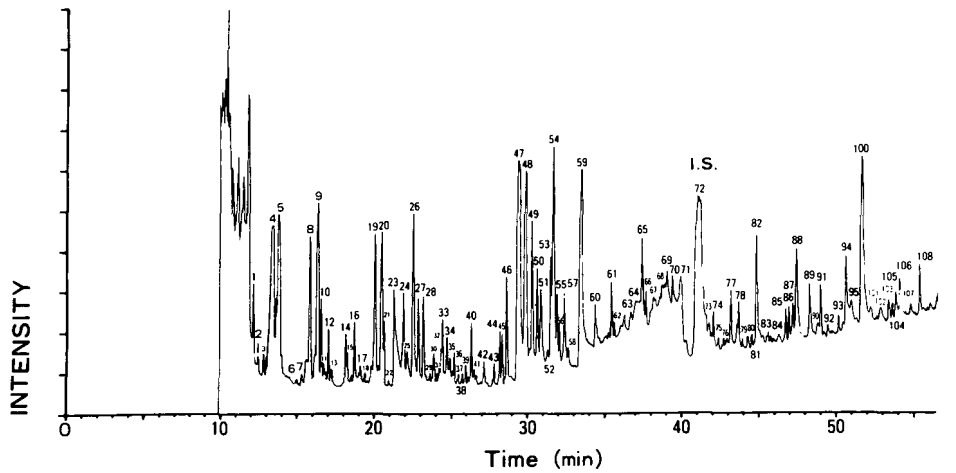

Fig. 1. Gas chromatogram of organic acids in renal tissue biopsy. The peaks were identified as follows: 3 = pyruvic acid; 4 = lactic acid; 5 = 2-hydroxyisobutyric acid (minor component), glycolic acid; 8 = 2-hydroxybutyric acid; 9 = 3-hydroxypropionic acid; 12 = 3-hydroxybutyric acid; 19 = 4-hydroxybutyric acid; 20 = diethylene glycol (artifact?); 25 = phosphoric acid; 26 = glycerol; 27 = 4-hydroxy-2-butenoic acid; 28 = succinic acid; 30 = methylsuccinic acid; 33 = 2-methylglyceric acid; 34 = glyceric acid; 36 = nonanoic acid; 37 = 4-deoxyerythronic acid; 42 = glutaric acid; 45 = 3-deoxytetronic acid; 47 = 2-deoxytetronic acid; 59 = 2,3-dideoxypentonic acid; 71 = isosaccharinolactone.

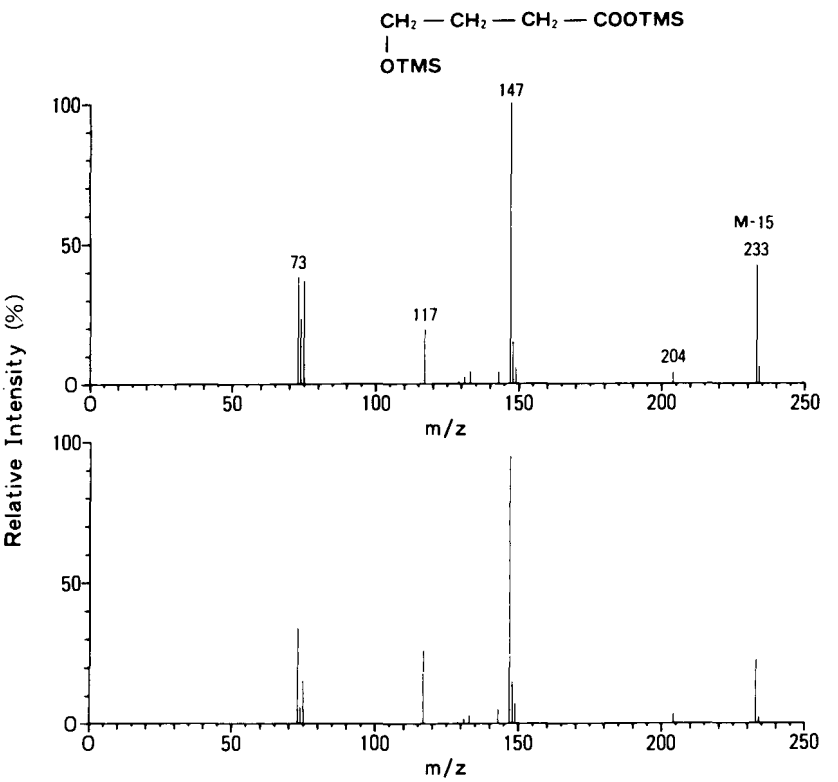

Fig. 2. EI-mass spectra of peak 19 in Fig. 1 (lower spectrum) and of trimethylsilylated 4-hydroxybutyric acid (upper spectrum).

(lower spectrum). The molecular ion of peak 19 was found to be *m/z* 248 by recording the chemical-ionization (CI) mass spectrum. High-resolution data of the *m/z* 233 ion indicated that the original molecular formula of the compound was $C_4H_8O_3$ and that the molecular structure was that of hydroxybutyric acid. The fact that the retention time of the compound on the gas chromatogram was delayed from 2-hydroxybutyric acid and 3-hydroxybutyric acid suggested the structure of 4-hydroxybutyric acid. The EI mass spectrum of trimethylsilylated 4-hydroxybutyric acid is shown in Fig. 2 (upper spectrum). Peak 19 was identified as trimethylsilylated 4-hydroxybutyric acid, since peak 19 and trimethylsilylated 4-hydroxybutyric acid showed identical retention times and identical mass spectra.

The compound of peak 27 in renal tissue was not detected in urine or serum at all. The EI mass spectrum of peak 27 is shown in Fig. 3 (lower spectrum). The CI mass spectrum of the peak indicated the molecular ion of *m/z* 246. High-resolution mass spectrometry of the *m/z* 246 ion showed an exact mass of 246.1089, an error of −1.6 millimass, an unsaturation of 2 and a probable composition of $C_{10}H_{22}O_3Si_2$. These data revealed the original composition of $C_4H_6O_3$ and the structure of hydroxybutenoic acid. The delayed retention time of peak 27 compared with 4-hydroxybutyric acid suggests the hydroxyl group at the C_4 position. The absence of an *m/z* 157 ion, $(M—OTMS)^+$, suggests unsaturation at C_2 not at C_3. 4-Hydroxy-2-butenoic acid was synthesized; the

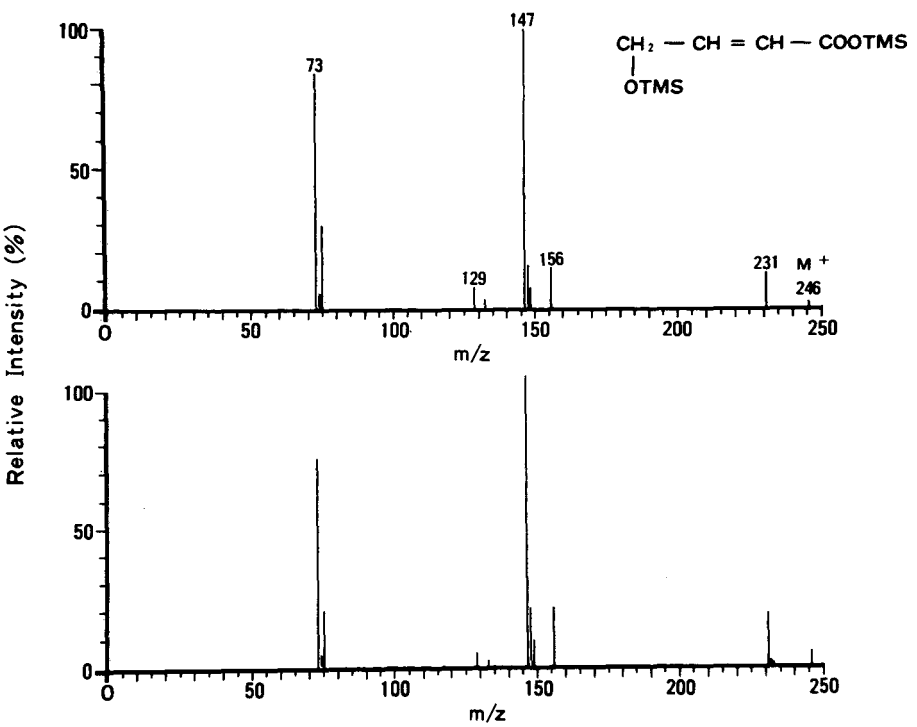

Fig. 3. EI mass spectra of peak 27 in Fig. 1 (lower spectrum) and of trimethylsilylated 4-hydroxy-2-butenoic acid (upper spectrum).

EI mass spectrum of the TMS derivative is shown in Fig. 3 (upper spectrum). Peak 27 was identified as trimethylsilylated 4-hydroxy-2-butenoic acid since peak 27 and trimethylsilylated 4-hydroxy-2-butenoic acid showed identical retention times and identical mass spectra.

DISCUSSION

By our profiling analysis of the renal tissue specimen, 4-hydroxybutyric acid and 4-hydroxy-2-butenoic acid were detected in renal tissue. So far 4-hydroxybutyric acid has been detected only in the human brain [4]. Recently, a case of inborn error of metabolism who excreted a large amount of 4-hydroxybutyric acid in the urine has been reported [5]. 4-Hydroxybutyric acid shows a pharmacological effect of central nervous system depression [6], and was synthesized as an intravenous anesthetic. The formation of 4-hydroxybutyric acid from 4-aminobutyric acid has been demonstrated in vitro [7] and in vivo [8], but this pathway seems to be a minor one for the degradation of 4-aminobutyric acid. The major portion of 4-aminobutyric acid is metabolized in brain and liver to succinic acid semialdehyde, which is further metabolized to succinic acid. 4-Aminobutyric acid is formed from glutamine via glutamic acid. The metabolic pathway of glutamine and glutamic acid is shown in Fig. 4. In the kidney, ammonia is produced from glutamine and glutamic acid, and excreted into the urine. The physiological significance of 4-hydroxybutyric acid in the kidney is at present unclear, but the formation of 4-hydroxybutyric acid in the kidney seems to be related to the ammonia production.

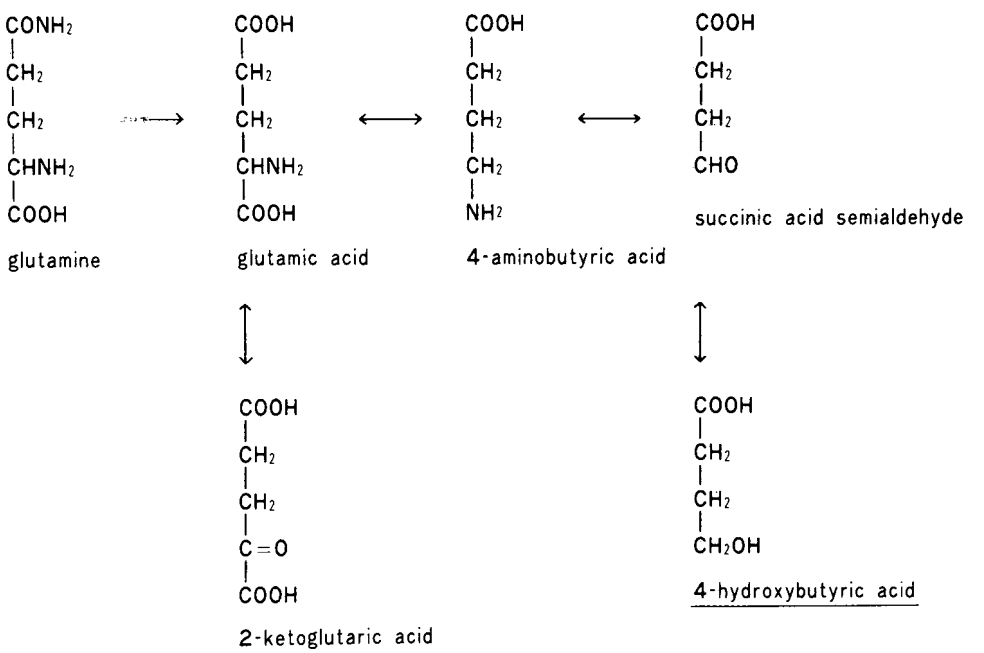

Fig. 4. Metabolic pathway of glutamine and glutamic acid.

4-Hydroxy-2-butenoic acid has not been reported to be present in human physiological fluids nor in tissues thus far. 4-Hydroxy-2-butenoic acid seems to be a metabolite of 4-hydroxybutyric acid. Walkenstein et al. [9] suggested the β-oxidation metabolic pathway of 4-hydroxybutyric acid (Fig. 5). The proposed pathway is considered to be a minor one, because the conversion of 4-hydroxybutyric acid via succinic semialdehyde to succinic acid seemed to be a more important pathway [10]. The detection of 4-hydroxy-2-butenoic acid as well as 4-hydroxybutyric acid in renal tissue, however, supports the β-oxidation pathway of 4-hydroxybutyric acid.

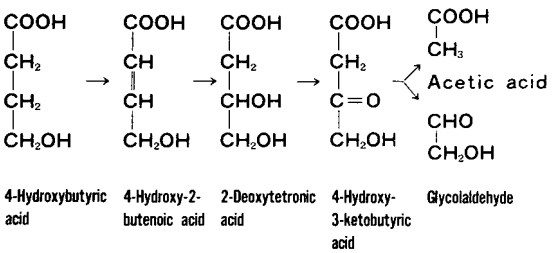

Fig. 5. Metabolic pathway of 4-hydroxybutyric acid.

The profiling analysis of organic acids in renal tissue biopsy shows the metabolic state mainly in the renal tubules, since most organic acids are known to be actively excreted into the urine by the renal tubular cells, and ammonia production is also performed in the renal tubular cells. The method may be useful for the investigation of renal disease which primarily affects the renal tubules, such as renal tubular acidosis and Fanconi's syndrome.

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CHROMBIO. 1246

ANALYSIS OF ORGANIC ACIDS IN THE HEARTS OF PATIENTS WITH IDIOPATHIC CARDIOMYOPATHY BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(Received December 24th, 1981)

SUMMARY

Organic acids in the hearts of patients with idiopathic cardiomyopathy, obtained by biopsy, were studied using gas chromatography—mass spectrometry. The profiling of organic acids was compared among eight cases of hypertrophic cardiomyopathy, three cases of congestive cardiomyopathy, and nine cases of other heart diseases, which were regarded as controls.

It was found that almost all organic acids, especially deoxyaldonic acids of 2-deoxytetronic acid, 2,3-dideoxypentonic acid, 3-deoxy-2-C-(hydroxymethyl)tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid, were accumulated in large amounts in the heart in congestive cardiomyopathy, while these acids were decreased in hypertrophic cardiomyopathy. It was therefore suggested that deoxyaldonic acid metabolism in the heart in congestive cardiomyopathy is quite different from that in hypertrophic cardiomyopathy.

INTRODUCTION

The cause of idiopathic cardiomyopathy (ICM) has not been clear, and few morphological and biochemical studies have been performed [1, 2]. The paucity of research might be related to difficulties in producing an

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experimental animal ICM that is the same as human ICM, and of finding out which compounds are characteristic in ICM.

Gas chromatography—mass spectrometry (GC—MS) has the capability of analyzing many compounds simultaneously, and is ideally matched to a wide-range survey of metabolism, as, for example, which compounds are characteristic in the heart. Moreover, it has already been reported that organic acids in the heart muscle have been analyzed by this technique [3, 4].

The present study was therefore undertaken to examine organic acids in the heart of patients with hypertrophic cardiomyopathy (HCM) and congestive cardiomyopathy (CCM) by the use of GC—MS, to find out whether or not biochemical changes in the organic acid metabolism occur in the hearts of patients with ICM.

MATERIALS AND METHODS

Chemicals

Reagents of lactic acid, glycolic acid, glyceric acid, palmitic acid and stearic acid were commercial products. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Tokyo Kasei Co. (Tokyo, Japan). All other reagents were of the highest purity available commercially.

Gas chromatography and gas chromatography—mass spectrometry

A Shimadzu GC-6A gas chromatograph with dual flame ionization detectors was used. A glass coiled column (2 m × 3 mm, I.D.) was packed with 3% OV-17 on Gas-Chrom Q (80—100 mesh). The column oven was maintained isothermally at 80° C for 2 min and then programmed at 6° C/min until 290° C. Peak areas and retention times were determined with an on-line Shimadzu Chromatopac 4-B computer. For identification of the compounds, a JEOL JMS-D 100 GC—MS system with an on-line JMA 2000 data acquisition system was used.

Mass spectra were recorded at an ionizing voltage of 75 eV with a 300-μA trap current, and an ion source temperature of 280° C. The magnet of the mass spectrometer was scanned repetitively over field strengths from *m/z* 50 to *m/z* 700 every 5 sec.

Sample preparation

A few milligrams of heart samples were obtained from patients with heart diseases by biopsy during cardiac catheterization. Eight samples were obtained from the right heart ventricle of patients with HCM, who ranged in age from 19 to 64 years (mean 49 years) and included seven males and one female. Three samples were from patients with CCM, including a 15-year-old male, a 38-year-old male and a 35-year-old female. Nine samples were obtained from controls, who ranged in age from 17 to 63 years (mean 49 years) and included five males and four females. The diagnosis of the controls was as follows: atrial septal defect, hypertensive heart diseases, sick sinus syndrome, ventricular premature contraction, and atrioventricular block.

The biopsied heart specimens were immediately frozen in dry ice–acetone and kept until analysis. The extraction procedure followed was that described

in ref. 4. The samples were thawed and rinsed in cold saline solution and then homogenized. Then 20 μg of heptadecanoic acid per 1 mg of protein (which was determined by the Bio-Rad protein assay method) were added to the homogenates as an internal standard. Centrifugation was performed at 25,000 *g* for 15 min. The supernatant collected was concentrated in order to remove the ethanol. Organic acid fractions were obtained by extraction with an equal volume of diethyl ether and ethyl acetate twice at pH 1 with 2 *N* hydrochloric acid. Organic solvent extracts were dried under a nitrogen stream.

The samples were trimethylsilylated by adding 200 μl of BSTFA to the residue. The mixtures were then heated to 60°C for 1 h. Aliquots of the samples were subjected to GC and GC—MS for analysis.

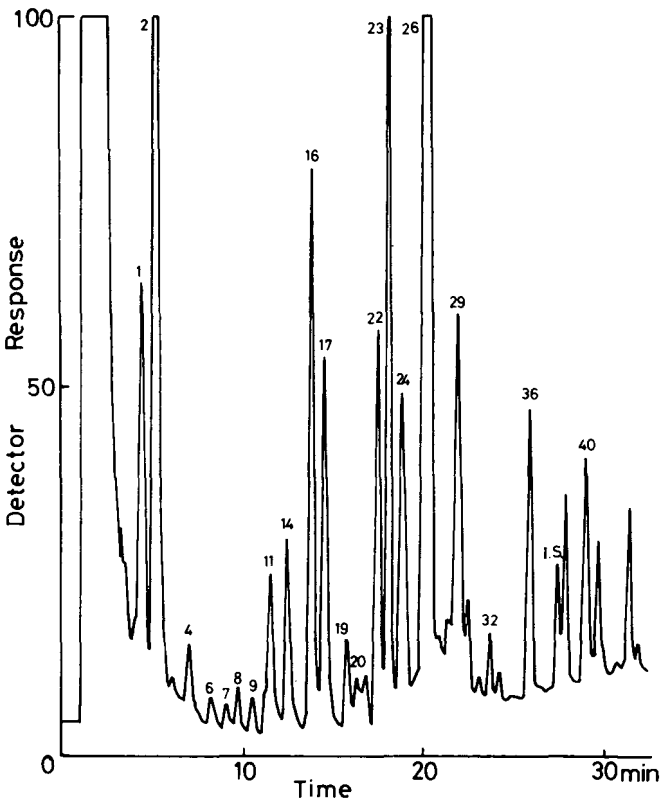

Fig. 1. Gas chromatogram of trimethylsilyl (TMS) derivatives of organic acids in heart biopsy of a patient with heart disease (hypertensive heart disease). Identified and tentatively identified compounds were as follows: 1 = lactic acid; 2 = glycolic acid; 4 = 3-hydroxypropionic acid; 7 = glycerol; 9 = 2-methylglyceric acid; 11 = glyceric acid; 16 = 2-deoxytetronic acid; 17 = 3-deoxy-2-C-(hydroxymethyl)tetrono-1,4-lactone; 20 = 2,3-dideoxypentonic acid; 22 = 3-deoxy-2-C-(hydroxymethyl)tetronic acid; 23 = 3-deoxypentono-1,4,-lactone; 24 = 3-deoxyerythropentonic acid; 26 = 3-deoxy-2-C-(hydroxymethyl)pentono-1,4-lactone; 28 = 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid; 36 = palmitic acid; 40 = stearic acid.

RESULTS

The profile of organic acids in human heart obtained by biopsy is shown in Fig. 1. Over 40 peaks were detected on the gas chromatogram. The peak which appears between peaks 36 and 40 is the internal standard, heptadecanoic acid. When the profile was compared with that obtained from rat heart muscle [4] the profiles were found to be quite similar to each other.

Identification of the peaks was performed by comparing their mass spectra and retention times with those of laboratory samples or literature references [5, 6]. The mass spectrum obtained from peak 16 is shown as an example (Fig. 2). The molecular ion at *m/z* 336 was not detected but an ion at *m/z* 321 $(M-15)^+$ was found as a relatively small peak. Other fragment ions, at *m/z* 246 $(M-90)^+$, at *m/z* 233 $(M-CH_2OTMS)^+$, at *m/z* 231 $(M-15-90)^+$ and at *m/z* 205 $(CH_2OTMS \cdot CH{=}OTMS)^+$ were detected. The base peak was observed at *m/z* 73. This mass spectrum and the retention time were consistent with that of ref. 4. Peak 16 was, therefore, identified as 2-deoxytetronic acid tri-TMS. In this way, each peak was identified, and the identified compounds are shown in the legend of Fig. 1.

Gas chromatograms of specimens obtained from patients with HCM and with CCM are shown in Figs. 3 and 4, respectively. The peak of lactic acid (No. 1), which increases in ischemic myocardium, appeared as a relatively small peak. Peaks of 2-deoxytetronic acid (No. 16) and 2,3-dideoxypentonic acid (No. 20), which accumulate in ischemic-like conditioned rat heart muscle [7, 8], were observed as small peaks in Fig. 3. However Fig. 4 shows that these peaks were detected as relatively large peaks.

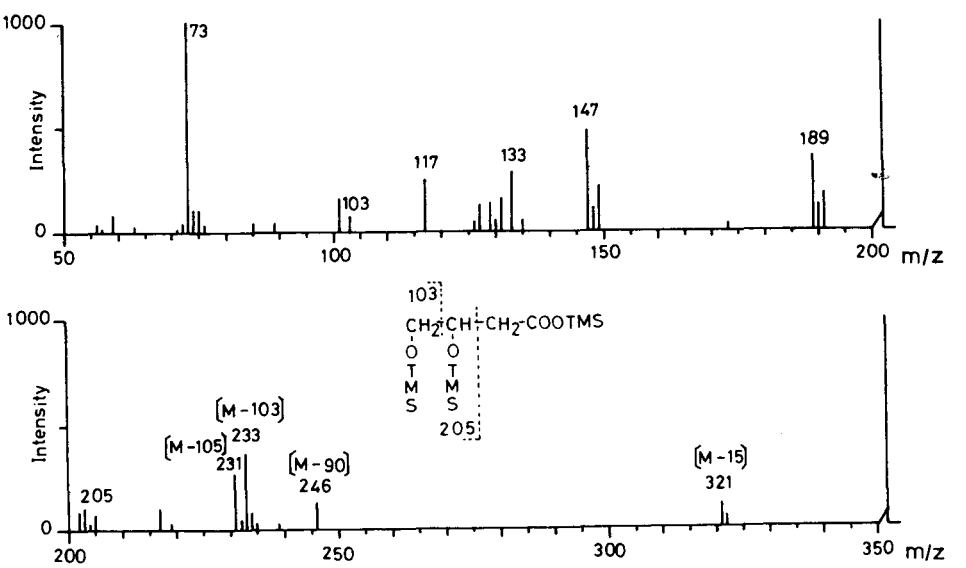

Fig. 2. Mass spectrum of the trimethylsilyl (TMS) derivative of 2-deoxytetronic acid, which was obtained from peak 16 in Fig. 1.

As it has been reported by Thompson et al. [9] that lactones are formed under acidic conditions during the extraction procedure, the lactones were therefore regarded as the corresponding acids. Peaks of deoxyaldonic acids, 3-deoxy-2-C-(hydroxymethyl)tetronic acid (Nos. 17 and 22), 3-deoxyerythropentonic acid (Nos. 23 and 24) and 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid (Nos. 26 and 28), were detected as large peaks, as shown in Fig. 4, compared with those of Fig. 3.

The peak of palmitic acid was detected as a very large peak because of contamination by an unknown compound which appeared at the same retention time as palmitic acid, as shown in Fig. 4 [7]. But the peak of palmitic acid (in Fig. 3) was detected as a relatively large peak, and peaks of stearic acids (shown in Figs. 3 and 4) were detected at almost the same height. It is considered that the content of fatty acids in HCM heart was almost the same as that of CCM heart.

Table I shows the relative peak height ratios of the major peaks to the internal standard among three groups — HCM, CCM and control. The ratios of almost all peaks of the CCM group were higher than those of the control group, and those of the HCM group were the lowest.

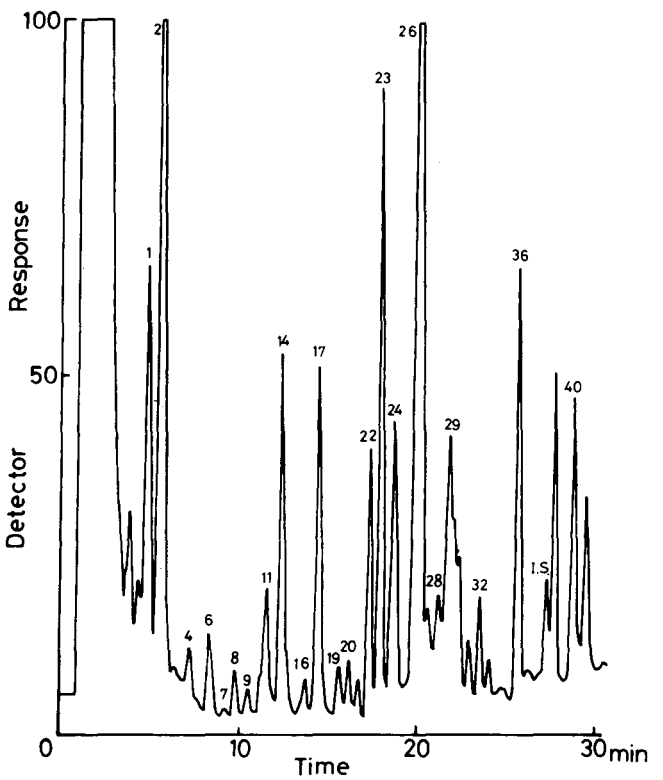

Fig. 3. Gas chromatogram of TMS derivatives of organic acids in heart biopsy from a patient with hypertrophic cardiomyopathy. The above peak numbers correspond to those in Fig. 1.

TABLE I

RELATIVE PEAK HEIGHT RATIOS (±S.D.) OF MAJOR COMPOUNDS TO THE INTERNAL STANDARD IN THE THREE GROUPS — HCM, CCM AND CONTROL

No.*	Compound	HCM (n=8)	CCM (n=3)	Control (n=9)
1	Lactate	0.418 (±0.372)	0.539 (±0.084)	0.587 (±0.415)
2	Glycolate	1.094 (±0.939)	2.339 (±1.327)	1.720 (±1.275)
4	3-Hydroxypropionate	0.309 (±0.158)	1.905 (±0.850)	0.875 (±0.809)
16	2-Deoxytetronate	0.270 (±0.343)	1.778 (±1.814)	0.870 (±1.023)
17 + 22	3-Deoxy-2-C-(hydroxymethyl)tetronate	0.157 (±0.184)	0.262 (±0.244)	0.195 (±0.159)
20	2,3-Dideoxypentonate	0.064 (±0.130)	0.946 (±0.764)	0.381 (±0.417)
23 + 24	3-Deoxyerthyropentonate	0.422 (±0.348)	3.267 (±2.488)	0.582 (±0.694)
26 + 28	3-Deoxy-2-C-(hydroxymethyl)erythropentonate	0.428 (±0.206)	0.946 (±0.764)	0.579 (±0.549)
36	Palmitate	0.883 (±0.511)	2.359 (±1.593)	1.075 (±0.622)
40	Stearate	0.906 (±0.455)	1.018 (±0.555)	1.112 (±0.726)

*The numbers refer to the numbered peaks in Fig. 1.

DISCUSSION

Profiling of organic acids in various tissues and fluids (rat heart and brain, human amniotic fluid and cerebrospinal fluid) have been reported using GC—MS [10, 11]. It is considered that the use of GC—MS is well adapted to this kind of study, because of its capabilities for simultaneous analysis and identification of compounds.

This is the first report of analysis of organic acids in human heart using a packed column. A capillary column is able to separate more peaks clearly, but this is not suitable for profiling because of its poor reproducibility and its use is time-consuming. Consequently, the packed column was used for the current study.

It might be supposed that the profiling of organic acids in human heart is similar to that in rat heart muscle [4] even though different ventricles were used (right in human and left in rat), and no characteristic compound different to those found in rat heart was detected in human heart.

Let us consider two points as to why deoxyaldonic acids were accumulated more in patients with CCM than in those with HCM. First, the number of patients with CCM was small (three cases). One must be cautious in comparing the ratios of peaks to internal standard in CCM with those in HCM. However, as Table I demonstrates that means of these ratios in CCM are clearly larger than those in HCM, the difference in case numbers may not be of importance in interpreting the increase in deoxyaldonic acids in CCM. Secondly, the proteir

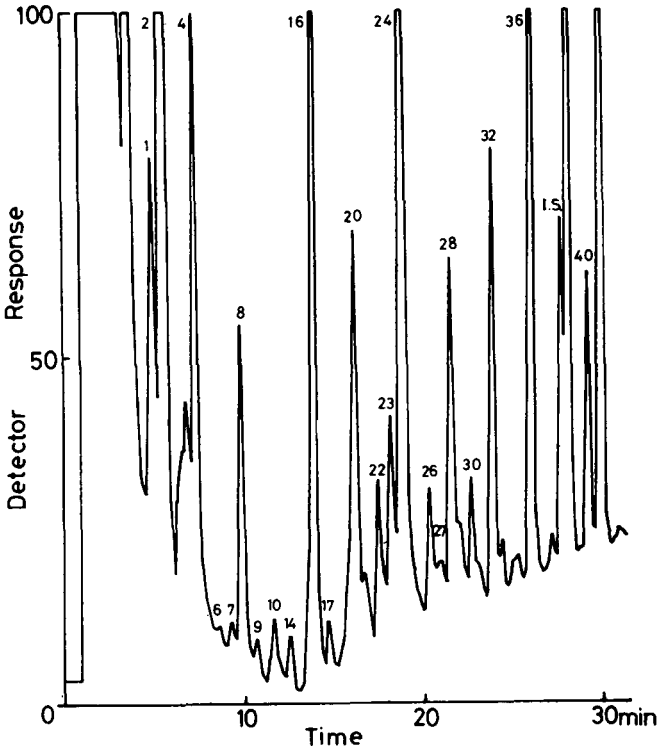

Fig. 4. Gas chromatogram of TMS derivatives of organic acids in heart biopsy from a patient with congestive cardiomyopathy. The peak numbers correspond to those in Fig. 1.

content in CCM appeared to be lower than that in HCM, the average values being 240 and 450 mg/g wet tissue, respectively. However, the ratio of protein content in CCM to that in HCM was only one half. And as the ratios of each peak in CCM were more than twice those in HCM, the increase in deoxyaldonic acids in CCM does not seem to be related to a difference of protein content in the hearts. It is concluded, therefore, that deoxyaldonic acid was actually accumulated in large amounts in CCM heart.

Haragachi et al. [8] reported that deoxyaldonic acids, which were accumulated in CCM heart, had been gradually accumulated during the time elapsed after decapitation in rat heart muscle. These results mean either that significant accumulation of deoxyaldonic acid might cause deterioration of the heart function, or that the accumulation might be induced by the heart damage. The relevance of these data to the present findings is unclear, because it is not known whether or not biochemical functions of the rat and human heart are completely identical. However, one possible interpretation is that the heart of CCM might be more dysfunctional or damaged than that of HCM, probably due to the accumulation of deoxyaldonic acids. In fact, in contrast to HCM patients, the prognosis of patients with CCM is generally bad and heart failure readily occurs.

As neither the function nor the metabolism of deoxyaldonic acids detected in heart muscle is clearly understood, the metabolic changes in CCM and HCM

have not been discussed biochemically. It is known, however, that nuclei of CCM heart muscle cells are morphologically damaged and the DNA content decreases. It may be possible to assume that 2-deoxyaldonic acids are derived from 2-deoxyribonucleic acid produced by decomposition of the nucleus. On the other hand, some researchers have reported that derivation of 2-deoxytetronic acid from carbohydrate was indirectly demonstrated [9, 12, 13]. It is presumed, therefore, that a change in carbohydrate metabolism, particularly of the pentose phosphate cycle which normally plays an important role in the heart [14], induced by unknown causes, might lead to increased deoxyaldonic acids in CCM and decreased acids in HCM.

ACKNOWLEDGEMENT

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DETERMINATION OF PROSTAGLANDINS AND THROMBOXANE AS THEIR PENTAFLUOROBENZYL-TRIMETHYLSILYL DERIVATIVES BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

The optimization of the parameters affecting the chromatographic properties and separation of prostaglandin pentafluorobenzyl derivatives by gas chromatography using electron-capture detection is described. The effects of composition and flow-rate of carrier gas, temperatures of detector and column, and nature of stationary phases on the detector response to different pentafluorobenzyl (both oxime and ester) trimethylsilyl ether derivatives of prostaglandins were systematically examined. The stability of some selected prostaglandin derivatives at −20°C was also determined. After standardizing these parameters, prostaglandins and related compounds from biological samples, e.g. semen, rat aorta, dog serum and trout gill were successfully analyzed. Identification of prostaglandins was confirmed by gas chromatography—mass spectrometry.

INTRODUCTION

Progress in the study of factors affecting the concentrations and types of prostaglandins (PGs), thromboxane (TX) and related compounds and in relating different species of PG to specific functions in tissue would be greatly facilitated by the availability of a rapid method for determining the PGs and related compounds occurring in biological tissues. Currently, radioimmunoassay (RIA), a sensitive technique, is most commonly used. However, it requires antisera for each PG, cross-reactivity is a problem and only one PG species is quantified per analysis [1]. Gas chromatography (GC) using a flame ionization detector separates and detects the major PGs and TX but lacks sufficient sensitivity to quantify the low concentrations of PGs found in most biological tissues [2]. However, using the electron-capture detector (ECD) and appropriate derivatization of PGs, a much greater sensitivity can be obtained

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[3—5]. This method is equivalent in sensitivity to GC—mass spectrometry (GC—MS) [1, 2] and should be more feasible and affordable for most research laboratories. However, this method has not yet been widely adopted because the ECD procedure requires several tedious and scrupulous multi-step preparations of sample which make quantitative recovery challenging. Secondly, preparation of a clean sample is essential for quantification from GC peaks [6]. Most importantly there is very limited information concerning the effects of operating parameters, i.e. carrier gas composition, flow-rates, detector temperature, column temperature and chromatographic stationary phases on the resolution capacity and sensitivity of the ECD to the pentafluorobenzyl derivatives of PG. This paper reports the systematic study of these parameters and describes the optimum GC conditions for better resolution and sensitivity of PG determination in biological samples. The use of this technique for profiling the PG and TX from biological tissues as confirmed by GC—MS was demonstrated.

MATERIALS AND METHODS

Methanol, ethanol, chloroform, anhydrous diethyl ether, light petroleum, hexane, sodium sulfate (Mallinckrodt, St. Louis, MO, U.S.A.); pyridine (Fisher Scientific, Pittsburgh, PA, U.S.A.); N-methyl-N-nitroso-*p*-toluenesulfonamide (Sigma, St. Louis, MO, U.S.A.); sodium borohydride ($NaBH_4$) (Alfa, Danvers, MA, U.S.A.); O-methylhydroxylamine hydrochloride, O-pentafluorobenzylhydroxylamine hydrochloride, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *tert.*-butyldimethylchlorosilane imidazole (Applied Science Labs., State College, PA, U.S.A.); pentafluorobenzyl bromide, diisopropylethylamine (Aldrich, Milwaukee, WI, U.S.A.) were used as received. The PGs and TX were gifts from Upjohn (Kalamazoo, MI, U.S.A.). 12-Hydroxyeicosatetraenoic acid (HETE) was provided by Dr. Edward Goetzl, Department of Medicine, Harvard University. The other chemicals used were analytical grade.

Stationary phases 3% SP-2100, 3% SP-2100 OH, 3% SP-2250, 10% SP-2340 (Supelco, Bellefonte, PA, U.S.A.), 15% Silar 10C, 10% EGSS-X (Applied Science Division, State College, PA, U.S.A.), 3% Dexil 300, 3% OV-1, 3% OV-101, 3% SE-30 and 3% OV-351 (Alltech Assoc., Deerfield, IL, U.S.A.) were used to test the effects of coating materials on the chromatographic resolution as well as detector performance.

Extraction of prostaglandins

Several different biological tissues were analyzed for PG. Semen was extracted as described previously [7]. The extraction procedure for the other samples was described in detail elsewhere [8]. Basically the tissue was homogenized in 0.9% saline, the homogenate was adjusted to pH 4.0—4.5 and the PGs were extracted with ethyl acetate [8]. Ten minutes equilibration time was allowed when internal standard procedure was used.

Thin-layer chromatography, using the solvent system described by Goswami and Kinsella [9], was performed to separate and purify the PG samples from tissues.

Derivatization for GC analyses

PGs and TX were esterified, oximated and silylated as described [4, 5]. A scheme of this derivatization is shown in Fig. 1. The recoveries for each step were monitored by tritium-labelled $PGF_{2\alpha}$ and PGE_2 (New England Nuclear, Boston, MA, U.S.A.).

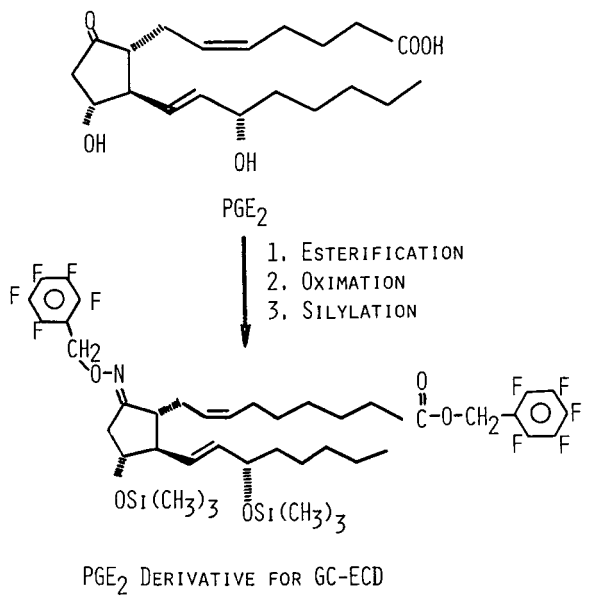

Fig. 1. Schematic illustration of the procedure for derivatization of prostaglandins and thromboxane for gas chromatography—electron-capture detection quantitation.

Gas chromatography—electron-capture detection

A Hewlett-Packard 5830A automated gas chromatograph equipped with a HP-18803A ^{63}Ni electron-capture detector was used. The retention times were automatically measured with an 18850A GC terminal. Silanized pyrex glass columns of 2.8 m × 0.32 cm and 4.7 m × 0.32 cm were used. The columns were packed with 3% OV-101 (100—120 mesh) and/or 1% SE-30 (100—120 meh) and conditioned for 24—48 h before use.

The conditions employed for the separation of the PG pentaflurobenzyl ester (PFBE) derivatives were: injector temperature 270°C, detector temperature 320°C and column temperature, unless otherwise specified, was maintained at 270°C for 10 min and then programmed at 5°C/min until 285°C. The carrier gas was set at a flow-rate of 17—20 ml/min.

Argon—methane carrier gas at either 95:5 or 90:10 ratio and nitrogen was used to test if the composition of carrier gas affected the response of the ECD.

Gas chromatography—mass spectrometry—selective ion monitoring

A Hewlett-Packard 5995A GC—MS system was used to conduct GC—MS—selective ion monitoring (SIM) analysis for the identification of PGs and hydroxy fatty acid from biological tissues. A fused silica capillary column (12

m × 0.2 mm) coated with methyl silicone (Hewlett-Packard) was used to separate the PG derivatives.

RESULTS AND DISCUSSION

Because stationary phases, column temperature, carrier gas composition, gas flow-rate and detector temperature are important parameters in determining the sensitivity and resolution of PGs for GC—ECD, we examined these parameters using a few PFBE derivatives of PG as model compounds.

GC stationary phases

The type and properties of stationary phases are critical for successful analyses of PG. Thus a series of stationary phases was tested and their suitability for PG analysis is summarized (Table I). The 3% SP-2100, 3% SP-2100 OH,

TABLE I

SUITABILITY OF STATIONARY PHASES TESTED FOR THE GC—ECD ANALYSIS OF PENTAFLUOROBENZYL DERIVATIVES OF PROSTAGLANDINS

+ = good performance; ± = intermediate; — = unsuitable.

Stationary phase	Selectivity	Stability	Temperature limit (°C)
10% SP-2340	+	±	275
3% SP-2250	—	—	250
3% SP-2100	+	+	350
3% SP-2100 OH	+	+	350
3% OV-101	—	—	275
3% OV-1	+	+	350
3% SE-30	+	+	300
3% Dexil 300	±	+	400
15% Silar 10C	+	—	275
10% EGSS-X	—	—	230

3% SE-30, 3% OV-1 stationary phases were most satisfactory for the general separations of all the PG pentafluorobenzyl derivatives. The 3% SP-2340 column was extremely good for the separation of PGF isomers. The 15% Silar 10C column, although selective for the PGF isomers, presented problems because of bleeding and contamination. Frequently, after a few runs on this column, the ECD becomes contaminated to the point where column bleeding interferes with the signal peaks. The stationary phases suitable for high-temperature runs are generally very non-polar. In order to separate derivatives differing slightly in functional structure or degree of unsaturation, capillary columns are necessary. There is a need for stationary phases which are more polar but cause less contamination of detector at high temperature.

Column temperature

To study the effect of column temperature on ketonic and non-ketonic PGs,

a 2.8 m × 0.32 cm 3% OV-101 glass column was used. The responses of the ECD to $PGF_{2\alpha}$-pentafluorobenzyl ester-trimethylsilyl (PFBE-TMS) and PGE_2-PFBE-TMS at different temperatures are shown in Fig. 2. As the column temperature was increased, the response to $PGF_{2\alpha}$-PFBE-TMS decreased slightly. The PGE_2-pentafluorobenzyl ester-pentafluorobenzyl oxime-trimethylsilyl (PFBE-PFBO-TMS) was not eluted below a column temperature of 260°C, probably because of the presence of the high-molecular-weight pentafluorobenzoxime group. From 270—285°C the ECD response to PGE_2-PFBO-PFBE-TMS increased due to the increase of volatility of the derivative. After several trials, we found that holding the column at 270°C then programming to 285°C at 5°C/min gave satisfactory resolution and a reasonably short running time of ca. 25 min.

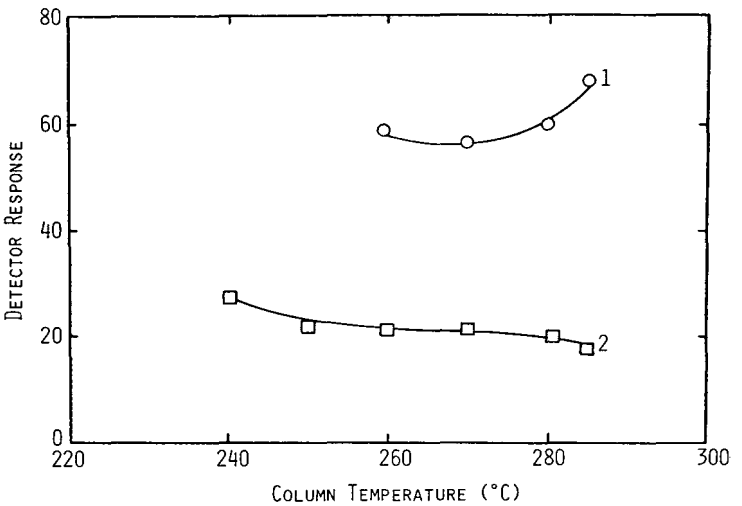

Fig. 2. The effects of column temperatures on the electron-capture detector response to PGE_2-PFBO-PFBE-TMS (1) and $PGF_{2\alpha}$-PFBE-TMS (2).

Detector temperature

The temperature dependence of ECD response to PG derivatives is represented by plotting $\ln A/n \times T^{3/2}$ versus $1/T$, where A is the peak area in arbitrary units, n the number of moles injected, and T the temperature in °K [10]. All the derivatives tested gave the same response patterns, i.e. as the detector temperature increased, the responses also increased (Fig. 3). The observed change in response is probably due to the variations of the electron absorption coefficient, k, with the temperature for each PG or TX derivative [11]. For the PFB derivatives of PG, it seems probable that the electron is initially captured by the resonance system, which has a high cross-sectional area for collisions and offers the possibility of interaction with other free electrons [10—12]. High detector temperature seems to facilitate this reaction. For highest sensitivity it is recommended that the detector temperature be set at 350°C. The maximum temperature for the Hewlett-Packard ^{63}Ni detector is 370°C [12].

From the above studies, the running conditions for optimum quantification

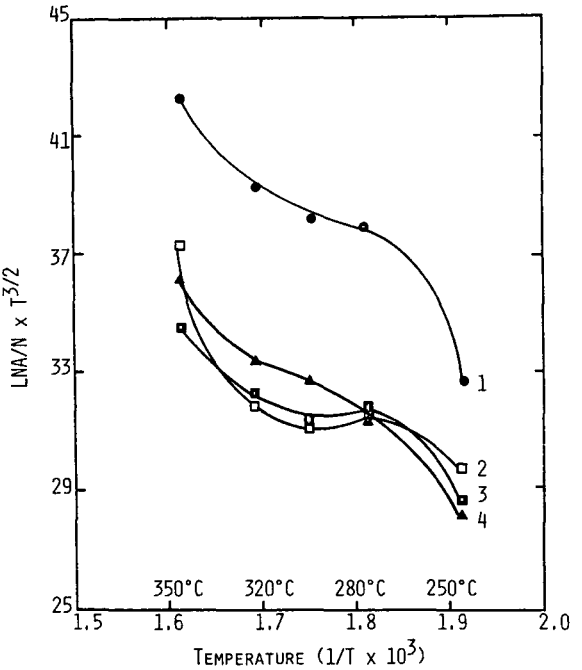

Fig. 3. The effects of detector temperatures on the ECD response to prostaglandin derivatives. (1) Cholanic acid-PFBE, (2) PGE_2-PFBO-PFBE-TMS, (3) $PGF_{2\alpha}$-PFBE-TMS, (4) PGE_1-PFBO-PFBE-TMS.

of the PG derivatives were detector temperature 350°C, flow-rate of carrier gas 16 ml/min and column temperature holding at 270°C for 10 min and then programmed at 5°C/min until 285°C when the high temperature stable non-polar stationary phases were used.

Effect of composition of carrier gas on ECD response

The effect of composition of carrier gas on ECD response to PG derivatives showed that argon—methane (95:5) resulted in slightly higher responses than did argon—methane (90:10). This is expected since the higher proportion of scavenger methane gas in the carrier gas trapped more thermal electrons [13]. Generally, the electronically excited species are deactivated by hydrocarbons and because electrons are generated in this reaction, a negative peak would result if the hydrocarbons were in the column effluent. By adding methane to the carrier gas the deactivation process in the ECD cell is continuous, and a constant number of electrons are added to the cell.

Nitrogen gas failed to give an acceptable baseline for GC—ECD analysis of PG. Theoretically the use of nitrogen as carrier gas results in an increase in the absolute sensitivity of the ECD but the noise increases proportionately so the minimum detectable level is not improved.

Effect of flow-rate of carrier gas

The gas flow-rate affected responses of ECD to different PG derivatives.

PGE_1-PFBO-PFBE-TMS, PGE_2-PFBO-PFBE-TMS and TXB_2-PFBO-PFBE-TMS all showed slight decreases in response as the flow-rate increased (Fig. 4A). However, the PGF-PFBE-TMS showed a sigmoidal type of response, i.e. at the higher and the lower flow-rates the response became drastically low and high, respectively, while at the intermediate flow-rates (10—16 ml/min) the responses of all the PGFs tested were rather constant. This phenomenon was not observed for all the keto-containing derivatives. Since the only difference between the PGEs and PGFs is the PFBO group, it is conceivable that the pentafluorobenzoxime may have stabilized the response of the ECD to change of flow-rates but the actual mechanism for the sigmoidal response of the PGF

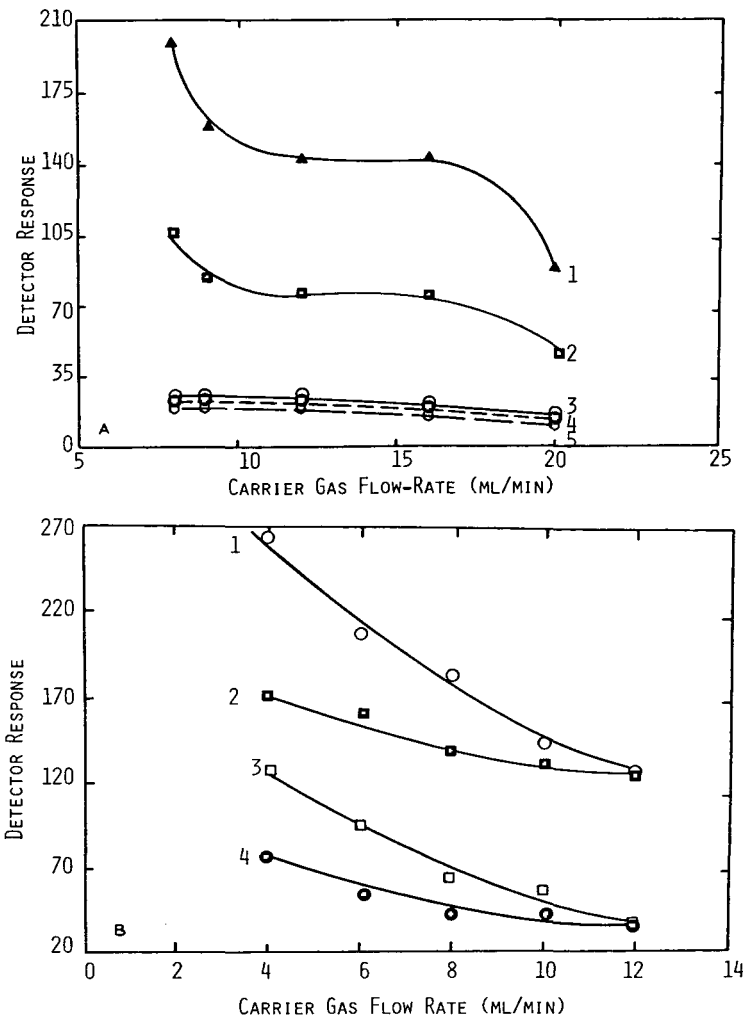

Fig. 4. The effects of carrier gas flow-rates on the ECD responses to different prostaglandin derivatives. (A) At flow-rates between 8 and 20 ml/min: (1) $PGF_{1\alpha}$-PFBE-TMS, (2) $PGF_{2\alpha}$-PFBE-TMS, (3) TXB_2-PFBO-PFBE-TMS, (4) PGE_2-PFBO-PFBE-TMS, (5) PGE_1-PFBO-PFBE-TMS. (B) At flow-rates between 4 and 12 ml/min: (1) PGE_1-PFBO-PFBE-TMS, (2) PGE_1-MO-PFBE-TMS, (3) $PGF_{2\alpha}$-PFBE-TMS, (4) cholanic acid-PFBE.

derivatives as the flow-rate changed is not understood. In general, at lower flow-rates there was a tendency for the ECD responses to increase. The ECD responses to other PG derivatives at slower flow-rates were plotted in Fig. 4B. These data show that as the flow-rates decreased, the ECD responses to all the derivatives consistently increased. Comparing responses at 4 ml/min to those at 12 ml/min, the ECD responses of $PGF_{2\alpha}$-PFBE-TMS and cholanic acid-PFBE were more than doubled.

The sensitivity of the ECD depends upon the instantaneous concentration of the sample molecules in the active sensing region of the detector cell because it is a concentration-dependent detector. Thus, the highest sensitivity is obtained at lower gas flow-rates [14]. However, at low flow-rates (<10 ml/min) increased noise levels and poor resolution are problems which affected both quantification and separation of the peaks. The best practical flow-rate was 16 ml/min.

Stability of the PG derivatives

The stability of PG derivatives is an important criterion in deciding which derivatives to use for GC analysis of PG from biological samples. Most of the PG derivatives tested were fairly stable (Fig. 5). The estimated half-lives ($t_{1/2}$) for $PGF_{2\alpha}$-PFBE-TMS, PGE_2-PFBO-PFBE-TMS and PGA_2-PFBO-PFBE-TMS were 30, 20 and 14 days, respectively. In general, the PGF-PFBE-TMSs were more stable than the keto-containing PG-PFBO-PFBE-TMSs.

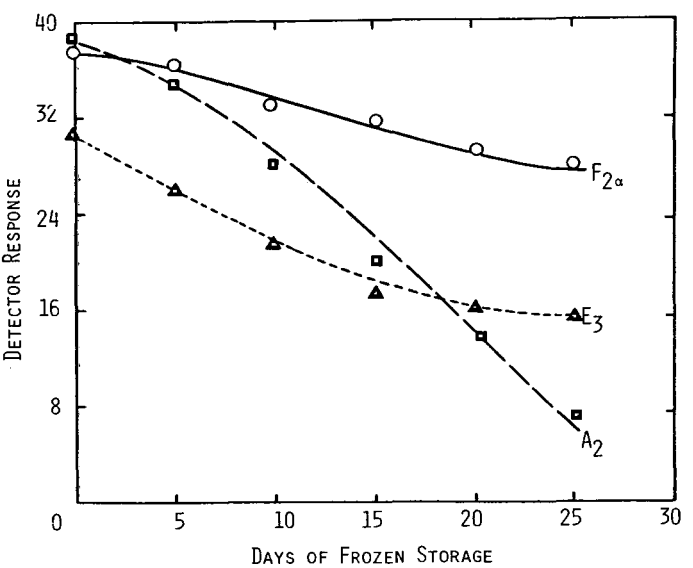

Fig. 5. The relative stabilities of PG-PFBE-TMS or PG-PFBO-PFBE-TMS derivatives during storage at −20°C.

Stability of the PG derivatives for GC detection has rarely been determined. Miyazaki et al. [15] reported that the stability of the dimethylethylsilyl ether of the $PGF_{2\alpha}$ methyl ester was better than the commonly used trimethylsilyl ether of $PGF_{2\alpha}$ methyl ester. The other advantage of dimethylethylsilyl ether

derivatives as claimed by the authors was that they give more characteristic fragmentation peaks upon mass spectroscopic analysis than the trimethylsilyl derivatives. However, the dimethylethyl silylating reagent is not commercially available.

Recently we have tried a more stable *tert.*-butyldimethylsilyl (tBDMS) ether derivative (1000 times more stable than TMS ether) for GC—ECD determination. While these were much more stable to hydrolysis the PFBE-(PFBO)-tBDMS ethers required much higher column temperature for successful GC—ECD separation.

The linearity of the detector response

The detector responses to $PGF_{2\alpha}$-PFBE-TMS at concentrations from 50 pg to 1000 pg were linear. This mass range encompasses the concentration of PGs and TX commonly found in most biological samples.

Standard curves for different PG derivatives

For quantification of PG from biological tissues, standard curves for known amounts of various PG derivatives were constructed. The area responses were plotted against the amounts used for derivatization, i.e. 0.2—10 ng. Except for the $PGF_{2\alpha}$-PFBE-TMS which gave lower response, the responses to other derivatives showed the same trend as reported earlier by Fitzpatrick et al. [16] using a glass capillary column coupled to the ECD. The lower response of $PGF_{2\alpha}$-PFBE-TMS may be related to the temperature used in the study which was 270°C instead of 250°C as used by Fitzpatrick et al. [16]. The relatively low response to TXB_2-PFBO-PFBE-TMS was expected because alkaline conditions (which favor the quantitative formation of PFBE) tend to open the acetal ring on the TXB_2 [5]. The responses of PGB-PFBO-PFBE-TMSs were also relatively low. Prostaglandin Bs have inherent electron-capturing properties and have been used for electron-capture detection after being derivatized to their methoxime methyl ester TMS ethers [17]. A derivatization of a compound having an inherent electron-capturing property with pentafluorobenzyl bromide may reduce rather than enhance the response to the ECD [18]. This was also observed by Fitzpatrick et al. [5], i.e. >400 pg of PGB_1-PFBO-PFBE-TMS and PGB_2-PFBO-PFBE-TMS are required for electron-capture detection. For some other PG derivatives, concentrations as low as 30 pg can be detected, e.g. PGA_1, PGD_2, PGE_1 and 15-keto-$PGF_{2\alpha}$.

A tabulation of response factors for quantitative work using internal standard procedure is included (Table II). Both 1a,1b-dihomo-$PGF_{2\alpha}$ and cholanic acid are appropriate internal standards to use.

The *syn-* and *anti*-isomers of all the PFBO-PFBE-TMS derivatives were resolved on the chromatogram except for PGBs and 6-keto-$PGF_{1\alpha}$ where the two isomers emerged as a single peak. The ratios of the first isomer to the second isomer are given in Table III. Except for the PGA derivatives, the ratios were very reproducible between the OV-101 and SE-30 columns over a range of 200 pg to 7.5 ng of sample injected. The PGE_3 derivatives gave three peaks and in this case the ratio was obtained by arbitrarily assigning the first peak to be one unit.

Since no GC method, so far, can satisfactorily separate all the PG derivatives

TABLE II

RELATIVE RESPONSE FACTORS OF SOME PENTAFLUORO DERIVATIVES OF PG COMPARED TO THE DERIVATIVES OF 1a,1b-DIHOMO-$PGF_{2\alpha}$ ON TWO STATIONARY PHASES, 3% OV-101 AND 1% SE-30, RESPECTIVELY

Columns used: 3% OV-101, 100—120 mesh, 2.8 m × 0.32 cm glass column; 1% SE-30, 100—120 mesh, 4.7 m × 0.32 cm glass column. GC conditions: 285°C isothermal, argon—methane (95:5) at 20 ml/min, detector temperature, 300°C. Amounts injected: 500 pg for each PG.

PG compounds	OV-101	SE-30
A_1	1.17, 1.40	0.74, 0.88
A_2	1.10, 1.30	0.70, 0.82
B_1	1.75	1.10
B_2	1.70	1.07
D_2	1.32, 1.51	0.85, 0.95
E_1	1.38, 1.64	0.88, 1.03
E_2	1.81, 1.54	0.82, 0.96
E_3	1.09, 1.30, 1.52	0.74, 0.83, 0.96
$F_{1\alpha}$	0.67	0.45
$F_{2\alpha}$	0.61	0.41
6-Keto-$F_{1\alpha}$	1.62	1.00
15-Keto-$F_{2\alpha}$	1.35, 1.44	0.86, 0.91
13,14-Dihydro-15-keto-$F_{2\alpha}$	1.38, 1.45	0.88, 0.92
TXB_2	1.79, 1.93	1.11, 1.18

TABLE III

RATIO OF *syn-* AND *anti-*ISOMERS OF SOME PENTAFLUORO DERIVATIVES OF PG SEPARATED BY GAS CHROMATOGRAPHY ON COLUMNS CONTAINING 1% SE-30 AND 3% OV-101

PG derivatives*	Ratio of first peak vs. second peak	
	1% SE-30	3% OV-101
A_1	1.30 ± 0.23	3.67 ± 0.38
A_2	1.65 ± 0.30	2.70 ± 0.47
D_2	0.25 ± 0.01***	0.26 ± 0.01
E_1	0.40 ± 0.03	0.42 ± 0.05
E_2	0.42 ± 0.00	0.47 ± 0.03
E_3 **	1:3.33:6.55 ± 0.10 ± 0.12	1:3.41:6.02 ± 0.60 ± 0.78
15-Keto-$F_{2\alpha}$	0.45 ± 0.03	0.44 ± 0.02
13,14-Dihydro-15-keto-$F_{2\alpha}$	0.97 ± 0.08	0.97 ± 0.08
TXB_2	0.56 ± 0.03	0.40 ± 0.10

*Pentafluorobenzyl oxime pentafluorobenzyl ester trimethylsilyl ether derivatives.
**Three instead of two isomers were observed. The exact structure of each isomer has not yet been identified.
***Mean ± S.D. from four samples of different concentration ranged from 7.5 ng to 950 pg.

and their isomers, knowing the ratio of the *syn*- and *anti*-isomers can help in calculating the individual amount of PG in a merged peak. A typical application of this calculation can be demonstrated clearly in Fig. 6. In Fig. 6A the complete separation of the $PGF_{1\alpha}$,$PGF_{2\alpha}$-PFBE-TMS and PGE_1,PGE_2-PFBO-PFBE-TMS and TXB_2-PFBO-PFBE-TMS is shown. On the upper right corner of Fig. 6A, the retention time of 6-keto-$PGF_{1\alpha}$-PFBO-PFBE-TMS is given as 20.13 min. This peak eluted after the second isomer of PGE_2 and before the second isomer of PGE_1. However, this peak of 6-keto-$PGF_{1\alpha}$ emerged coincidentally with the second isomer of PGE_1 as shown in Fig. 6B. With the information given in Table II, one can calculate the quantity of 6-keto-$PGF_{1\alpha}$ in the merged peaks by subtracting the amount of the second isomer of PGE_1 as calculated from the first PGE_1 isomer.

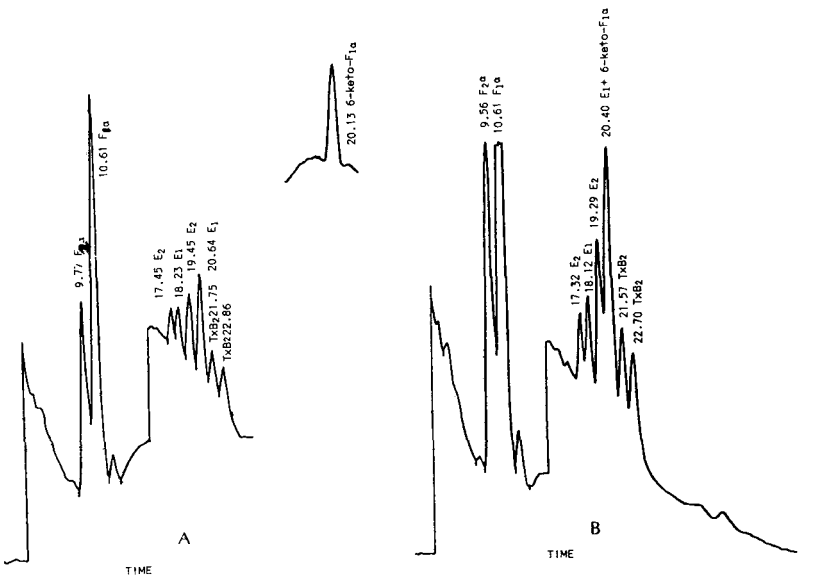

Fig. 6. Examples of separation of some major prostaglandins and thromboxane standards. Column: 2.8 m × 0.32 cm, 3% OV-101 (100—120 mesh) column. (A) $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_1, PGE_2 and TXB_2 were well resolved. (B) Merging of 6-keto-$PGF_{1\alpha}$ with the second isomer of PGE_1.

Analyses of PGs and TX from biological samples

After we developed and standardized the GC—ECD procedures, we analyzed the PGs, HETE and TX extracted from human semen, dog serum, trout gill and rat aorta. Each of the organs contain different levels and types of PGs so they were useful in evaluating the applicability of the GC—ECD system to biological samples generally. Preliminary identification of PGs was done by comparing the retention times of the suspected PGs with those of the authentic PGs. Confirmation of the identity of specific PGs was achieved by GC—MS—SIM.

Identification of PG by GC—MS—SIM

The PGs present in the biological samples were derivatized into either methyl

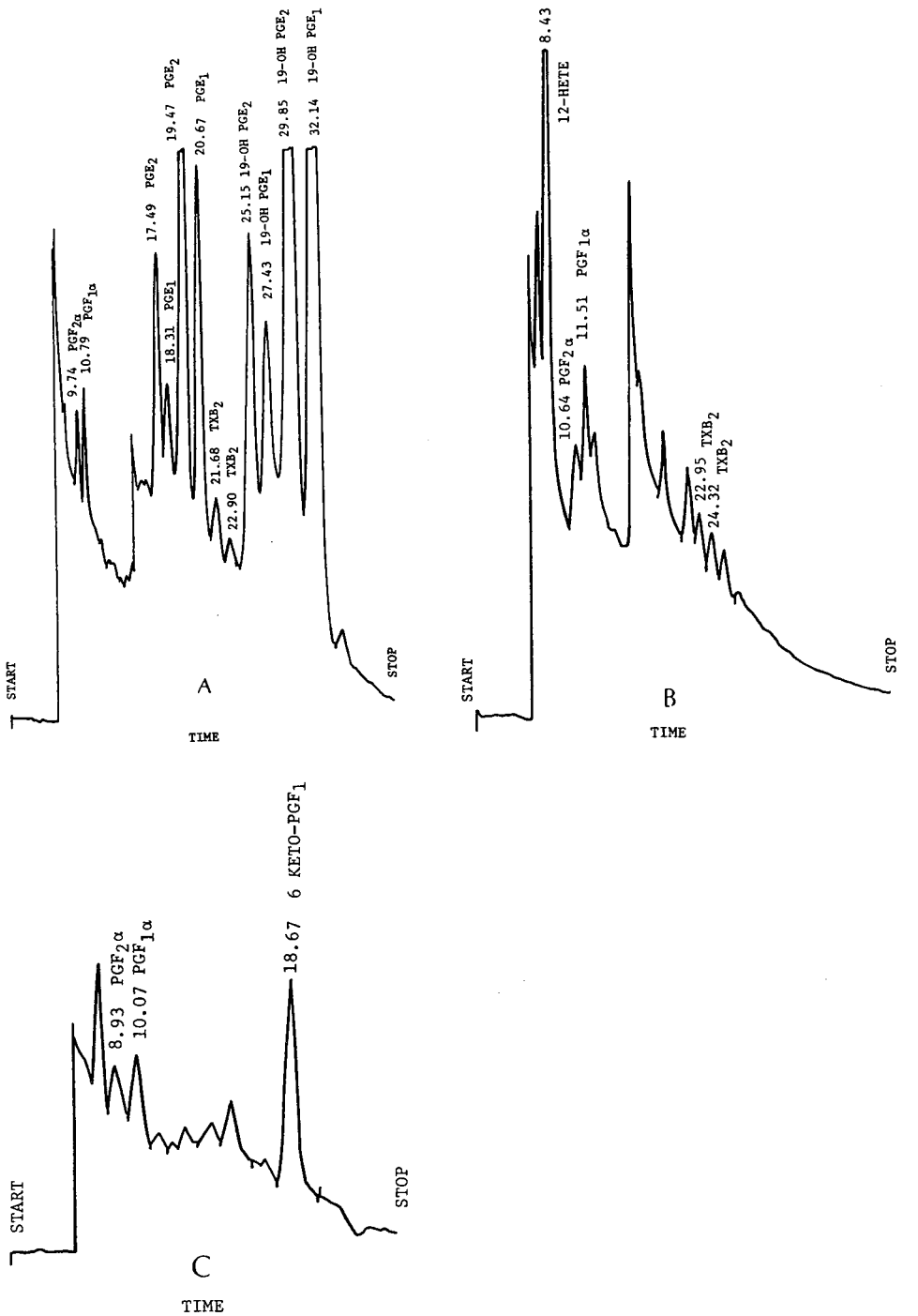

Fig. 7. (A) Separation of derivatives of human semen prostaglandins and thromboxane by GC—ECD. (B) GC—ECD profile of prostaglandin derivatives prepared from dog serum. (C) Prostaglandins from extract of rat aorta incubation. 2.8 m × 0.32 cm, 3% OV-101 (100—120 mesh) glass columns. Conditions are detailed in the text.

ester (methoxime) trimethylsilyl ether or methyl ester (methoxime) *tert.*-butyldimethyl ethers and identified by GC—MS—SIM technique. The details of this GC—MS—SIM technique are described elsewhere [19,20].

Semen prostaglandins. Seven species of PGs and TX are found in human semen (Fig. 7A). These PGs are $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_1, TXB_2, 19-OH-PGE_2 and 19-OH-PGE_1. The ratios of PGE_2 to PGE_1 and 19-OH-PGE_2 to 19-OH-PGE_1 were 1.7 and 1.0, respectively. PGFs and TXB_2 were comparatively minor components in human semen.

Dog serum prostaglandins. PGs were extracted from dog serum, derivatized and resolved by GC—ECD (Fig. 7B). The most abundant peak was 12-HETE tentatively identified from an authentic standard. Also identified were $PGF_{2\alpha}$, $PGF_{1\alpha}$ and TXB_2. No significant PGE_2 peaks were observed. Several unknown peaks were observed.

Rat aorta prostaglandins. A significant amount of 6-keto-$PGF_{1\alpha}$ was extracted from rat abdominal aorta after 15 min incubation in 0.05 *M* phosphate buffer (pH 7.4) (Fig. 7C). $PGF_{2\alpha}$ and $PGF_{1\alpha}$ were also identified.

Trout gill. $PGF_{3\alpha}$ was the major PG found in trout gill tissue followed by PGE_3 (Fig. 8A). A chromatogram for authentic standard is also provided for comparison (Fig. 8B). We also found an unknown peak (retention time 14.25 min) which eluted between $PGF_{3\alpha}$ and PGE_3. Using the GC—MS procedure, we further determined the structure of this compound as a PG containing four

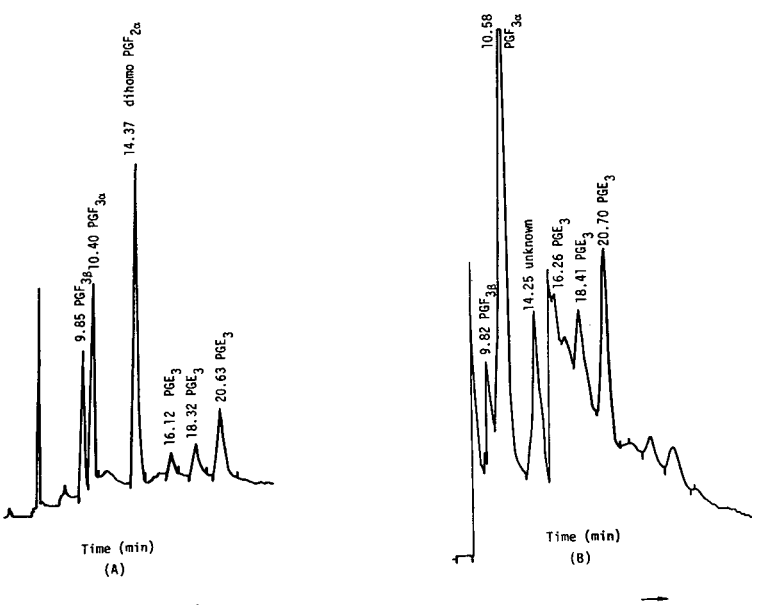

Fig. 8. Separation of prostaglandin derivatives from trout gill. (A) Authentic PG standards; (B) trout gill PG extract.

double bonds tentatively identified as C_{22}-$PGF_{4\alpha}$ [21], the first report of a PG with four double bonds.

This paper demonstrates the systematic calibration of GC—ECD and the successful use of this method for determining PGs from biological samples containing different concentrations and types of PGs. The technique should greatly facilitate the routine quantification of PG from biological materials and is being used in our laboratory for studying the effects of dietary *trans*-linoleic acid on the production of PGs and TX in different organs of rats.

ACKNOWLEDGEMENTS

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CHROMBIO. 1242

DETERMINATION OF METHYLPREDNISOLONE IN CENTRAL NERVOUS TISSUE AND PLASMA USING NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific high-performance liquid chromatographic technique is described for the quantitative measurement of the synthetic glucocorticoid methylprednisolone in central nervous tissue (spinal cord) and plasma. Following intravenous administration, methylprednisolone is extracted from spinal cord tissue with diethyl ether—methylene chloride (60:40, v/v). The extract is washed sequentially with alkali, acid and water, concentrated, then chromatographed on an NH_2 column using a mobile phase of methylene chloride—isopropanol (85:15, v/v). Steroid elution is monitored with an ultraviolet detector set at 254 nm. Such a system has a detection limit of 2.8 ng methylprednisolone. Extraction of methylprednisolone from spinal cord tissue is linear with tissue concentration and the recovery is around 70%. Endogenous hydrocortisone or other metabolites in the tissue do not interfere with the methylprednisolone peak. A description of the quantitation of methylprednisolone in cat lumbar spinal cord and plasma samples after single intravenous doses of methylprednisolone sodium succinate is given.

INTRODUCTION

Recent studies from this laboratory have demonstrated acute effects of the synthetic glucocorticoid methylprednisolone (MP) on certain neurophysiological [1, 2] and neurochemical [3—5] parameters of spinal cord function. In order to correlate the amount of MP at the site of action with these effects, a reliable method was needed for the extraction and determination of glucocorticoid concentrations in the spinal cord of treated animals.

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Methods have been presented for assaying MP in plasma samples utilizing both reversed-phase [6, 7] and normal-phase [8—10] high-performance liquid chromatography (HPLC). However, to adapt any of these methods to the use of tissue samples, an effective extraction procedure is required and the assay must be of sufficient sensitivity to detect the smaller amount of the drug that would likely be present in the tissue compared to plasma. In addition, the method must resolve MP and its internal standard from all the other steroidal compounds endogenously present in the central nervous system (CNS). In this report we describe a simple method using normal-phase HPLC with UV detection for the measurement of MP in both CNS tissue and plasma samples.

EXPERIMENTAL

Materials

Triamcinolone acetonide (TA) and hydrocortisone (HC) were obtained from Sigma (St. Louis, MO, U.S.A.). Methylprednisolone sodium succinate (Solu-Medrol) (MP) and methylprednisolone were gifts from Upjohn (Kalamazoo, MI, U.S.A.). The HPLC solvent, methylene chloride and isopropanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Other reagents were from Fisher (Cleveland, OH, U.S.A.) and were the highest grade available.

Instrumentation

A Varian (Walnut Creek, CA, U.S.A.) high-performance liquid chromatograph Model 5020 consisting of a gradient capacity pump, a Valco loop injector, Micropak NH_2 column (30 cm × 4 mm, 10 μm particle size), a fixed-wavelength (254 nm) ultraviolet detector, a CDS 111 integrator and a Model 9176 recorder were used in these studies. The mobile phase was an isocratic mixture of methylene chloride—isopropanol (85:15) pumped through the column at a flow-rate of 2 ml/min.

Animal preparation and administration of drugs

Adult mongrel cats of either sex weighing 1.8—4.0 kg were anesthetized with alpha chloralose from Sigma (80 mg/kg intravenously). A dorsal laminectomy was performed to expose the lumbar spinal cord from the 1st through the 4th lumbar levels. Methylprednisolone sodium succinate (Solu-Medrol) in a concentration of 60 mg/ml was injected into a brachial vein as a single dose per animal of either 15, 30, 60 or 90 mg/kg. Samples (1 ml) of carotid arterial blood were collected in heparinized syringes just prior to the removal of approximately a 1.5-cm cross-section of the lumbar spinal cord at one hour after MP administration. The section of spinal cord was immediately frozen in liquid nitrogen and stored at —70° C until assay. Blood samples were immediately centrifuged at 1000 *g* for 5 min and the plasma was transferred to clean tubes, frozen and stored at —70° C until assay.

Sample preparation

A 200—400 mg portion of frozen spinal cord was homogenized with a Polytron Homogenizer (Brinkman Instruments, Westbury, NY, U.S.A.) at 4° C

in 5 ml of diethyl ether—methylene chloride (60:40). The homogenate was incubated at room temperature for 15 min with shaking to facilitate extraction of the lipophilic MP from the highly lipid nervous tissue. The extracted homogenate was centrifuged at 700 *g* for 5 min and the resulting supernatant fraction was sequentially washed with 1-ml volumes of 0.1 *N* sodium hydroxide, 0.1 *N* hydrochloric acid and glass-distilled water. After each washing, the samples were centrifuged at 700 *g* for 5 min and the organic phase was transferred to a clean tube for the next washing. The final extract was transferred to a small centrifuge tube where it was evaporated to dryness under a stream of dry nitrogen. The residue was reconstituted in 0.3 ml of methylene chloride—isopropanol (85:15) containing 500 ng of TA as an internal standard, and a 100-μl aliquot was injected into the chromatographic system.

Plasma methylprednisolone was extracted by adding 0.5 ml plasma to 5 ml diethyl ether—methylene chloride (60:40). The sample was then extracted and prepared as described for the tissue samples, dried, dissolved in 500 μl of methylene chloride—isopropanol (85:15) containing 10 μg TA as an internal standard, and a 25-μl aliquot was injected into the chromatographic system.

Standard solutions of TA, HC and MP in isopropanol were prepared from stock solution of 0.4 mg/ml. MP was quantitated on the basis of the ratio of the peak area of MP to that of TA. The results were expressed as μg of MP per g wet tissue or per ml plasma.

RESULTS AND DISCUSSION

The general purpose of this study was to develop a technique which would allow a determination of glucocorticoid concentrations in central nervous tissue using HPLC with UV detection. In the present instance, the glucocorticoid in question is the synthetic MP and the tissue of interest is the spinal cord. In addition to the usual criteria for good chromatography, a suitable technique for this purpose must include: (1) the choice of a suitable internal standard with good separation from the glucocorticoid to be measured, (2) a clear separation of the measured glucocorticoid from other steroids such as endogenous HC (i.e., cortisol) which is concentrated in brain and other tissues [11], and (3) an extraction procedure capable of removing the lipophilic steroid from neuronal membranes and myelin. The method described in this report meets these criteria.

Fig. 1 shows a chromatogram of a mixture of 100 ng each of TA, HC and MP demonstrating the clear separation of these three glucocorticoids using the column and mobile phase described above. The longest retention time was approximately 6 min for MP, the least polar of the three. Furthermore, the detector response to MP was found to be linear over a ten-fold range of MP ($r = 0.99$).

Fig. 2 shows a typical chromatogram of a lumbar spinal cord sample from an untreated cat which had been spiked with 500 ng each of TA, HC and MP. Chromatograms of unspiked spinal cord extracts from untreated animals (not shown) showed no major endogenous peaks which would interfere with the resolution of the three glucocorticoids in question.

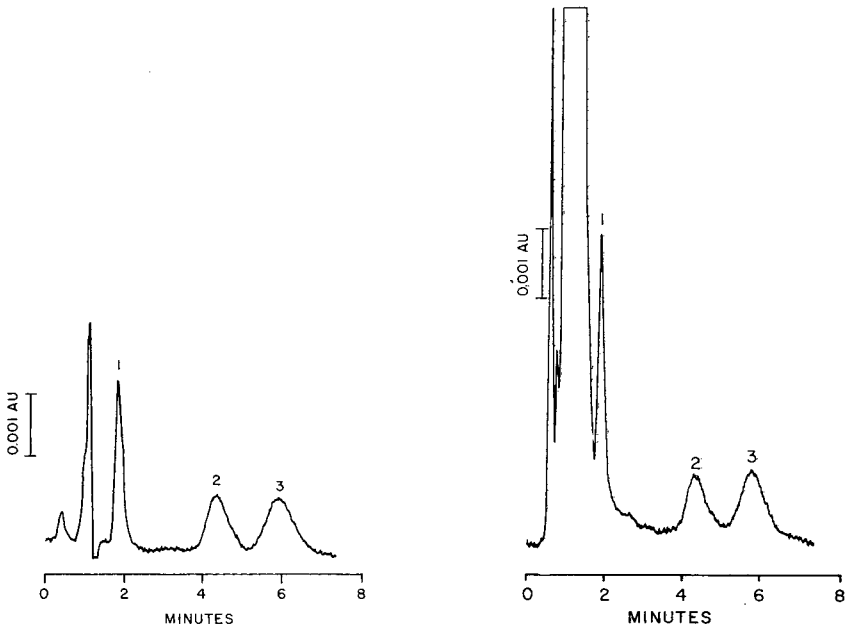

Fig. 1. High-performance liquid chromatogram showing separation of a mixture containing 100 ng each of TA = 1 (internal standard), HC = 2, and MP = 3. Injection volume was 100 μl.

Fig. 2. High-performance liquid chromatogram of a 200-mg cat lumbar spinal cord extract spiked with 500 ng each of TA (internal standard) = 1, HC = 2, and MP = 3. Injection volume was 100 μl.

Fig. 3 displays a chromatogram of a spinal cord extract from an animal injected intravenously with 90 mg/kg of the sodium succinate ester of MP 1 h prior to cord removal. There is no discernable HC peak because the amount endogenously present in the spinal cord was below the sensitivity required to measure the MP. For MP, the detection limit using this assay, determined as that quantity measured at twice the noise level, was 2.8 ng in a 100-μl injection volume or 8.4 ng in a 200-mg spinal cord sample. The intra-assay variability with triplicate determinations of a single sample was approximately 4%. The MP measured in the extract was determined to be the free non-esterified form since the retention time corresponded to that obtained for the MP standard. Fig. 4 shows a typical chromatogram of MP extracted from the plasma of a cat injected with 90 mg/kg of the sodium succinate ester of MP 1 h before sampling.

The extraction procedure with diethyl ether—methylene chloride (60:40) was arrived at by trial and error. Use of methylene chloride alone as described by others [6—10] for extraction of glucocorticoid from plasma samples was not effective in removing the highly lipophilic MP from the cord tissue samples. The effectiveness of the extraction described here is demonstrated in Fig. 5 which shows a linear relationship between cord sample size and MP content.

The recovery of MP from the tissue was estimated by adding 500 ng MP to a 200-mg spinal cord sample from an untreated cat either before or after the

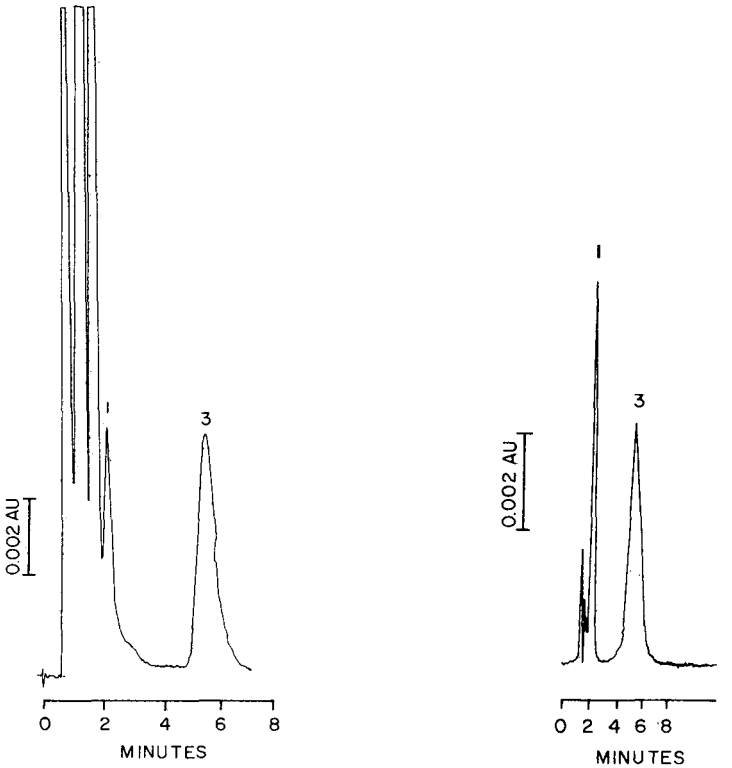

Fig. 3. High-performance liquid chromatogram of a lumbar spinal cord sample from a cat injected intravenously with 90 mg/kg MP sodium succinate at 1 h before cord removal. TA (internal standard) = 1, and MP = 3. Injection volume was 100 μl.

Fig. 4. High-performance liquid chromatogram of a plasma sample from a cat injected intravenously with 90 mg/kg MP sodium succinate at 1 h before sampling. TA (internal standard) = 1, and MP = 3. Injection volume was 25 μl.

tissue extraction procedure. Peak height ratios of MP to the TA internal standard in both instances were then compared. The mean recovery of MP determined accordingly was 70%.

Finally, Fig. 6 displays the mean concentrations of MP in 200-mg cat spinal cord samples as a function of dose at one hour after injection. The variability among animals may reflect differences in spinal cord blood flow or differences in the amount of body fat (i.e., peripheral uptake of the MP). Inter-assay variability does not seem to be a factor, since repeated assays of the same sample over several days showed negligible variation.

The procedure described in this report is accurate and sensitive. The method is currently being used to measure the levels of MP in plasma and spinal cord after single large intravenous doses of MP for the purpose of providing a pharmacokinetic correlation with previously reported pharmacological data [1—5].

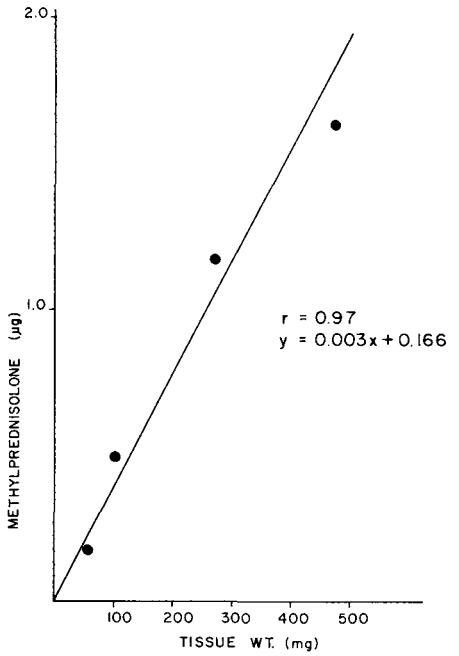

Fig. 5. Linearity of the MP content in spinal cord tissue following an intravenous injection of 90 mg/kg MP as a function of spinal cord sample size. Injection volume was 100 µl. All samples were run in duplicate.

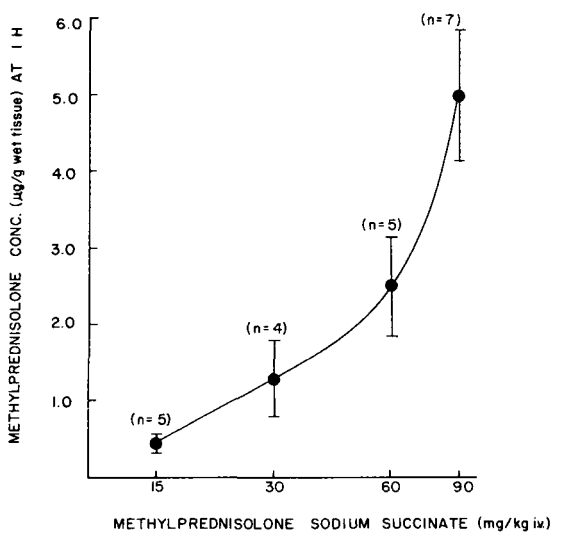

Fig. 6. Concentrations of MP in cat lumbar spinal cord samples at 1 h after intravenous administration of the dose of MP sodium succinate indicated. Values represent mean ± S.E. of duplicate determination from the number of animals indicated.

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CHROMBIO. 1234

DETERMINATION OF PLASMA LEVELS OF SPIRORENONE, A NEW ALDOSTERONE ANTAGONIST, AND ONE OF ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the determination of plasma concentrations of spirorenone, a new aldosterone antagonist, and one of its metabolites, chromatographically characterized as 1,2-dihydro-spirorenone, is described. The assay utilizes high-performance liquid chromatography with UV detection. Reproducible results can be obtained with standard deviations of about 5% and the limit of detection is less than 5 ng/ml. Plasma levels of drug and metabolite have been measured after oral doses of 10 and 40 mg, respectively, administered to two male volunteers.

INTRODUCTION

Spirorenone (6β,7β,15β,16β-dimethylene-1,4-androstadiene-[17(β-1′)-spiro-5′]perhydrofuran-2′,3-dione) is a newly synthesized aldosterone antagonist which is reported to be more than five times as potent in rats than spironolactone [1]. Currently the drug is under investigation in man using an aldosterone infusion model with constant oral water load of the test subjects. During these studies blood samples were drawn for the analysis of spirorenone levels. Another point of investigation was the possible appearance of the 17(α-1′) compound (Fig. 1) in blood, which was known from in vitro studies to be the acid-catalyzed rearrangement product of spirorenone [2] and therefore might have been formed in the stomach. The 17(α-1′) form is pharmacologically inactive and so, if substantial amounts of this compound are to be detectable in blood, a pharmaceutical formulation resistant to gastic juice must be developed.

Therefore the aim of the present study was to establish an assay procedure capable of detecting low plasma concentrations and which was able to separate the 17(α-1′) and 17(β-1′) forms described above.

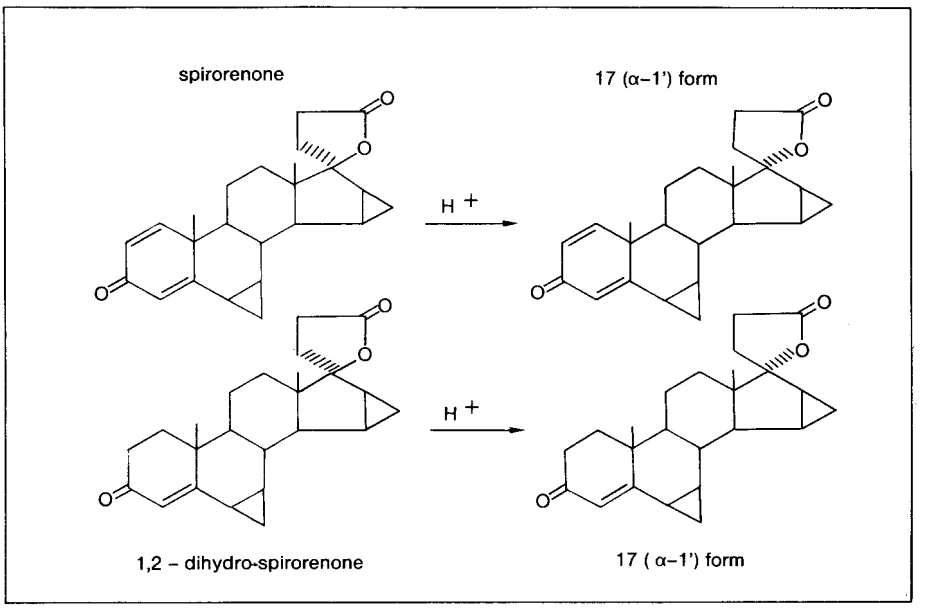

Fig. 1. Acid-catalyzed rearrangement of spirorenone and its 1,2-dihydro derivative.

EXPERIMENTAL

Subjects and medication

Two healthy male volunteers (24 and 21 years of age, 67 and 74 kg body weight, respectively) were given 10 mg of spirorenone in tablet form during constant aldosterone infusion (1 mg per 12 h) and oral water load (3250 ml per 12 h). Blood samples were taken at 0, 0.5, 1, 1.5, 2, 3, 6 and 11.5 h after drug administration. The samples were immediately centrifuged and the plasma kept frozen until analysis. One week later the same subjects were given 40 mg of spirorenone under the same experimental conditions.

Chemicals

Methanol, *n*-hexane, toluene and acetic acid were all of analytical-reagent grade (Merck, Darmstadt, G.F.R.) and were used without further purification.

Spirorenone and 1,2-dihydro-spirorenone were stored dissolved in methanol in concentrations of 10 μg/ml.

Extraction procedure

Three millilitres of plasma were pipetted into a 10-ml stoppered test tube and 3 ml of *n*-hexane–toluene (1:1, v/v) were added. After thorough mixing on a Vortex mixer for 1 min and centrifugation at 1200 *g* for 5 min, the organic phase was removed. The residue was extracted once again with 3 ml of the solvent described above and the two organic phases were combined and taken to dryness under a slight stream of nitrogen. The dry extract was taken up in 200 μl of the high-performance liquid chromatography (HPLC) mobile phase; 150 μl were injected for analysis. Alternatively, for the low-dose study, 5 ml of plasma were extracted three times each with 2 ml of solvent.

The extraction efficiencies were determined with 3-ml plasma samples containing 200 ng/ml spirorenone and 30 ng/ml 1,2-dihydro-spirorenone, and with 5-ml plasma samples containing 20 ng/ml and 10 ng/ml, respectively.

Chromatographic systems

High-performance liquid chromatography. The HPLC system consisted of a solvent delivery pump (Waters, Königstein, G.F.R.; type 6000 A), a LiChrosorb RP-18 chromatographic column (10 μm particle size, 250 × 4.6 mm; Knauer, Berlin, G.F.R.) and a UV detector with fixed wavelength (254 nm; Knauer). Alternatively, a UV detector with variable wavelength (Schoeffel SF 770) was used. The detector signals were converted to a chromatographic trace by a W + W recorder (Basle, Switzerland). Injection was accomplished with a Rheodyne RH 7120 system. The mobile phase consisted of methanol—water (60:40, v/v). The eluent was degassed at reduced pressure before use. The chromatographic system was operated at ambient temperature with an eluent flow-rate of 2 ml/min.

For in vitro acid-catalyzed rearrangement studies of spirorenone two LiChrosorb RP-18 columns (10 μm particle size, 250 × 4.6 mm) in series were used; the mobile phase consisted of methanol—water (60:40, v/v) with 0.01 *M* sodium dodecyl sulphate and 2 ml of acetic acid per litre. Injection was accomplished with an automatic sampling device (WISP, Waters).

Standard curves were constructed with 3-ml blank plasma samples containing 0, 25, 50, 100, 200, 400 and 800 ng of spirorenone and 20, 40, 80, 160 and 320 ng of 1,2-dihydro-spirorenone. These samples were extracted by the method described above. Peak heights as determined by HPLC analysis were measured and plotted against the amount of the compound studied. Five-millilitre samples were handled likewise, since it had been ascertained previously that there was no difference in recoveries.

The overall accuracy of the HPLC assay was calculated from five consecutive determinations of two concentrations (see Table I).

Thin-layer chromatography. For thin-layer chromatography (TLC) silica gel precoated plates (Merck 60 F_{254}, 20 × 20 cm, layer thickness 0.25 cm) were used. Plasma extracts obtained by the procedure described above were dissolved in chloroform and spotted onto the plates. Development was performed twice with chloroform—methanol (96:4, v/v). Thereafter the upper half of each plate was cut off and run again in toluene—*n*-hexane (50:50, v/v). TLC spots were analyzed with a Zeiss scanner (KM 3) in the remission mode.

In vitro rearrangement

Two millilitres of 0.1 *N* aqueous hydrochloric acid solution were added to 500 μg of spirorenone and its 1,2-dihydro derivative in a sampling vial of the WISP. After short ultrasonic treatment 20 μl of the solution were repetitively injected into the HPLC system.

RESULTS

Assay of plasma levels

A highly sensitive and selective method for the determination of the new aldosterone antagonist spirorenone and one of its metabolites in plasma is described utilizing HPLC with UV detection (Fig. 2). Extraction from biological samples is performed with *n*-hexane—toluene (1:1). The recovery using this

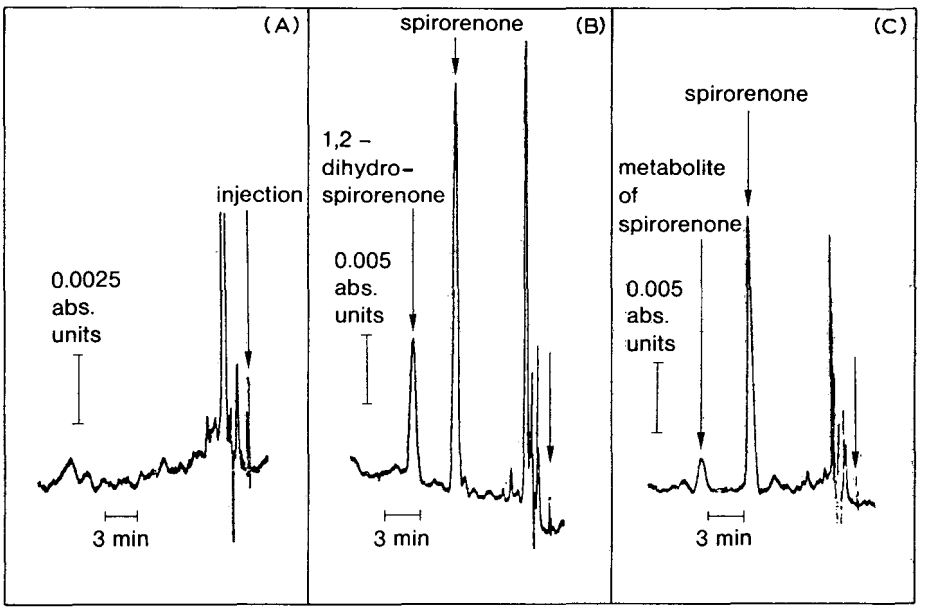

Fig. 2. HPLC chromatograms of (A) blank plasma samples, (B) plasma spiked with 133 ng/ml spirorenone and 53 ng/ml 1,2-dihydro-spirorenone, and (C) a plasma sample obtained from subject No. 2, 3 h after an oral dose of 40 mg of spirorenone.

TABLE I

EXTRACTION RECOVERIES OF DRUG AND METABOLITE

Recoveries were determined by extracting 3 or 5 ml of plasma spiked with different amounts of spirorenone and 1,2-dihydro-spirorenone, and comparing the peak heights measured to those of non-extracted material.

Spirorenone				1,2-Dihydro spirorenone			
200 ng/ml (3 ml)		20 ng/ml (5 ml)		30 ng/ml (3 ml)		10 ng/ml (5 ml)	
Peak height* (mm)	Recovery (%)	Peak height** (mm)	Recovery (%)	Peak height* (mm)	Recovery (%)	Peak height** (mm)	Recovery (%)
47	73	36	76	10	73	23	83
52	81	37	78	10	73	25	89
52	81	38	80	11	79	25	89
54	85	39	83	11	79	25	89
53	83	42	90	11	79	26	91
52 ± 3	81 ± 4	38 ± 2	82 ± 6	11 ± 1	76 ± 4	25 ± 1	88 ± 3

* 0.04 absorbance units.
** 0.01 absorbance units.

procedure was found to be about 80% independent of the volume of the test samples (Table I).

Matrix constituents and metabolites of spirorenone are then separated from the drug by HPLC using a reversed-phase system. The retention times of spirorenone, its 1,2-dihydro derivative and of the corresponding α forms were 9.2 min, 13.4 min, 10.5 min and 15.9 min corresponding to k' values of 9.2, 13.9, 10.7 and 16.7, respectively. Spirorenonic acid, the compound obtained after hydrolyzing the lactone ring, had a retention time of 2.7 min, corresponding to $k' = 2.0$.

Unknown concentrations of spirorenone and of its metabolite, tentatively characterized as 1,2-dihydro-spirorenone (cf. below), were determined by comparing their peak heights with those of spiked plasma samples. Linear calibration curves corresponding to the following equations were obtained:

spirorenone: peak height (mm) = 0.896 + 0.567 drug amount (ng)

1,2-dihydro-spirorenone: peak height (mm) = 1.871 + 0.462 drug amount (ng)

Correlation coefficients were calculated to be $r = 0.995$ and $r = 0.990$, respectively.

The overall accuracy of the assay expressed as standard deviation of five consecutive determinations of 200 and 20 ng/ml of drug were 5.2% and 6.0%, respectively; 20 and 10 ng/ml of the metabolite were determined with an accuracy of 5.2% and 4.4%, respectively. The detection limit of the assay is less than 5 ng/ml for both compounds of interest.

In vitro rearrangement

Spirorenone and its 1,2-dihydro derivative are unstable towards acid-catalyzed lactone ring isomerization (Fig. 3). On incubating the two compounds with 0.1 *N* hydrochloric acid, about 80% is converted into the α forms reaching a plateau at about 400 min after beginning (Fig. 4). However, the process of rearrangement was relatively slow compared to possible absorption rates in the stomach (cf. below).

Study of plasma levels

Spirorenone was absorbed with a half-life of 0.5—0.7 h (Table II) and reached its maximum plasma concentration 3 h after administration at a level of 41 ± 1 ng/ml (10 mg dose) and 105 ± 9 ng/ml (40 mg), respectively (Fig. 5). Until 11.5 h after administration the concentration of the drug diminished with half-lives of 6—9 h. The area under the plasma concentration—time curve (AUC) was 290 ± 16 ng h ml^{-1} (10 mg dose) and 851 ± 102 ng h ml^{-1} (40 mg dose), respectively.

The lactone rearrangement product of spirorenone was not detectable in the plasma, suggesting that the absorption process was much faster than the acid-catalyzed isomerization of the drug.

The compound obtained by opening of the lactone ring, a metabolite found after administering spironolactone [3—5] was hard to detect in plasma extracts using the HPLC system described above because of its short retention time. A

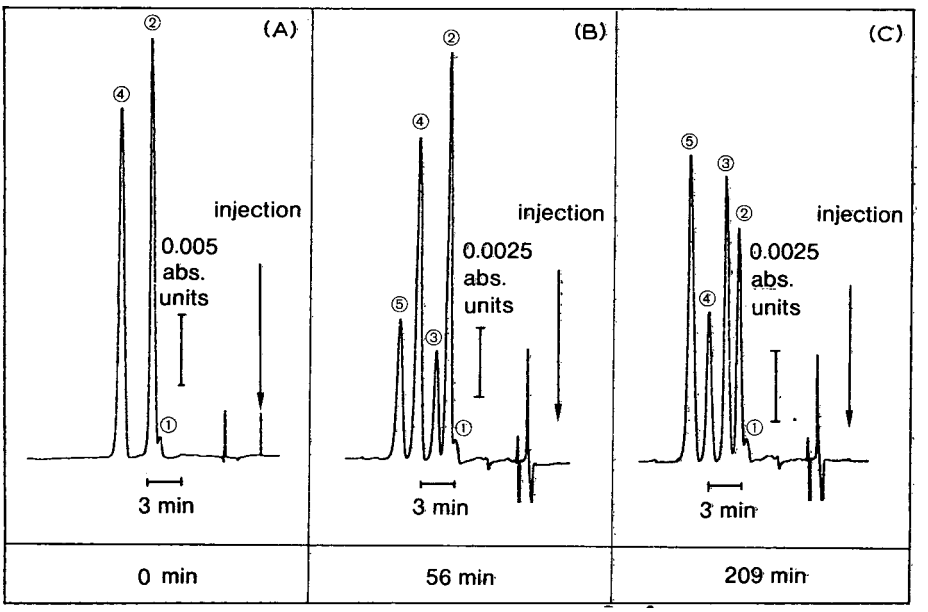

Fig. 3. HPLC chromatograms of incubates of spirorenone and its 1,2-dihydro derivative in 0.1 *N* HCl at t = 0 (A), t = 56 min (B), and t = 209 min (C). 1 = Impurity of spirorenone; 2 = spirorenone; 3 = 17(α-1') form of spirorenone; 4 = 1,2-dihydro-spirorenone; 5 = 17(α-1') form of 1,2-dihydro-spirorenone.

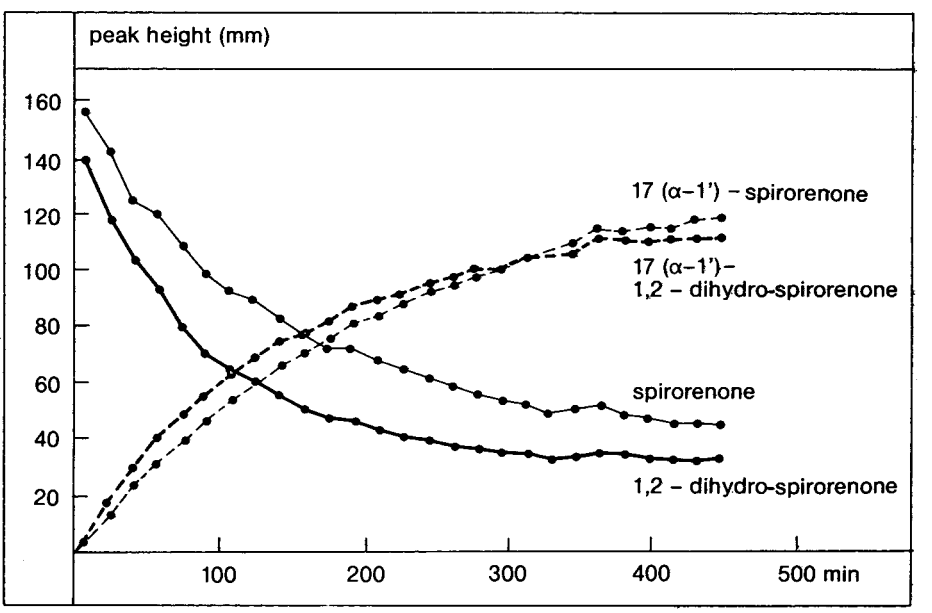

Fig. 4. Time course of acid-catalyzed rearrangement of spirorenone and 1,2-dihydro-spirorenone.

TABLE II

INDIVIDUAL PHARMACOKINETIC PARAMETERS OF TWO TEST SUBJECTS AFTER ORAL ADMINISTRATION OF 10 AND 40 mg OF SPIRORENONE, RESPECTIVELY

Dose (mg)	Test subject	Absorption $t_{1/2}$ (h)	Maximum (h)	Concentration (ng/ml)	Elimination $t_{1/2}$ (h)
10	1	0.4	2	49	5.3
	2	1.0	3	40	5.6
40	1	0.5	3	111	8.9
	2	0.5	3	98	9.0

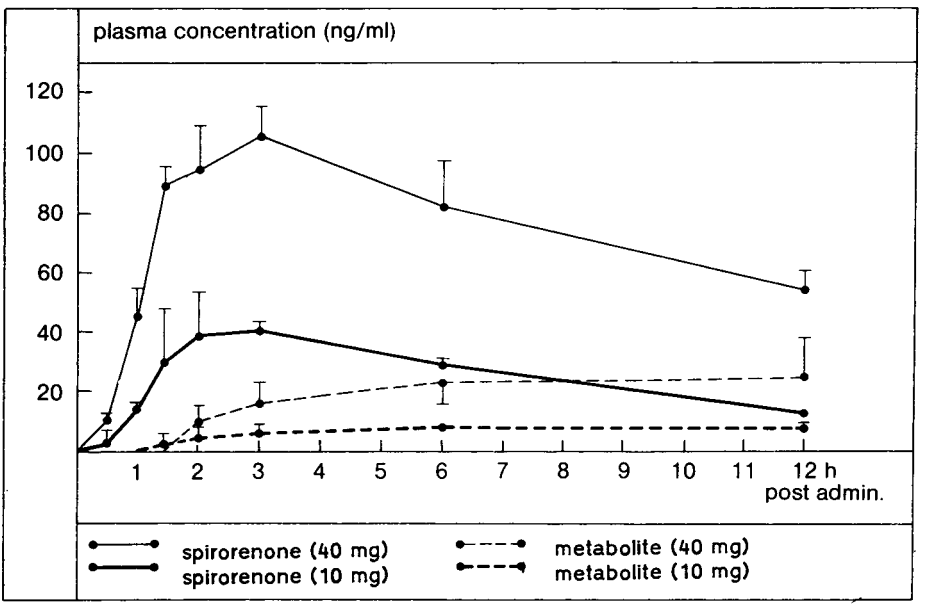

Fig. 5. Plasma levels of spirorenone and its metabolite (means) after oral administration of 10 and 40 mg of spirorenone to two male volunteers.

lot of matrix constituents were observed at this range of retention times so that this possible metabolite should not be regarded further.

A metabolite of spirorenone, however, chromatographically characterized as 1,2-dihydro-spirorenone (cf. below), could be measured in the plasma of the test subjects. It was detectable only after at least 1.5 h after drug administration, suggesting a relatively low rate of formation. Its concentration then constantly rose up to the end of the study period (Fig. 5).

Metabolite characterization

On HPLC analysis of plasma samples from test subjects having received oral doses of spirorenone, a metabolite was observed which had the same retention time as 1,2-dihydro-spirorenone (Fig. 2). On TLC analysis of the same plasma samples in the eluent system described above the metabolite again co-chroma-

tographed with the 1,2-dihydro derivative. And, moreover, the remission spectra on TLC plates of pure substance and of the metabolite after separation from plasma constituents were both identical with a UV maximum at 280 nm. Spirorenone, on the other hand, showed two maxima, at λ(1)= 250 nm and λ(2) = 290 nm (Fig. 6). Therefore it is quite probable that the metabolite observed in the HPLC chromatrogram corresponds to 1,2-dihydro-spirorenone.

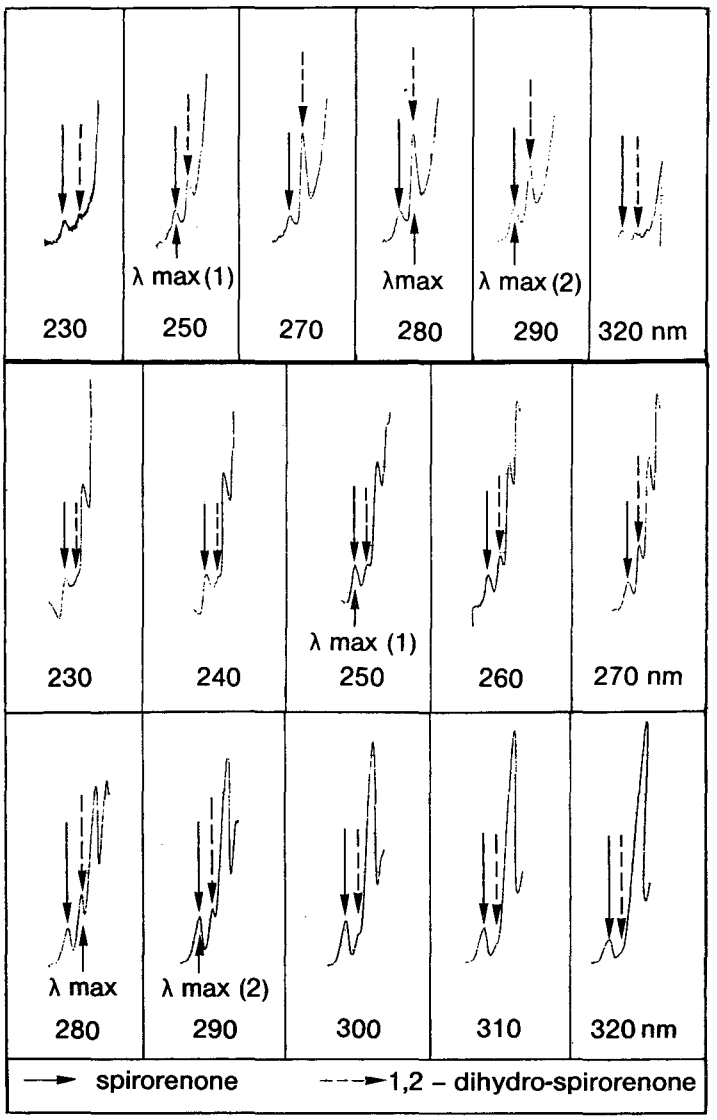

Fig. 6. UV absorption spectrum of spirorenone and its metabolite on a TLC plate after sepration from a plasma sample obtained from test subject No. 2, 11.5 h after oral administration of 40 mg of spirorenone (upper part) and as pure substances (lower part).

DISCUSSION

The present paper describes an assay procedure for the determination of spirorenone plasma levels to be expected after therapeutic drug administration. Simultaneously the concentration of a metabolite can be measured that was chromatographically characterized as 1,2-dihydro-spirorenone. Further studies including the isolation and final identification of this compound, however, still have to be performed. They are under progress at the present time.

1,2-Dihydro-spirorenone has been demonstrated to have an anti-aldosterone activity [1] about five times that of spironolactone. So if its identity could be verified in human plasma this would mean the appearance of an active metabolite probably prolonging the pharmacological activity of spirorenone itself.

The concentrations of the metabolite have been measured in this study by comparison with a standard curve of 1,2-dihydro-spirorenone although there was no explicit identification of the compound of interest. This was, however, possible since the UV spectrum of the metabolite and of 1,2-dihydro-spirorenone were identical. So if after ultimate isolation and identification of the metabolite it should — against all expectation — prove not to be the 1,2-derivative, the concentrations would nevertheless have been exactly measured.

Apart from HPLC and TLC with UV detection we had tried other methods for the determination of spirorenone in biological samples including fluorescence and electrochemical procedures and gas chromatography—mass spectrometry. However, the high temperature to volatilize the compound (300°C) or the unfavourable fluorescence and electrochemical data showed that UV detection combined with HPLC separation was the only way to measure those very low concentrations. In conclusion, the HPLC method described above seems to be a sensitive and selective assay suitable for further pre-clinical and clinical trials with spirorenone.

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URINARY PROTEIN PROFILING BY HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

We describe a new application of high-performance aqueous gel permeation chromatography for the analysis of human proteinuria. Separations of urinary proteins from normal subjects and patients with renal impairment were performed with TSK G 3000 SW columns. The effects of pH and ionic strength of the eluent on the separation of urinary proteins were investigated. Albumins were selectively separated from urine by affinity chromatography on Blue Sepharose CL-6B. According to the results of clinical investigations, urinary protein pattern derived from gel permeation chromatography revealed a good prediction of the site of renal involvement. Predominant excretion of proteins with lower molecular weight than albumin correlated with tubular damage. Albumin and higher molecular weight protein patterns were associated with glomerular disease. Absorbance measurements of the eluent at 280 nm were used for quantitative determination of total urinary protein. Gel permeation chromatography was compared to sodium dodecyl sulfate—polyacrylamide gel electrophoresis and the resulting protein patterns are in good agreement.

INTRODUCTION

Proteinuria (the excretion of proteins in excess of 100—200 mg/day) usually signifies either increased permeability of the glomerular capillary membrane and/or diminished tubular reabsorption. Different localizations of renal lesions are characterised by different molecular weight distributions of the urinary proteins. Therefore methods based on separation according to molecular size should be used for the characterisation of the urinary protein patterns. Sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS—PAGE) has been proved to be a valuable tool in the diagnosis of kidney diseases [1—5]. First attempts with gel chromatography on Sephadex were too time-consuming for routine laboratory use [6]. Recently, gel permeation chromatography (GPC) columns packed with microspheres of chemically modified silica gel (TSK GEL, type SW; Varian, Darmstadt, G.F.R.), have become commercially available. These columns can be used under high pressure in aqueous systems

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and possess a large number of theoretical plates.

High-performance liquid chromatography (HPLC) with these columns has been studied for the analysis of many biological substances, such as polypeptides, proteins, lipoproteins and enzymes [7—12]. However, these GPC columns have not yet been applied to the analysis of human urinary proteins and for characterisation of proteinuria.

In the present paper we investigated the value of GPC for differentiating proteinurias and for monitoring the follow-up of a diagnosed kidney disease. In addition, the new applied analytics were compared with an established method.

EXPERIMENTAL

Apparatus

Urinary proteins were separated with column systems consisting of a precolumn TSK GWSP (10 cm × 7.5 mm I.D.) and two TSK G 3000 SW columns (particle size 10 ± 2 μm, 30 cm + 50 cm × 7.5 mm I.D.) in series with 50 mm × 0.23 mm I.D. stainless-steel tubing (Varian). The HPLC system consisted of a Waters Model 6000 A solvent delivery system and an automatic sample injection system (Model 710 WISP, Waters, Königstein, G.F.R.). The UV absorbance of all proteins was measured at 280 nm with an ISCO Absorption Monitor Model UA-5 (Colora, Lorch, G.F.R.). The detector output was also connected to a Waters Data System M 730 for the determination of retention times and peak areas.

SDS—PAGE was carried out in a vertical slab gel using a Hoefer Scientific Instruments Model SE 500 (San Francisco, CA, U.S.A.).

Chromatography

For removal of interferences of low molecular mass we used scaled-down gel chromatography of urine samples in small columns (PD 10, Pharmacia, Freiburg, G.F.R.) originally filled with Sephadex G-25. The medium was drained and refilled to a height of 5.5 cm with hydrated Sephadex G-50 fine (Pharmacia) giving a total bed volume of 9.1 ml.

Normally 100 μl of gel-filtrated urine were injected automatically in time intervals of 35 min. The mobile phase consisted of 1/15 *M* phosphate buffer (pH 6.8) containing 0.1 *M* NaCl and $6 \cdot 10^{-4}$ *M* NaN_3 and was pumped through the G 3000 SW columns at a flow-rate of 1 ml/min.

SDS—PAGE was carried out according to the description of Laemmli [13]. Fifty microliters of gel-filtrated urine were mixed with SDS containing Tris buffer and separated by electrophoresis in a 10% polyacrylamide vertical slab gel at 25 mA/gel at 10°C. The gels were fixed with 20% sulphosalicylic acid for 1 h, stained with a solution of 1.25 g of Coomassie Brilliant Blue R 250 in 454 ml of 50% methanol and 46 ml of acetic acid for 1 h, and destained by diffusion in a solution of 875 ml of water, 75 ml of acetic acid and 50 ml of methanol.

Reagents

Thyroglobulin (porcine), ferritin (horse spleen) ovalbumin, chymotrypsinogen, myoglobin (horse) and cytochrome *c* were obtained from Serva Fein-

Biochemica (Heidelberg, G.F.R.). Human γ-globulin, Cohn fraction II, was from Sigma Chemie (Taufkirchen, G.F.R.); human albumin and transferrin were from Behringwerke (Marburg, G.F.R.).

Precinorm® U was obtained from Boehringer Mannheim (Mannheim, G.F.R.). Eluent and protein solutions were filtered with 0.45-μm mean pore size filters, type HA (Millipore, Neu-Isenburg, G.F.R.).

Samples and procedures

Urine was collected with sodium azide (0.3 g/l) as preservative. Under these conditions it can be stored for several days at 4°C or for several weeks at −20°C. After centrifugation 2 ml of urine were placed on top of Sephadex G-50 columns and washed with 0.8 ml of phosphate buffer used also in the HPLC gel permeation procedure. The protein-containing fractions were eluted in 4 ml of this buffer. The columns were rinsed with 20 ml of eluent to prepare them for re-use.

We used the group-specific adsorbent Blue Sepharose CL-6B (Pharmacia) for removing albumins selectively from urine samples; 0.5 ml of urine was mixed with a suspension of Blue Sepharose CL-6B, resulting in dilution by a factor of 2.

For quantitative determination of total urinary protein we used the biuret method described by Weichselbaum [14], as well as UV spectrometry after gel chromatography. The individual peak areas eluting between 13 and 34 min were determined separately and summarized at the end of a run as a measure for total protein concentration. Precinorm® U (1:50 dilution) was used as standard for both methods.

RESULTS AND DISCUSSION

A mixture of standard proteins was examined using a G 3000 SW column (30 cm + 50 cm), and their separation patterns monitored at 280 nm are shown in Fig. 1. Though the column gave very sharp peaks, h-transferrin and h-albumin eluted as one peak.

A calibration curve of the same series of protein standards with apoferritin and myoglobin added is plotted semilogarithmically in Fig. 2. With G 3000 SW columns the exclusion limit for proteins is above a molecular weight of 600,000, and best separation efficiency is in the molecular weight range below approximately 60,000. Since retention times are printed out by the integrator, this calibration curve allows a reliable determination of the molecular weight of separated urinary proteins. For more accurate molecular-weight determinations of proteins by GPC, proteins are usually denaturated by the addition of SDS to the mobile phase. But then the exclusion limit of G 3000 SW columns decreases [15] and no separation of IgG and higher molecular weight proteins is possible.

We used gel chromatography for quantification of proteinuria by measuring absorbance at 280 nm of all eluated proteins. Interfering substances of lower molecular weight than 10,000 are separated on small Sephadex G-50 columns. Sample volume and column dimensions are adjusted to minimize the dilution during column passage to a factor of 2. The analytical recovery of diluted

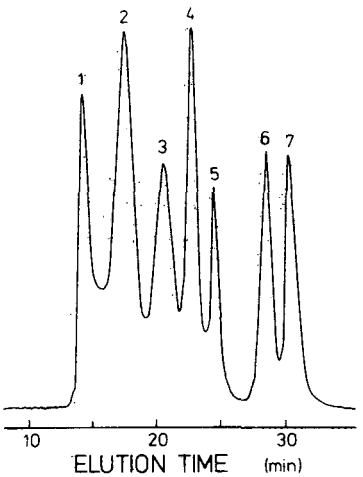

Fig. 1. Separation of a mixture of standard proteins: 1 = thyroglobulin (porcine); 2 = ferritin (horse spleen); 3 = h- γ-globulin; 4 = h-transferrin + h-albumin; 5 = ovalbumin; 6 = chymotrypsinogen; 7 = cytochrome *c*. Column: G 3000 SW, 7.5 mm I.D., 30 cm + 50 cm with precolumn. Flow-rate: 1 ml/min. Solvent: 1/15 *M* potassium phosphate buffer, pH 6.8, containing 0.1 *M* NaCl and $6 \cdot 10^{-4}$ *M* NaN_3. Sample load: 20—80 μg; charge 100 μl. Detector: UV 280 nm, 0.01 a.u.f.s.

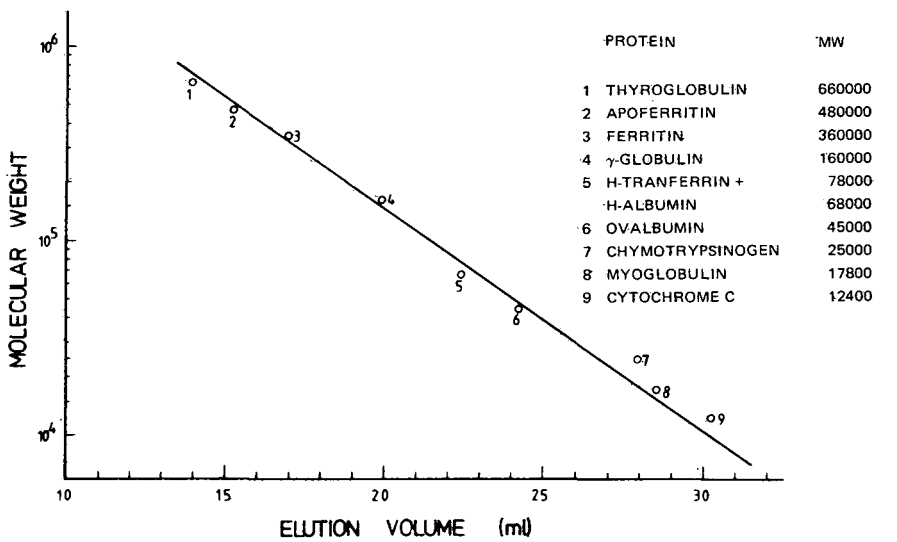

Fig. 2. Relationship between molecular weight of proteins and elution volume for G 3000 SW type columns. Conditions as in Fig. 1.

Precinorm® U (1.04 g/l) added to six different columns was 98.9%. To confirm the results obtained with the HPLC—280 nm method we also measured urinary protein concentration by the biuret method (see Fig. 3). The regression line of 62 paired measurements corresponds to the equation $y = 1.010x - 0.102$ with a correlation coefficient of 0.967, though in urine samples with a high content of lower molecular weight proteins (< 68,000) higher concentrations were

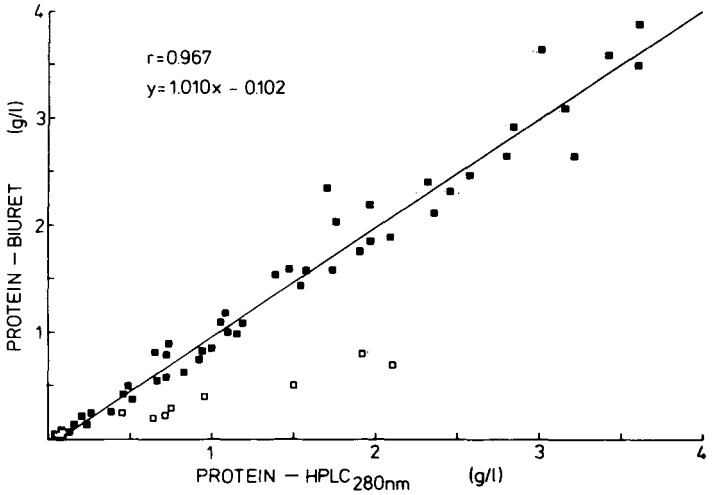

Fig. 3. Comparison of biuret and HPLC—280 nm techniques for the measurement of total urinary protein concentration. Open symbols indicate samples with more than 50% low molecular weight (< 68,000) proteins, which were included in the determination of the regression line. When these samples are excluded from the calculation, the regression line is $y = 1.024x - 0.037$ with a correlation coefficient $r = 0.988$.

found with the HPLC—280 nm method. This is probably caused by the high absorbance of a protein around 45,000 daltons at 280 nm scarcely visible in SDS—PAGE after staining with Coomassie Brilliant Blue R 250. However, there was a high correlation for quantification of Bence-Jones proteinurias using both methods.

The reproducibility of the method was tested also with diluted Precinorm® U solution (1.04 g/l). There was a within-run imprecision (C.V.) of 3.4% (n = 19) and a between-day imprecision of 3.65% (n = 13), which includes the error inherent in gel chromatography on G-50 columns. With an injection volume of 100 µl protein excretions from 40 mg/l up to 3 g/l can be measured. Since peak area does not depend on sample load in the range of 20—200 µl, optimal volumes can be injected according to the results of semiquantitative protein determinations obtained using test sticks.

For better differentiation of post-renal IgG secretion and IgG excretion caused by glomerular alteration, separation of transferrin and albumin is necessary. In both diseases albumin is always secreted, but transferrin only due to glomerular damage. In gel chromatography separation of these two proteins on the basis of their similar molecular size (78,000 vs. 68,000) can not be accomplished. However, the gel permeation media contain certain amounts of negatively charged groups which cause an additional ion-exclusion effect [16]. Since transferrin is more basic than albumin (pI 5.8 vs. 4.9), a better separation seems to be possible using a mobile phase of low ionic strength and high H^+ concentration. The elution patterns of a urine sample containing immunoglobulin, transferrin, albumin and different proteins of lower molecular weight using various eluents are shown in Fig. 4. The elution profiles with each eluent were similar, but the elution time of proteins increased with decreasing pH

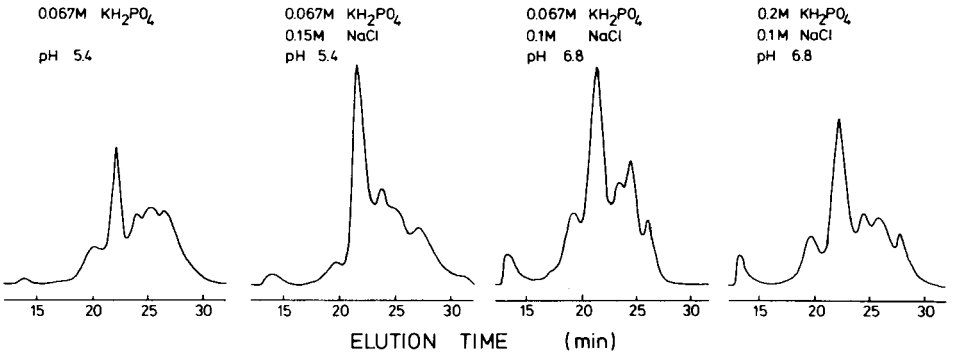

Fig. 4. Effect of pH and salt concentration of the mobile phase on separation of urinary proteins for one urine sample. Load volume: 80 µl. Detector: UV 280 nm, 0.01 a.u.f.s.

value, addition of sodium chloride and higher phosphate concentration. Absorbance of all proteins depends on the mobile phase. By decreasing the pH value further to 4.5 we observed a slight resolution between albumin and transferrin at low salt concentration but a loss in resolution in the area of microproteins. Since this condition was also not suitable for the column, we consider 1/15 *M* phosphate buffer, pH 6.8, containing 0.1 *M* NaCl as optimal for urinary protein separation.

We found affinity chromatography on immobilized Cibacron Blue E 3G-A an exceedingly efficient method for removing albumin selectively not only from plasma [17] but also from urine. Fig. 5 illustrates the removal of albumin from two urine samples with different transferrin concentrations by chromatography on Blue Sepharose CL-6B with consequent gel permeation on a G 3000 SW column.

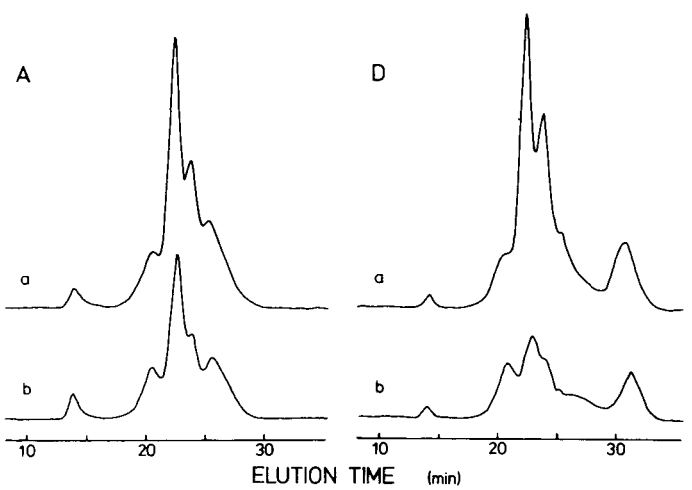

Fig. 5. Urinary pattern of proteinuria caused by glomerular and tubular involvement before (a) and after (b) treatment with Blue Sepharose CL-6B. Conditions as in Fig. 1. Sample A (50 µl injected) and sample D (100 µl injected) were also used for SDS—PAGE in Fig. 6. Dilution of samples by treatment with Blue Sepharose CL-6B was corrected for by injecting the doubled volume.

The increase in absorbance about 22 min after injection must be caused solely by transferrin (see Fig. 5, patterns b). This was judged by SDS—PAGE using samples of different protein concentrations. As can be seen from Fig. 6, albumin is removed completely by affinity chromatography on Blue Sepharose CL-6B. Urinary protein patterns using SDS—PAGE were in good agreement with the results obtained by gel chromatography on G 3000 SW columns, though resolution is higher with SDS—PAGE (see Figs. 5 and 6, samples A and D). Comparing peak heights in gel chromatography at a retention time of about 22 min before and after treatment with Blue Sepharose CL-6B as a measure of transferrin concentration is difficult to perform since the specific molar extinction coefficient is more than twice as high for transferrin as for albumin. But differences in peak areas can be used for determination of albumin concentrations.

Typical protein patterns for different types of proteinuria are shown in Fig. 7. In renal tubular diseases (Fig. 7b and c) proteins with a molecular weight lower than 70,000 are greatly increased, caused by the insufficiency of the tubules to reabsorb proteins filtered by the normal glomerulus. Among them albumin, α_1-antitrypsin (MW 54,000), free and dimer light chains (MW 22,000, 44,000), retinol binding protein (MW 21,000) and β_2-microglobulin (MW 12,400) have been identified [18].

Damage to glomerular basal membrane or degenerative diseases are associated with excretion of albumin and macroproteins in relative amounts similar

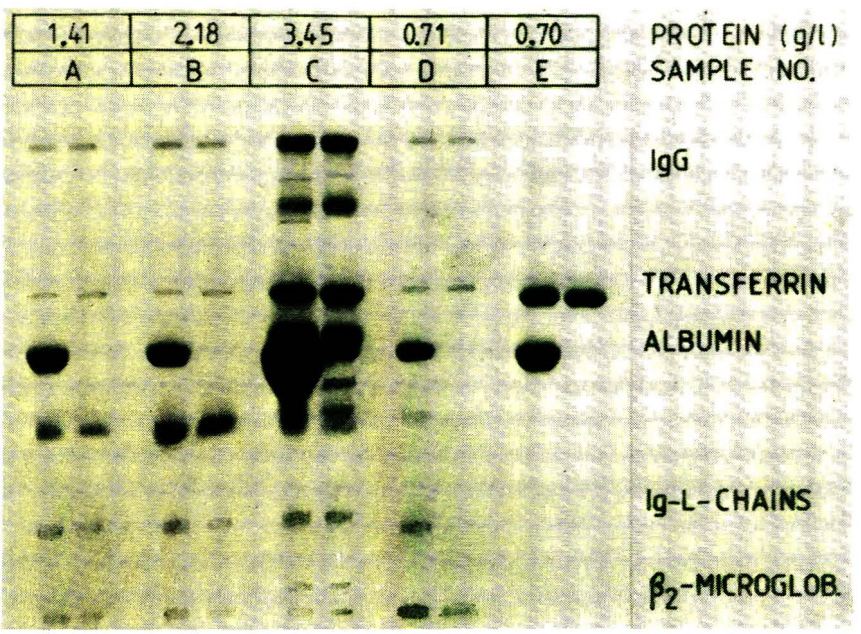

Fig. 6. SDS—PAGE patterns of different samples before and after chromatography on Blue Sepharose CL-6B. Samples A—D: mixed proteinuria with variable protein concentrations. Sample E: mixture of 0.5 g/l albumin and 0.2 g/l transferrin. Dilution by chromatography on Blue Sepharose CL-6B is corrected for by doubling the sample load.

to serum (Fig. 7d). Since the exclusion limit of the column is around MW 600,000, it is not possible to distinguish between α_2-macroglobulin and IgM. The most frequent proteinurias found were caused by glomerular and tubular alterations (Fig. 7f). Within the distinguishable tubular, glomerular, or mixed proteinurias, protein patterns can be different depending on the etiology (see Fig. 7b and c) but proved to be qualitatively constant during the course of a kidney disease. Patients with pure tubular dysfunction were much less frequent than those with glomerular disease and protein excretion was then always below 1.5 g per 24 h.

Patients with multiple myeloma often have Bence-Jones proteins passing freely across the normal glomerular wall. GPC of such urine samples (Fig. 8) seems to be particularly useful in the diagnosis of this disease and may be used for monitoring the therapy of the disease by quantification of the proteinuria. Additionally, glomerular and/or tubular alterations may be detected simultaneously (Fig. 8c).

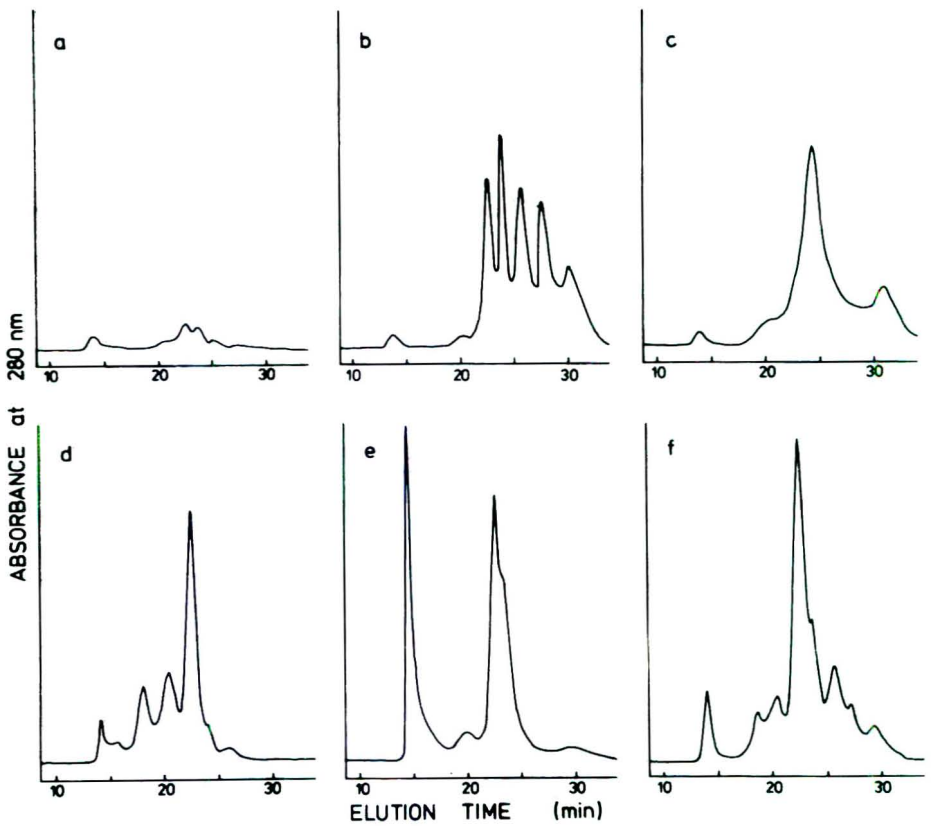

Fig. 7. Different patterns of renal proteinurias after separation on GPC: (a) physiological proteinuria; (b) tubular proteinuria in interstitial nephritis, MW 70,000—10,000; (c) tubular proteinuria in kaliopenic nephropathy, MW 50,000—10,000; (d) unselective glomerular proteinuria, MW 600,000—60,000; (e) glomerular proteinuria in plasmocytoma with excretion of albumin and polymer IgA (immunological proved); (f) mixed tubular and glomerular proteinuria in chemotherapy of a bronchial carcinoma, MW 600,000—10,000.

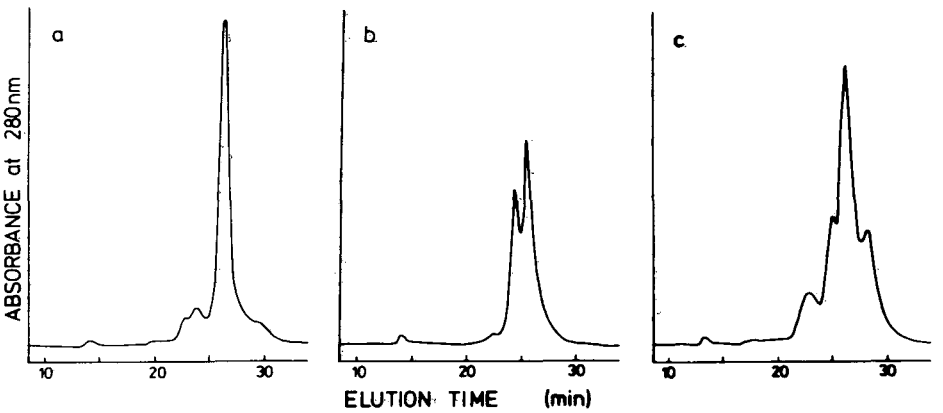

Fig. 8. Urinary pattern of patients with prerenal proteinuria: (a) plasmocytoma IgG, type kappa with excretion of monomer light chains; (b) plasmocytoma IgD, type lambda with excretion of monomer and dimer light chains; (c) mutiple myeloma IgA, type lambda with excretion of monomer and possible dimer light chains with simultaneous tubular alteration.

The chromatographic method we have described seems well suited for urinary protein profiling. Both relative and absolute changes in the protein pattern can be identified. GPC has been found to be as valuable as SDS—PAGE in differentiating physiological, tubular and glomerular types of proteinuria. Though protein discrimination might be further improved, the speed of analysis, the ability for automation, the favourable qualitative and quantitative results advocate this GPC method for routine use.

ACKNOWLEDGEMENTS

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DETECTION AND MEASUREMENT OF OPIUM ALKALOIDS AND METABOLITES IN URINE OF OPIUM EATERS BY METHANE CHEMICAL IONIZATION MASS FRAGMENTOGRAPHY

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SUMMARY

A gas chromatographic—mass spectrometric assay for eight opium alkaloids in human urine following opium ingestion is described. The compounds were extracted from urine with methylene chloride—isopropanol (7:3, v/v) at pH 9.5, evaporated, derivatized with Tri-Sil Z and analyzed by methane chemical ionization mass fragmentography. The method is sensitive to ca. 0.01 μg/ml for morphine and codeine and ca. 0.05 μg/ml for the other compounds. Adsorption problems on the gas chromatography column prevented obtaining reproducible results for the measurement of noscapine. Extraction efficiencies over the pH range of 8—11 for the eight compounds are reported. Retention times of the opium alkaloids were determined using five different liquid phases (3%) on Gas-Chrom Q (100—120 mesh) and two column lengths (36 cm and 183 cm). The 36-cm column packed with OV-210 was selected for use in the assay. Ions were selected for monitoring for each component from their methane chemical ionization spectrum to provide the needed sensitivity and specificity for analysis of a multi-component mixture. The assay was used for the analysis of an "opium eater's" urine. Morphine, codeine, nomorphine, norcodeine and noscapine were detected; however, no evidence was obtained for thebaine, papaverine or oripavine. Unconjugated morphine (0.64 μg/ml) was present at nearly twice the concentration of codeine (0.37 μg/ml) and normorphine and norcodeine were present in equal amounts (ca. 0.15 μg/ml).

INTRODUCTION

Opium, the dried exudate of the poppy plant *Papaver somniferum*, contains some twenty-five or more alkaloids which are responsible for its pharmacological activity. The major alkaloids, morphine (MOR), codeine (COD), thebaine (THE), papaverine (PAP) and noscapine (NOS), account for 0.3—10% of dry weight with the remaining alkaloids occurring in trace amounts [1].

Numerous assays have been developed for measurement of these compounds in opium preparations by gas chromatography (GC) [2—5] and high-performance liquid chromatography [6—11]. Combined gas chromatography—mass spectrometry (GC—MS) has been used for the forensic identification of opium constituents [12]. A single report has appeared on the urinary excretion of MOR and COD following the consumption of opium in which the compounds were separated and measured on thin-layer chromatography (TLC) [13]. However, none of the more sensitive and specific assays for the opium alkaloids have been applied to the study of the urinary excretion profiles of "opium eaters". This report describes the use of methane chemical ionization (CI) mass fragmentography (MF) for the detection and measurement of opium alkaloids and metabolites (See Fig. 1) in human urine following opium ingestion.

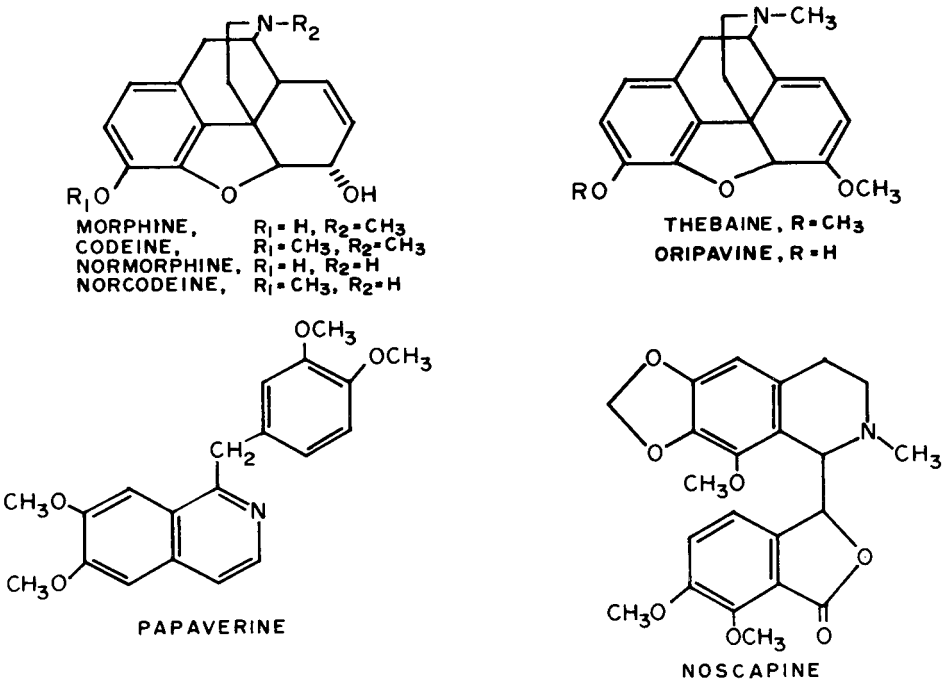

Fig. 1. Structures of opium alkaloids.

EXPERIMENTAL

Drug standards

MOR, COD and NOS were purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Normorphine (NOM) and norcodeine (NOC) were purchased from Merck (Rahway, NJ, U.S.A.). PAP was purchased from Sigma (St. Louis, MO, U.S.A.). THE and α-isocodeine (internal standard, IS) were obtained from the Research Technology Branch, Division of Research, National Institute on Drug Abuse (Rockville, MD, U.S.A.). Oripavine (ORI) was a gift from McFarlan Smith (Edinburgh, Great Britain). All compounds were analyzed by TLC and GC—MS for purity and structural verification.

Instrumentation

Gas chromatography. Analyses were performed on a Varian Model 2700 gas chromatograph equipped with a flame ionization detector. Glass columns (0.36 m or 1.83 m × 2 mm I.D.) were packed with Gas-Chrom Q (100—120 mesh) coated with 3% liquid phase. The injector and detector were maintained at 190°C and 275°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 20 ml/min. The column temperature was programmed as indicated in Table I. The reported retention times represent the mean of triplicate determinations.

Gas chromatography—mass spectrometry. Mass spectra were obtained with a Finnigan Model 4021 Automated GC/MS/DS system operating in the CI mode. The gas chromatograph consisted of a glass column (0.36 m × 2 mm I.D.) packed with 3% OV-210 on Gas-Chrom Q (100—120 mesh). The temperature of the injector and source were 190°C and 260°C, respectively. The GC oven was programmed as follows: 1-min hold at 170°C; program 170°C to 260°C at 10°C/min; 3-min hold at 260°C. A venting valve was opened for 30 sec following sample injection to allow volatiles and solvent to be diverted from the GC—MS source. Methane was used as carrier and reagent gas at a flow-rate of 20 ml/min. The electron energy was set at 70 eV and the multiplier voltage at 1.4 kV. Total ion scans were collected over the range of 80—600 a.m.u.

Mass fragmentography. Quantitative analyses were performed by MF with the same conditions as those for GC—MS. The ions selected for monitoring and confirmation of each substance were as follows: MOR, *m/e* 340 and 414; COD, *m/e* 282 and 372; NOM, *m/e* 326 and 400; NOC, *m/e* 268 and 358; THE, *m/e* 312 and 340; ORI, *m/e* 370 and 354; PAP, *m/e* 340 and 368; NOS, *m/e* 220 and 195; IS, *m/e* 372. Standard curves were prepared for MOR (0—20 μg/ml), COD (0—10 μg/ml) and the remainder of the compounds (0—2 μg/ml). Linear relationships of peak intensity of drug to IS vs. concentration were obtained for all compounds with the exception of NOS. The response for NOS was variable and appeared to be related to the age of the column.

Extraction procedure

An aliquot (5 ml) of each urine specimen was mixed with IS (30 μg) and the pH was adjusted to 9.5 with 2 *N* sodium hydroxide. Sodium chloride (1.0 g) and 15 ml of methylene chloride—isopropanol (7:3, v/v) solution were added and the contents were shaken for 20 min. Following centrifugation, the aqueous layer was discarded and 13 ml of the organic phase were transferred and evaporated to dryness under nitrogen. Tri-Sil Z (Pierce, Rockford, IL, U.S.A.) (0.1 ml) was added and the tube was sealed and incubated at room temperature for 1 h. From 1—3 μl were removed for analysis by GC—MS.

Extraction efficiency and pH studies

Standard buffered solutions of the opiate alkaloids (10 μg/ml) were prepared in the pH range of 8—11. Aliquots were extracted in triplicate as described in the extraction procedure with the IS added in the evaporation step. Following derivatization, the extracts were analyzed by GC (36 cm × 2 mm column packed with 3% OV-210 on Gas-Chrom Q). Comparison of peak height ratios of extracted standards to those of unextracted standards provided percent recoveries.

TABLE I

INFLUENCE OF LIQUID PHASE AND COLUMN LENGTH ON THE GC RETENTION TIMES OF EIGHT OPIUM ALKALOIDS

The values represent relative retention times and are the mean of triplicate determinations. Values in brackets represent uncorrected retention time in min.

Compound	GC column phases and length*									
	OV-101		OV-17		OV-210		OV-225		Silar-5CP	
	36 cm	183 cm	36 cm	183 cm	36 cm	183 cm	36 cm	183 cm	36 cm	183 cm
MOR-TMS_2	1.00 (6.8)	1.00 (8.0)	1.00 (5.3)	1.00 (18.8)	1.00 (2.5)	1.00 (2.8)	1.00 (5.3)	1.00 (16.3)	1.00 (4.4)	1.00 (9.2)
COD-TMS	0.91	0.87	1.01	1.02	1.01	1.01	1.09	1.05	1.20	1.50
NOM-TMS_2	1.00	1.00	1.07	1.05	1.16	1.17	1.13	1.08	1.27	1.31
NOC-TMS	0.90	0.90	1.07	1.07	1.18	1.20	1.23	1.15	1.45	1.77
ORI-TMS	0.99	1.05	1.18	1.18	1.14	1.24	1.22	1.25	1.53	1.10
THE	0.99	0.97	1.34	1.30	1.43	1.48	1.54	1.46	1.93	1.19
PAP	1.34	1.66	1.90	2.15	2.76	2.91	2.42	2.68	3.03	—
NOS	1.70	2.89	2.91	—	4.17	6.83	5.47	—	—	—

*Retention times were determined on the 36-cm columns with a temperature program of 170° C to 260° C at 10° C/min and on the 183-cm columns with a temperature program of 220° C to 260° C at 10° C/min.

RESULTS AND DISCUSSION

Extraction of opium alkaloids from urine

A single, one-step extraction procedure was adopted for the recovery of the opium alkaloids from urine. Further sample clean-up by back extraction with dilute mineral acid resulted in significant losses of THE and PAP. With the exception of NOM, all compounds were efficiently extracted with methylene chloride—isopropanol (7:3, v/v) from solution in the pH range of 8.5—10.5 (Fig. 2). At pH 9.5 recoveries ranged from 83% to 91%. Recovery for NOM at pH 9.5 was 39%.

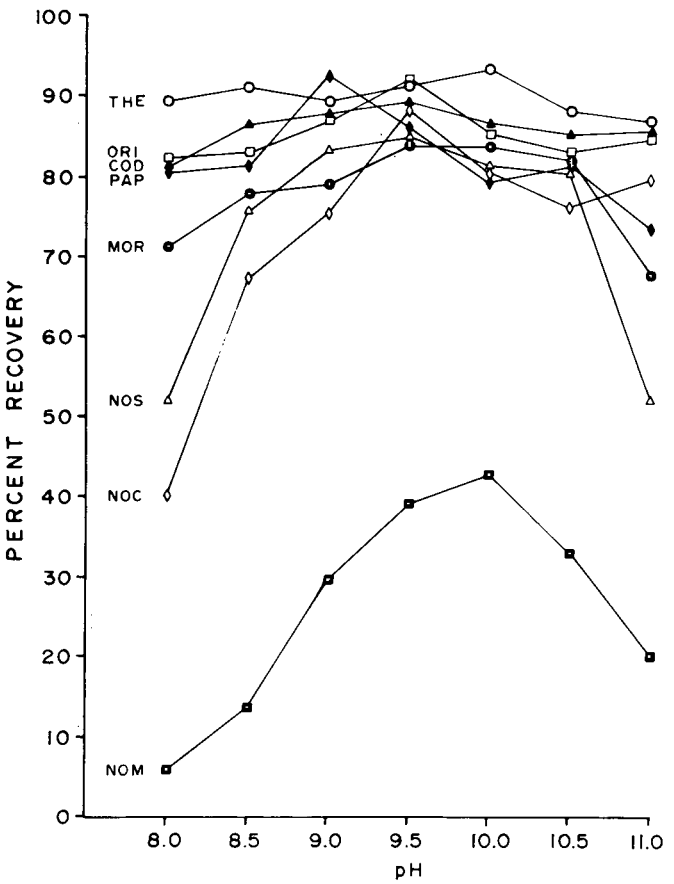

Fig. 2. Efficiencies of methylene chloride—isopropanol (7:3, v/v) extraction of opium alkaloids at pH 8—11.

GC separation of opium alkaloids

Resolution of the opium alkaloids [trimethylsilyl (TMS) derivatives] was attempted by GC with five different liquid phases (3% by weight on Gas-Chrom Q, 100—120 mesh). Two column lengths were employed; a short column (36 cm) and a longer column (183 cm) were packed with each of the liquid phases noted in Table I (listed in order of increasing polarity). The most complete

separation of the components was obtained on Silar-5CP, the most polar liquid phase tested. Unfortunately, NOS did not elute from either the short or long column with this packing, nor did it elute from the long columns packed with OV-17 or OV-225, making these columns unacceptable for further use. Resolution of all components was not complete on any of the other columns examined; however, since complete chromatographic resolution was not required for MF assay, considerations in selection of column length were given to reduction of assay time and background bleed response. The 36-cm column provided the shortest retention times in all cases as well as substantial reductions in bleed rate. Also, resolution on the 36-cm column was almost equal to the 183-cm column, hence the short columns were used in all further assay work.

Selection of the liquid phase was made on the basis of assay time and resolution. The most acceptable phase was OV-210, intermediate in polarity of the phases tested. The GC tracings for the opium components and IS on OV-210 are shown in Fig. 3. The same sample was injected on both columns using the indicated temperature programs. All other instrumental settings were the same. On both columns, MOR and COD were unresolved and eluted as one peak; likewise NOM, NOC and ORI were unresolved and eluted following the MOR/COD peak. IS, THE, PAP and NOS were well resolved. The reduction in retention times and bleed rates as well as an enhanced response for NOS on the short column are apparent.

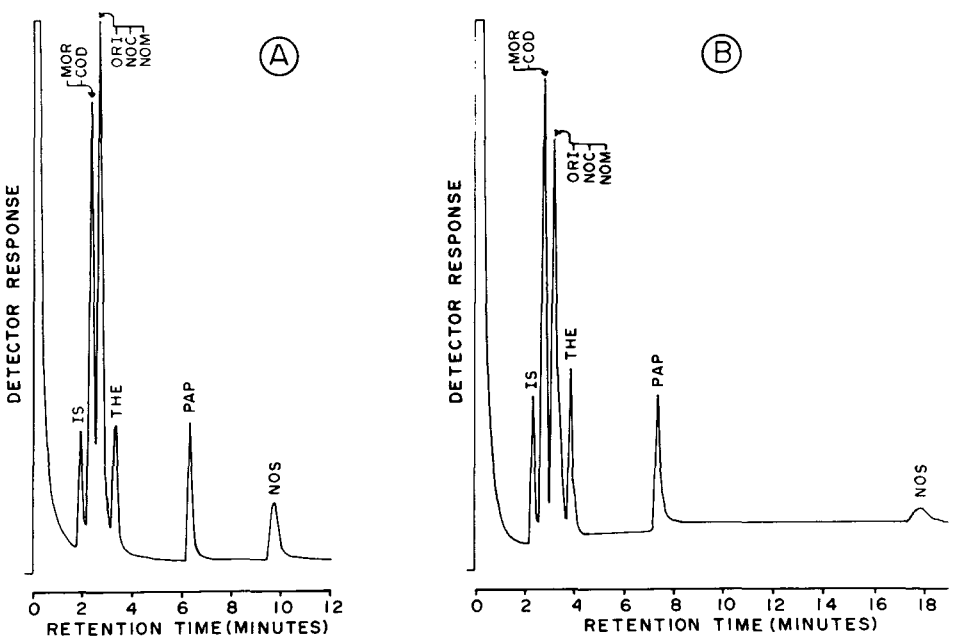

Fig. 3. GC separation of eight opium alkaloids and internal standard on 3% OV-210. (A) 36-cm column with a temperature program of 170° C to 260° C at 10° C/min, (B) 183-cm column with a temperature program of 220° C to 260° C at 10° C/min. All other instrumental settings were the same for both columns.

Methane CI—MS of opium alkaloids

Following derivatization the eight alkaloids and IS were analyzed by GC—MS under CI conditions with methane as carrier and reagent gas. The spectra are summarized in Table II. Six of the nine compounds formed TMS derivatives. All of the components, with the exception of NOS, displayed a strong $(M+1)^+$ ion and $(M+29)^+$ ion. The most abundant ion for NOS was *m/e* 220 indicating a loss of the isobenzofuranone ring from the parent compound. Characteristically, the most abundant ions for MOR-TMS_2 were represented by the $(M-15)^+$ ions, whereas those for COD-TMS and NOC-TMS were represented by the $(MH-90)^+$ ions [loss of $HOSi(CH_3)_3$]. The $(M+1)^+$ ion was the most abundant ion in the spectra of ORI-TMS, THE, PAP and IS-TMS.

MF of opium alkaloids

In the development of an assay for opium constituents in urine it was presumed that any of the eight compounds could be present in urine in varying amounts. Since the chromatographic columns tested did not resolve all components, the specificity and sensitivity of the MF assay depended on the ions selected for monitoring. Ideally the selected ions should be present in high relative abundance and be unique for each compound.

The ions selected for monitoring for the opium alkaloids were the two most abundant ions in the spectrum for each component. Monitoring of these ions for THE, PAP, NOS and IS at their respective retention times offered the required sensitivity and specificity since the OV-210 column adequately resolved these from the other compounds of interest. The remaining substances eluted on OV-210 as two clusters of unresolved compounds (Fig. 3), one containing MOR and COD and the other ORI, NOC and NOM. Consequently, it was necessary to assess the potential interference of each member of the cluster of compounds on the remaining members of that cluster. By injecting each individual compound singly followed by MF analysis of all selected ions of interest, it was possible to determine the relative potential interference for each component at its corresponding scan number (SN, analogous to retention time). These data are shown in Table III. For example, interference in the measurement of COD-TMS at SN = 42 using the ion at *m/e* 372 could occur from MOR-TMS_2 at SN = 43. Although the percent relative abundance (%RA) of this interference from MOR-TMS_2 is only 6%, at low concentrations of COD-TMS and high concentrations of MOR-TMS_2 the interference could be very significant since these two compounds are unresolved. On the other hand, contributions to this ion from the remaining compounds at SN = 32, 48 and 52 are not potential interferences since they are well resolved from COD-TMS. Selection of the ion at *m/e* 282 for monitoring of COD-TMS is an even better choice since there is no interference from MOR-TMS_2. The ions of choice for each compound are underlined in Table III. No serious interferences were present for any of the compounds. The secondary ions were used for confirmation.

A typical MF scan of opium standards extracted from normal urine is shown in Fig. 4A. Peak intensity ratios of drug to IS were measured in the range of 0—20 μg/ml for MOR, 0—10 μg/ml for COD and 0—2 μg/ml for the other compounds. Lower limits of sensitivity for MOR and COD were ca. 0.01 μg/ml and

TABLE II

METHANE CHEMICAL IONIZATION SPECTRA OF EIGHT OPIUM ALKALOIDS AND INTERNAL STANDARD

Compound	Mol. wt.	Methane CI spectra*			
		$(M+29)^+$	$(M+1)^+$	M^+	Prominent fragment ions
MOR-TMS_2	429	458 (19)	430 (70)	429 (55)	416 (15), 415 (34), 414 (100), 371 (18), 341 (22), 340 (91)
COD-TMS	371	400 (16)	372 (51)	371 (36)	356 (31), 313 (19), 283 (20), 282 (100)
NOM-TMS_2	415	444 (18)	416 (74)	415 (49)	401 (32), 400 (100), 371 (16), 326 (90), 222 (15)
NOC-TMS	357	386 (14)	358 (50)	357 (28)	342 (34), 313 (13), 269 (19), 268 (100), 164 (11)
ORI-TMS	369	398 (17)	370 (100)	369 (41)	355 (24), 354 (82), 353 (11), 108 (24)
THE	311	340 (13)	312 (100)	311 (47)	—
PAP	339	368 (22)	340 (100)	339 (11)	—
NOS	413	442 (1)	414 (6) 413 (0)	221 (14)	220 (100), 195 (13), 137 (8)
IS-TMS	371	400 (17)	372 (100)	371 (39)	357 (11), 356 (43), 282 (34)

*m/e (percent abundance). The ions underlined are those slected for monitoring in the MF assay.

TABLE III

POTENTIAL INTERFERENCES IN THE MASS FRAGMENTOGRAPHIC ASSAY OF UNRESOLVED MIXTURES OF OPIUM ALKALOIDS

Compound (SN)*	Selected ions (RA)**	Percent relative abundance of interfering ion from other compounds (SN)***						
		MOR-TMS$_2$ (43)	COD-TMS (42)	NOM-TMS$_2$ (52)	NOC-TMS (52)	ORI-TMS (48)	THE (66)	IS-TMS (32)
MOR-TMS$_2$	340 (91)	91	0	4	2	1	13	0
(43)	414 (100)	100	0	11	0	0	0	0
COD-TMS	282 (100)	0	100	0	0	0	0	34
(42)	372 (51)	6	51	4	1	9	0	100
NOM-TMS$_2$	326 (90)	0	0	90	0	2	0	0
(52)	400 (100)	2	16	100	0	2	0	17
NOC-TMS	268 (100)	0	0	0	100	0	0	0
(52)	358 (50)	0	2	0	50	0	0	5
ORI-TMS	370 (100)	0	15	0	0	100	0	25
(48)	354 (82)	0	0	4	0	82	0	0
THE	312 (100)	0	0	3	0	2	100	0
(66)	340 (13)	91	0	4	2	1	13	0
IS-TMS	372 (100)	6	51	4	1	9	0	100
(32)	282 (34)	0	100	0	0	0	0	34

*Scan number (corresponding to retention time) at which the compound is monitored.
**Percent relative abundance of ions selected for monitoring. Ions used for quantitation are underlined.
***Scan number at which the interference will occur.

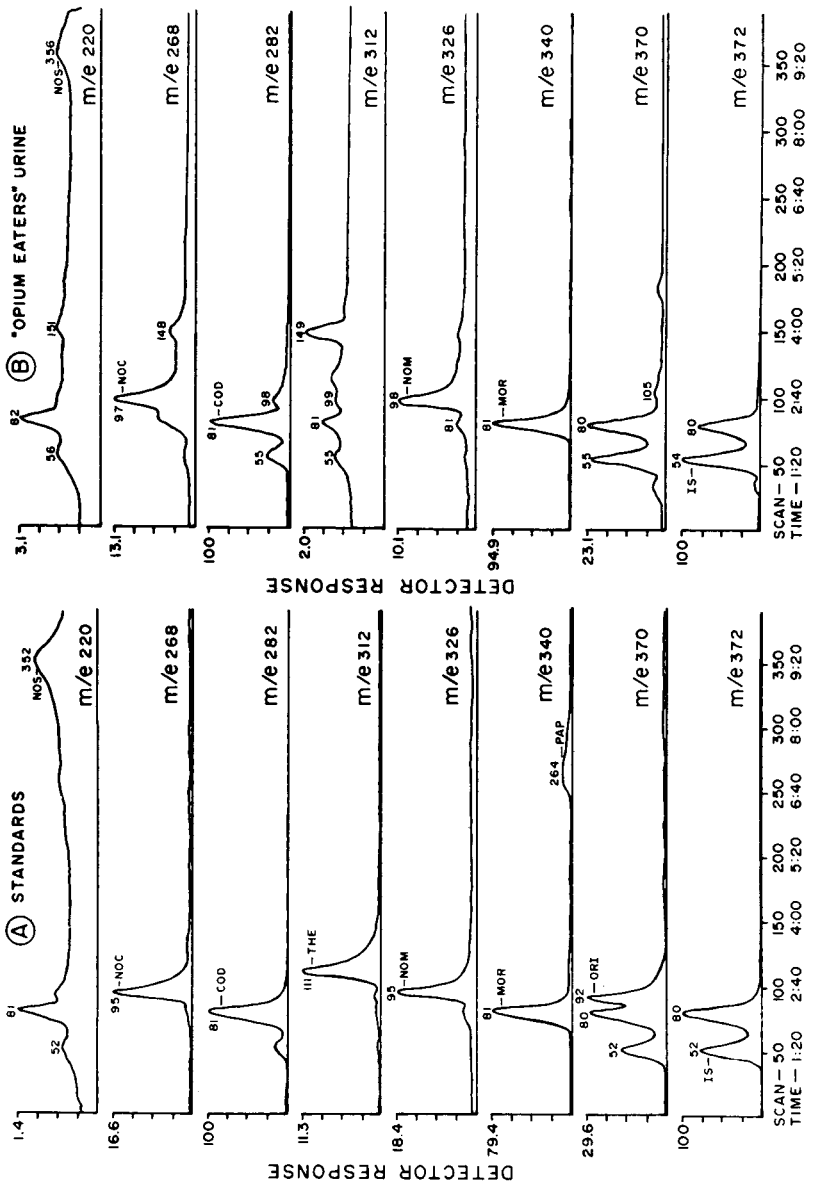

Fig. 4. Selected ion recordings of urinary extracts of standards (A) and "opium eater's" urine (B).

ca. 0.05 μg/ml for the remainder of the compounds. Plots of peak intensity ratios versus concentration were linear with correlation coefficients (r) ⩾ 0.97. An exception to this was NOS which gave variable responses which seemed to be related to the age of the GC column. The strongest responses were obtained with freshly packed columns. After several weeks of column use the response for NOS often became erratic and weaker, an indication of probable adsorption on active sites of the column. Retention times also varied from column to column as indicated by the SN for the IS (see Table III and Fig. 4). This was not a problem, however, since relative retention times were reproducible.

MF analysis of an "opium eater's" urine

Urine was obtained from a male "opium eater" hospitalized for treatment of cancer of the esophagus. The patient was ingesting approximately 1 g per day of a dark resinous material which he identified as dross from the opium pipe or "sukhteh" [14]. The urine samples were extracted as described and analyzed by MF. Fig. 4B shows the response from an unhydrolyzed urine extract. MOR, COD, NOM, NOC and NOS were detected; however, no evidence was obtained for the presence of THE, PAP or ORI. The concentration of MOR (0.64 μg/ml) was approximately twice that of COD (0.37 μg/ml), whereas NOM and NOC were present in equal amounts (ca. 0.15 μg/ml). Treatment of the urine sample with β-glucuronidase enzyme produced a greater than tenfold increase in concentration for the four compounds, a finding consistent with the extensive conjugation normally observed in opiate metabolic studies.

It is anticipated that this method could serve as a useful assay for further metabolic studies on "opium eaters", a large population of drug users which have received very little attention.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE METABOLISM OF PRIMAQUINE AND THE IDENTIFICATION OF A NEW MAMMALIAN METABOLITE

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SUMMARY

Using rats that had been dosed with 20 mg/kg of primaquine diphosphate (11.4 mg/kg free base), it was found that the drug underwent a metabolic oxidative deamination to give 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline. The presence of this new mammalian metabolite was verified using high-performance liquid chromatographic, gas chromatographic, and mass spectral methods. A quantitative high-performance liquid chromatographic method for the determination of primaquine and the carboxylic acid metabolite in plasma using only 50 μl of whole blood from the rat was developed and the method could be used to detect levels as low as 0.05 μg/ml of the metabolite. Following intravenous administration of the drug, it was found that the plasma levels of primaquine fell very rapidly and after 30 min, the levels of the metabolite were much higher than those of primaquine.

INTRODUCTION

Primaquine (I) is often used in combination with other antimalarial drugs for prophylaxis in endemic areas. Primaquine has a fairly low therapeutic index and its use is frequently associated with hemolytic anemia, particularly in individuals with a deficiency in glucose 6-phosphate dehydrogenase [1]. In vitro studies using both normal and glucose 6-phosphate deficient erythrocytes

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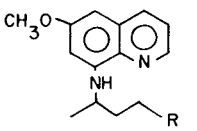
have indicated that this effect is caused by a primaquine metabolite rather than primaquine itself [2—4].

Earlier studies in these laboratories using microorganisms as models for mammalian metabolism had shown that 23 of 77 microorganisms studied could convert primaquine to II (Fig. 1) and/or III [5]. The primary objective of the present study was to develop analytical methods for these metabolites in plasma samples and to determine if these metabolites were formed in mammalian systems.



I $-CH_2NH_2$
II $-CH_2NHCOCH_3$
III $-CO_2H$
IV $-CO_2CH_3$

Fig. 1. Primaquine and metabolites.

EXPERIMENTAL

Reagents

Primaquine diphosphate was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was used as received. The reference standards of 8-(4-acetamido-1-methylbutylamino)-6-methoxyquinoline (II) and 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline (III) were prepared in these laboratories using a previously reported procedure [5]. All other materials were of reagent grade quality and were used as received.

Administration of primaquine and collection of blood samples

Primaquine diphosphate at a dosage of 20 mg/kg (11.4 mg/kg free base) was administered through the penile vein of male Wistar rats that had been anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneal). A single rat was used for the time—course study rather than using pooled data from several rats. However, the complete time—course study was repeated using a total of five rats. This method was made possible by the extremely small sample size required by the analytical procedure. Using 50-μl heparinized hematocrit tubes, blood samples were collected from the intraorbital sinus vein of a single rat at 1 min, 3 min, 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h after administration of primaquine diphosphate.

High-performance liquid chromatographic analysis of plasma samples

The 50-μl samples of whole blood were centrifuged for 2 min using a standard hematocrit centrifuge, then a 20.0-μl sample of the plasma was injected into the high-performance liquid chromatograph. A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, Model U6K injector, and Model 440 ultraviolet detector (254-nm and 280-nm dual detectors plumbed in series)

were used. A Whatman (Clifton, NJ, U.S.A.) C-18 guard column (with the first frit removed) was used between the injector and the analytical column. It was usually necessary to change the packing of the guard column every 20—30 injections.

A 30 cm × 3.9 mm I.D. octadecyl reversed-phase analytical column (Waters Assoc., μBondapak C_{18}, 10 μm particle size) was used with a flow-rate of 1.0 ml/min. The mobile phase consisted of 6.6 g K_2HPO_4, 8.4 g KH_2PO_4, 4.0 g N,N-dimethyl-octylamine, 2.4 l of methanol, and 1.6 l of water. The retention characteristics of primaquine and its metabolites (Table I) were quite consistent when aged columns were used. When new columns were first put into service, III was observed to elute (retention time, 10 min) after primaquine. After the column had aged, III (retention time, 7.44 min) was observed to elute before primaquine. This aging process usually took 7—10 days after which the retention times were very stable.

Quantitation of primaquine and metabolites was based on a direct comparison of the height of the chromatographic peaks for the plasma samples and freshly prepared pure reference standards of the compounds. The sensitivity of the procedure was primarily limited by the presence of naturally occurring plasma constituents. The quantity of III in the sample shown in Fig. 2 was 0.39 μg/ml. The peak for III was easily distinguished from adjacent chromatographic peaks by the marked differences in the A_{254}/A_{280} ratios.

Gas chromatographic analysis using a nitrogen-selective detector

A 2.0-ml sample of plasma was extracted with 2 × 3 ml of ethyl acetate and then the combined extracts were evaporated under a stream of nitrogen. The extract was then treated with 1.0 ml of diazomethane in diethyl ether at room temperature for 30 min. The ether was removed with a stream of nitrogen and the residue was taken up in 30 μl of methanol. Then 1.0 μl of the extract was injected into a Perkin-Elmer Model 900 gas chromatograph equipped with dual rubidium-bead nitrogen-selective and flame ionization detectors. A 183 cm × 2 mm glass column packed with 3% OV-17 on 110—120 mesh Anachrom ABS support was used at 265°C with a flow-rate of 30 ml/min. The ratio of the response of the two detectors (response index) was measured using a previously published procedure [6] and was used as a means of characterizing the materials in addition to their retention times.

TABLE I

HPLC CHARACTERIZATION OF PRIMAQUINE AND METABOLITE REFERENCE STANDARDS

Compound	Retention time (min)	A_{254}/A_{280}*
III	7.44	3.79 (3.74)
Primaquine, I	8.40	4.41 (4.26)
II	12.5	4.18

*Values in brackets are the absorbance ratios observed for the compounds as detected in the plasma of the test animal.

Gas chromatography—mass spectroscopy of metabolites

The plasma of a rat that had been given primaquine and one that had not received any drugs other than the anesthetic agent were extracted and derivatized as indicated in the previous section. The two extracts were chromatographed on a 3% OV-17 column in a Finnigan Model 3200 mass spectrometer with an INCOS data system. Ionization was by electron impact at 70 eV. Because of coelution of the metabolite and compound common to the extracts of the treated and control animals, a mass spectrum of the metabolite could not be obtained that was completely free of the peaks associated with the interfering substance. The gas chromatographic—mass spectrometric (GC—MS) data files of the treated animal and control animal were then re-examined by reconstructing single ion chromatograms (Fig. 4) at *m/e* 159, 186, 201, 215, and 288 which are characteristic of IV [5].

RESULTS AND DISCUSSION

The high-performance liquid chromatograms of plasma of the rats that had been given the intravenous (I.V.) dose of primaquine (Figs. 2 and 3) clearly indicate the rapid disappearance of the drug and the formation of metabolite III. The chromatograms of the plasma of a rat that had not received primaquine showed peaks at 6.80, 9.28, 11.32, and 15.10 min, but did not exhibit peaks at the same retention times for III (7.44 min) or I (8.40 min). Analysis of plasma samples taken 3 min after the injection of primaquine (Fig. 2) showed that metabolite III was formed very rapidly and by 3 h (Fig. 3) the concentration of the metabolite far exceeded that of the drug. Other than I and III, the only peaks observed in the chromatograms corresponded to natural constituents also found in the control plasma.

In chromatograms of complex matrices such as these, identification of metabolites through comparison of retention times alone will lead to misidentifications fairly frequently. In the present study however, the components in the plasma were characterized by the retention time and by the A_{254}/A_{280} values for each compound (Table I). Using this approach, it was fairly easy to differentiate between the natural plasma constituent (retention time 6.80 min, $A_{254}/A_{280} \approx 120$) and the reference standard of metabolite III (retention time 7.44 min, $A_{254}/A_{280} = 3.74$) even though the retention times of the two were fairly close.

Since III was a new mammalian metabolite of primaquine, additional studies were conducted to verify the production of the metabolite. The gas chromatogram of a pure reference standard of III that had been derivatized to its methyl ester IV was found to contain a peak at 5.0 min and a nitrogen-detector response index of 0.27 was observed. When the pure reference standard of III was derivatized and injected, a second peak was also observed that is the lactam form of III that probably results from the on-column elimination of methanol and cyclization. The lactam form of III was observed to have a retention time of 6.1 min and a nitrogen-detector response index of 0.19 was observed. Previous studies [6] on the correlation of chemical structure and the nitrogen-detector response index have shown that secondary amines are usually observed to have an index value of 0.18—0.32 while amides have values of 0.08—0.17

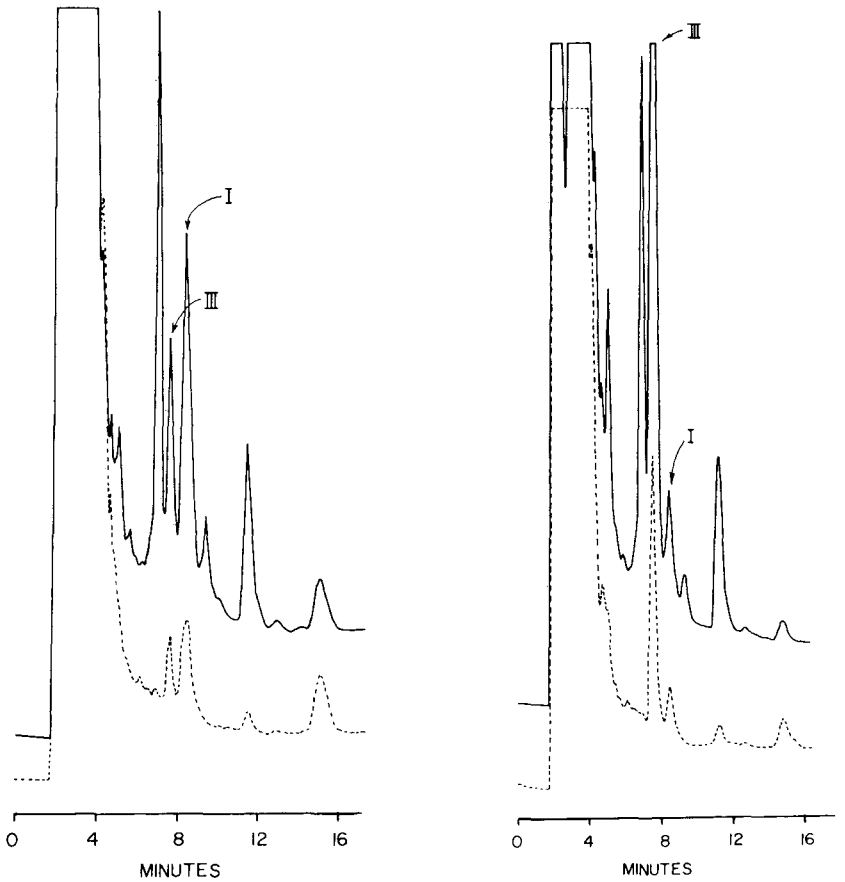


Fig. 2. High-performance liquid chromatogram of 3-min plasma sample. The solid line represents the response of the 254-nm UV detector (0.01 a.u.f.s.) and the dotted line represents the response of the 280-nm detector. The sample was found to contain 3.59 μg/ml I and 0.39 μg/ml III.

Fig. 3. High-performance liquid chromatogram of 3-h plasma sample. Conditions were the same as in Fig. 1. The sample was found to contain 1.21 μg/ml I and 2.31 μg/ml III.

which is consistent with the peak assignments of the ester and lactam. The mass spectrum of the GC peak of IV gave a spectrum [288 (M^+, 8%), 256 (7%), 215 (17%), 201 (100%), 186 (26%), 159 (45%), and 115 (29%)] that was consistent with its structure. The mass spectrum of the GC peak for the lactam [256 (M^+, 12%), 228 (16%), 213 (16%), 200 (70%), 186 (50%), 159 (100%), and 115 (40%)] was also consistent with its structure.

The identification of metabolite III was also substantiated by a GC analysis of the plasma of a rat 4 h after it had been given a 20 mg/kg dose of primaquine diphosphate. In this analysis, the derivatized extracts of the test animal and a control animal plasma were examined using a gas chromatograph equipped with a nitrogen-selective detector and a flame ionization detector. The nitrogen-selective detector showed a very strong response at 5.0 min corresponding to the derivative of III and a smaller peak at 6.1 min corresponding to the lactam in the sample from the treated animal. The

nitrogen-detector response of the derivatized extract of the control animal did not show any response during the 3.0—9.0 min portion of the chromatogram. Unfortunately, the nitrogen-detector response index of the two chromatographic peaks could not be measured because the flame ionization detection—gas chromatograms of the test animals and the control animal showed a large number of peaks in the 3.0—9.0 min region. In order to measure the nitrogen-detector response index of a peak, the peak must be clearly identifiable with both the nitrogen-selective and flame ionization detectors. In this case the metabolite peaks were very easily identified using the nitrogen-selective detector, but the flame ionization detector peaks of the metabolites were completely obscured by a large number of naturally occurring components in the region where the metabolite would elute. The reconstructed total ion chromatogram (6th and 12th tracings in Fig. 4) of the derivatized extracts of the test animal and control animal plasma were outwardly identical and they both matched their flame ionization detection—gas chromatograms. The most direct method of demonstrating the presence of the metabolite in this complex mixture was through the single-ion monitoring technique. The ions that were selected as being most characteristic were *m/e* 159, 186, 201, 215 and 288 and the result of this analysis is shown in Fig. 4 for the test sample and the control sample. Each tracing in Fig. 4 represents the intensity observed for a specific ion as a function of time (scan number) during the GC analysis. The dotted vertical line in Fig. 4 represents the scan number in which IV appeared. In the test sample, all five ion monitoring channels showed a response at the appropriate retention time, while only one of the ion monitoring channels showed a significant response for the derivatized extract of the plasma of the control animal. It was also observed that the scan of the extract of the test animal (scan 104) contained all of the MS peaks associated with the pure sample of IV, but there were additional ions in the spectrum that came from some other material.

The identity of III as a metabolite of primaquine was thus established by comparing HPLC retention times, A_{254}/A_{280} ratios, GC retention times using a nitrogen-selective detector, and mass spectra of the metabolite and an authentic standard of III. For a pharmacokinetic analysis of the time—course of the formation of the metabolite, the HPLC method was found to be the most useful. In order to determine the precision of the HPLC method, a 1.0-ml plasma sample containing approximately 10 μg/ml III was subdivided and assayed four times giving a coefficient of variation of ±4.3%. Concentrations as low as 0.05 μg/ml of III were detected in plasma samples using the HPLC procedure. The detection of III was limited by the presence of a naturally occurring plasma constituent that eluted just before the metabolite. The signal-to-noise ratio of HPLC system was not a limiting factor in the detection of III.

Following i.v. administration of primaquine diphosphate (Fig. 5), the plasma levels of the drug fall extremely rapidly. If one assumes that the dose was uniformly distributed in the blood at the moment of injection, a 200—400 μg/ml plasma level would be expected. Based on the observation of the plasma levels of nine rats 1 min following the injection, levels of only 2—10 μg/ml were observed and these values were rapidly falling. Studies still underway in these laboratories using ^{14}C-labeled primaquine have also shown that less than 1% of the dose remains in the blood when sampled just after the injection. The half-

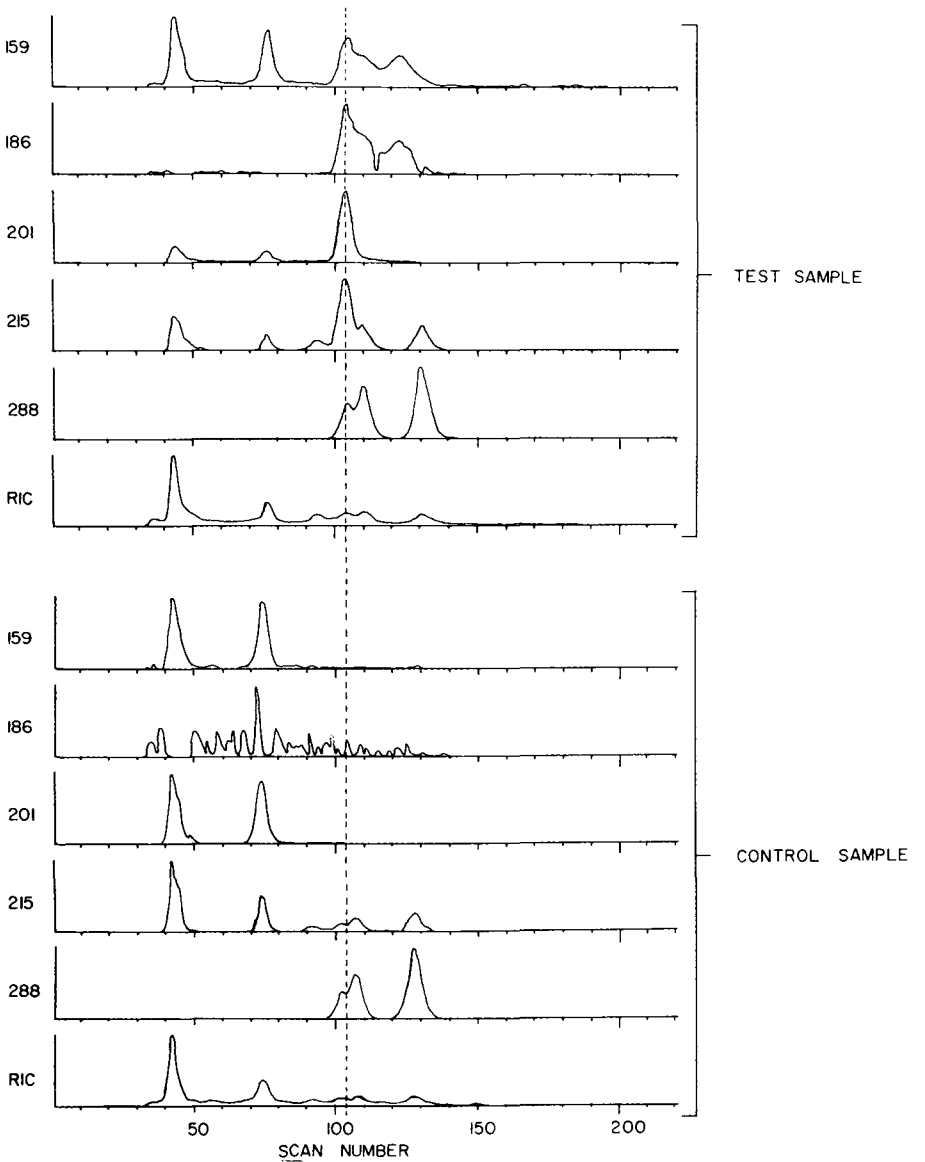


Fig. 4. Single-ion chromatograms of derivatized extracts of plasma samples. The upper 5 ion chromatograms were obtained from the derivatized extract of the plasma of a rat 4 h after it had received a 20 mg/kg i.v. dose of primaquine diphosphate. The lower 5 ion chromatograms were obtained from an animal that had not been given the drug. The dotted line represents the retention time obtained for an authentic standard of the methyl ester of III.

life of this rapid elimination would appear to be less than 1—2 min. During the 1—4.0 h period, the plasma level continues to fall with a half-life of 1.4—2.4 h, but the rate of decline appears to slow down after this period. The level of the metabolite (III) rises very rapidly (Fig. 5) and significant levels were observed 3.0 min following the injection (Fig. 2). From 2 to 4 h following

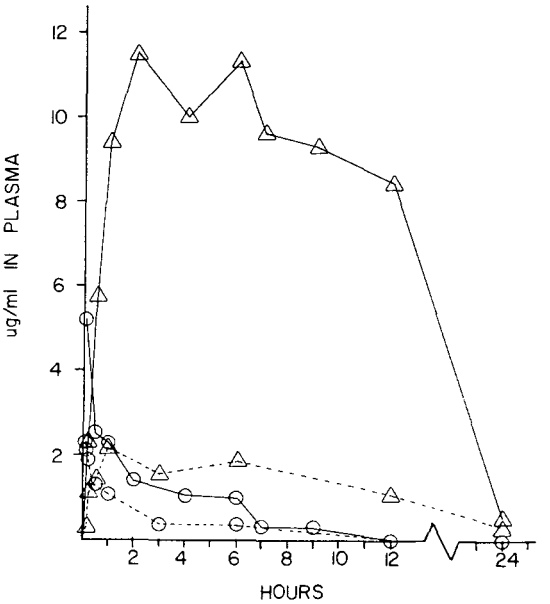


Fig. 5. Plasma concentration of primaquine diphosphate and III following a 20 mg/kg intravenous dose. ○, Primaquine diphosphate, △, III. The dotted lines were obtained using a second test animal.

the injection, the levels of III remained fairly constant and much higher than primaquine diphosphate. Even after 24 h, 0.2—0.5 μg/ml levels of the metabolite were still observed.

As can be seen in data for the two rats given the i.v. dose (Fig. 5), there was a considerable variation in the amount of III formed. More extensive pharmacokinetic studies with a larger number of animals are still in progress and will be reported at a later date. The data for the two animals shown in Fig. 5 represent the extremes of the data that have been observed to date.

In summary, for the first time in mammals, primaquine has been found to be metabolized to III and the concentration of this metabolite in the plasma is considerably higher than that of the parent drug except for the first few minutes after administration. In contrast to the metabolism by fungi, the N-acetyl derivative (II) was not found to be a metabolite of primaquine in rat. The HPLC method that was developed requires only 50 μl of whole blood for the analysis of primaquine and the metabolite. The low limit of detectability of III is about 0.05 μg/ml and this limit arises primarily because of a naturally occurring peak that elutes just before the metabolite.

ACKNOWLEDGEMENTS

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DETERMINATION OF THIABENDAZOLE AND 5-HYDROXYTHIABENDAZOLE IN HUMAN SERUM BY FLUORESCENCE-DETECTED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have developed a rapid, sensitive and precise high-performance liquid chromatographic method using fluorescence detection for the simultaneous determination of thiabendazole and unconjugated 5-hydroxythiabendazole in serum. Sample pretreatment consists only of protein precipitation with acetonitrile containing the internal standard, 2-methylindole. Detection limits were found to be 0.1 μg/ml serum for thiabendazole and 0.4 μg/ml serum for 5-hydroxythiabendazole. Between-day analytical precision coefficients of variation for serum-based controls were 7% and 11% for thiabendazole levels of 1 and 5 μg/ml, respectively; and 43% and 8% for 5-hydroxythiabendazole levels of 6 and 60 μg/ml, respectively. We also devised a microenzymatic method for the conversion of the glucuronide and sulfate esters of 5-hydroxythiabendazole using β-glucuronidase [EC 3.2.1.31] and sulfatase [EC 3.1.6.1]. Thus, quantitation of the separate metabolites was possible. We also utilized a special adaptation of the chromatographic procedure for the determination of the 5-hydroxythiabendazole metabolites in the sera of uremic patients, which can contain large amounts of interfering fluorescent substances. The method should be particularly useful for monitoring thiabendazole therapy in patients unable to eliminate the potentially toxic metabolites.

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INTRODUCTION

Thiabendazole, 2-(4-thiazoyl)-1H-benzimidazole (TBZ) is a broad-spectrum anthelmintic agent that is also extensively used as a fungicide for the post harvest protection of citrus fruits and bananas. Several high-performance liquid chromatographic (HPLC) methods for the quantitation of TBZ have recently been reported with regard to its use as a fungicide [1—4]. However, for two reasons, none of these methods are directly applicable to monitoring the therapeutic use of TBZ. First, they are not designed for the determination of TBZ in serum. Second and more importantly, they do not address the quantitation of the metabolites of TBZ. That monitoring of the metabolites' concentrations is important is evident from a recent study in which accumulation of the metabolites was linked with toxicity in a patient who was unable to eliminate the metabolites because of impaired renal function [5]. We report here the development of a simple and precise HPLC method for the determination of TBZ and 5-hydroxythiabendazole (5OHTBZ) in human serum, as well as the quantitation of the glucuronide and sulfate esters of 5OHTBZ following an in vitro enzymatic conversion to 5OHTBZ. We have successfully utilized the method in monitoring TBZ therapy and metabolite elimination in an anephric patient receiving hemodialysis and hemoperfusion [6].

EXPERIMENTAL

Chemicals

Purified TBZ and 5OHTBZ were kindly supplied by the Merck Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). Dibasic potassium phosphate, phosphoric acid (85%), sodium acetate and hydrochloric acid were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). β-Glucuronidase [EC 3.2.1.31] (Type B-1 from bovine liver), sulfatase [EC 3.1.6.1] (Type H-1 from Helix pomatia) and 2-methylindole were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol (both glass distilled) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

HPLC mobile phase preparation

Water for the mobile phase was passed through a Milli-Q Reagent Grade Water System (Millipore, Bedford, MA, U.S.A.) until a resistance of 16—18 MΩ cm or greater was achieved. Phosphate buffer, 0.01 mol/l was prepared from K_2HPO_4 and adjusted to pH 7.00 ± 0.05 with phosphoric acid. This phosphate buffer was stored up to four weeks at 4°C. Mobile phase was prepared daily by mixing phosphate buffer with methanol. Two mixtures were employed for these studies. The first was used for TBZ and 5OHTBZ determination in normal human sera and consisted of a mixture of buffer—methanol (50:50, v/v). The second was used specifically for 5OHTBZ quantitation in serum samples from a renally compromised patient, and consisted of a mixture of buffer—methanol (55:45, v/v). After mixing, the mobile phase was filtered and degassed through a Millipore GS membrane (0.22 μm, Millipore).

HPLC apparatus

A Waters Model 6000A solvent delivery system and Model U6K injector (Waters Assoc., Milford, MA, U.S.A.) were connected to a Waters precolumn (containing 37—50-μm Bondapak C_{18}/Corasil) and a 30 cm × 4 mm μBondapak C_{18} column, particle size 10 μm (Waters Assoc.). The Model FS970 L.C. Fluorometer (Schoeffel, Westwood, NJ, U.S.A.) fitted with a GM970 excitation monochromator, a Corning 7-54 excitation prefilter and emission cutoff filters was used. Output was monitored using an Omniscribe Recorder (Houston Instruments, Austin, TX, U.S.A.). Excitation was set at 305 nm, while emission was monitored using either a 370-nm or 470-nm cutoff filter. Fluorometer settings included a time constant of 6 sec and range setting of 0.2 to 1.0 μA, depending upon the size of the eluting peaks. The flow-rate was 1.0 ml/min.

Sample preparation

The internal standard (IS), 2-methylindole [2], was dissolved in acetonitrile at concentrations of 50 μg/ml (for an emission cutoff of > 370 nm) and 2000 μg/ml (for an emission cutoff of > 470 nm). These solutions were stored at 4°C. A 50-μl aliquot of patient serum or aqueous standard was pipetted into an 0.5-ml polypropylene micro test tube and 50 μl of acetonitrile—IS were then added. The mixture was vortexed to facilitate mixing and protein precipitation. The tubes were then centrifuged for 2—4 min at 7500 *g* (Fisher Centrifuge Model 59, Pittsburgh, PA, U.S.A.). A 10—20 μl aliquot of the supernatant was injected into the chromatographic system.

Preparation of controls and standards

TBZ and 5OHTBZ were added to optically clarified [7] pooled human sera to obtain two control pools; a high and a low pool spanning the expected range of concentrations for specimens from a dialysis patient [5] (high: 6 μg TBZ per ml, 60 μg 5OHTBZ per ml; low: 1 μg TBZ per ml, 5 μg 5OHTBZ per ml). These were aliquoted (50 μl) into micro test tubes (as described above for patient sera and standards), capped and frozen at −20°C. Each day, a high and low tube was thawed, to which 50 μl of acetonitrile—IS was added.

A series of aqueous standards, each containing TBZ and 5OHTBZ, were prepared from methanolic stock standards prepared gravimetrically at 100 μg TBZ per ml and 1000 μg 5OHTBZ per ml. The concentrations of the stock standards were then checked spectrophotometrically after dilution with methanol (TBZ) or 0.1 *N* hydrochloric acid (5OHTBZ) using the published extinction coefficients (TBZ: $\epsilon_{311\,nm}$ = 23,300 l/mol · cm; 5OHTBZ: $\epsilon_{318\,nm}$ = 17,794 l/mol · cm [8]) and a GCA/McPherson spectrophotometer (Acton, MA, U.S.A.). The spectrophotometrically determined concentrations were 99 μg TBZ per ml and 880 μg 5OHTBZ per ml (approximately consistent with 5OHTBZ being supplied as a monohydrate). Since recovery of both compounds from a serum matrix was virtually 100% (see Results and Discussion) the working standards were prepared in water. These aqueous standards were stored at 4°C and aliquoted fresh each day.

Quantitation of TBZ and 5OHTBZ

Only two standards were run routinely, the remaining standards being used to check the range of linearity (see Results and Discussion). Peak height was determined for TBZ and 5OHTBZ and the IS; and the ratios of peak heights, TBZ/IS and 5OHTBZ/IS, plotted vs. the spectrophotometrically determined concentrations. A linear least squares analysis was then performed using these two data points and the origin. The derived slope and intercept were used to calculate concentrations for patient or control samples.

Enzymatic degradation of the glucuronide and sulfate esters of 5OHTBZ

A modification of previously published procedures [8, 9] for the enzymatic hydrolysis of glucuronide and sulfate esters of 5OHTBZ was devised in order to minimize the dilution of sample in the reaction mixture. A mixture of β-glucuronidase and sulfatase was prepared by suspending 10 mg of β-glucuronidase (at 600,000 U/g) and 500 mg of sulfatase (21,000 U/g) in 0.1 mol/l sodium acetate, pH 5.0. A β-glucuronidase solution was also prepared by dissolving 10 mg of the enzyme in 5 ml of 0.1 mol/l sodium acetate, pH 5.0. Measurement of the 5OHTBZ peak following treatment with the combined enzyme mixture corresponded to the total 5OHTBZ metabolites (unconjugated 5OHTBZ + glucuronide ester + sulfate ester). Measurement of the 5OHTBZ peak following treatment with just β-glucuronidase yielded unconjugated 5OHTBZ + glucuronide ester. Measurement of the 5OHTBZ without any enzymatic treatment yielded the value for the unconjugated 5OHTBZ; therefore, appropriate subtraction of the various measurements gave separate values for each of the three metabolites. Sulfatase could not be used alone to measure the sulfate conjugate concentration since the Helix pomatia preparation also contains β-glucuronidase.

The conditions used for the enzymatic hydrolyses were as follows. A 50-μl aliquot of serum was pipetted into a micro test tube. To this were added 50 μl of 1.0 mol/l sodium acetate followed by 25 μl of either the combined enzyme mixture or β-glucuronidase preparations described above. The micro test tubes were capped and gently vortexed. They were then placed in a 37°C Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, IL, U.S.A.) for 18 h. Completion of the enzymatic conversion using the combined enzyme mixture was checked by noting the complete loss of conjugate peaks in a patient's serum. At the end of the incubation period the tubes were removed and stored at −20°C until analysis. At the time of analysis, the tubes were thawed, 100 μl of acetonitrile—IS were added and the mixture vortexed. These samples required centrifugation immediately prior to supernatant injection because of the ready re-suspension of fine particles. This procedure resulted in a dilution of the serum sample which was nominally 2.25 times greater than the dilution occurring with samples not taken through an enzymatic step. As a check on this dilution factor, control samples were taken through this procedure several times and found to have concentrations 1/2.32 lower than those not subjected to the procedure. Since this factor is close to that expected on the basis of the additional dilution with disodium acetate, enzyme and acetonitrile (1/2.25), we conclude that the volumes of mixing are negligible and that the concentration of unconjugated 5OHTBZ is unaffected by the treatment.

Thus, standards were not processed through this procedure. Instead, concentrations derived from the ordinary standard curve for specimens taken through an enzymatic conversion were multiplied by this dilution factor (2.32).

RESULTS AND DISCUSSION

Fig. 1a presents a typical chromatogram of the low control made from pooled human sera. This chromatogram was run using buffer—methanol (50:50), 50 μg IS per ml acetonitrile, and the 370-nm emission cutoff filter. Retention times were 5.5 min for 5OHTBZ, 10.5 min for TBZ and 13.5 min for IS. Peak-to-trough noise level was equivalent to 0.07 μg/ml TBZ and 0.2 μg/ml 5OHTBZ per ml serum after a 1:1 dilution with acetonitrile—IS, so the detection limits could be defined as approximately twice these levels. Fig. 1b illustrates serum from a uremic patient 1 h following the patient's first dose of TBZ (25 mg/kg orally). As is clear from Fig. 1b, large amounts of fluorescent substances present in the serum of our patient made it impossible to accurately quantitate

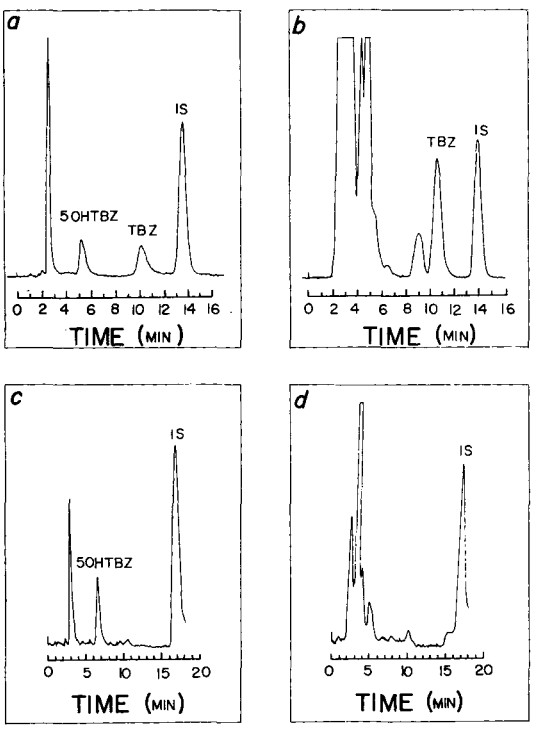

Fig. 1. Typical chromatograms of: (a) low serum control material, 1 μg TBZ per ml, 6 μg 5OHTBZ per ml, and (b) renal patient serum 1 h following an oral TBZ dose (25 mg/kg orally), TBZ concentration determined to be 5.0 μg/ml. Conditions: buffer—methanol (50:50) mobile phase, 50 μg IS per ml acetonitrile, 370-nm cutoff filter. (c) Low serum control material, 6 μg 5OHTBZ per ml, and (d) renal patient serum blank (i.e. before TBZ dosage) taken through the combined enzyme procedure described in the text. Conditions: buffer—methanol (55:45) mobile phase, 2000 μg IS per ml acetonitrile, 470-nm cutoff filter: range setting for IS is double that of the remaining chromatogram. Abbreviations: TBZ = thiabendazole; 5OHTBZ = 5-hydroxythiabendazole; IS = internal standard.

the level of 5OHTBZ. This is probably not unique to our patient: it is well known that uremic patients have significant amounts of fluorescent substances in their serum and urine [10—12]. This problem was solved by using the 470-nm cutoff filter which dramatically reduced the fluorescence from the endogenous interfering substances (reported in one study to have a fluorescence maximum emission at 430 nm following excitation at 342 nm [12]), TBZ and IS, but not that of 5OHTBZ (which has emission maxima at 425 nm and 525 nm following excitation at 325 nm [8]). In addition, a slight change in the mobile phase to a 55:45 mixture of buffer and methanol further improved the resolution of the 5OHTBZ peak in our patient serum. The IS concentration had to be increased to 2000 μg/ml for a suitable signal level using the 470-nm cutoff filter. Figs. 1c and d are chromatograms run under these modified conditions. Fig. 1c shows the low control material and Fig. 1d the same uremic patient's serum prior to any TBZ dose after having been subjected to combined enzyme treatment. It is clear that under these conditions, 5OHTBZ could be readily resolved and quantitated. Retention times under these conditions were 7.0 min for 5OHTBZ and 17 min for IS. Peak-to-trough noise level was equivalent to 1.7 μg 5OHTBZ per ml, the detection limit therefore being 3 μg 5OHTBZ per ml.

Typically then, specimens from the uremic patient were divided into eight 50-μl aliquots and frozen for later use. Two were used for duplicate TBZ quantitation under the first set of conditions described. Two each were used for 5OHTBZ quantitation under the second set of conditions, following either no enzymatic treatment, combined enzyme treatment or β-glucuronidase treatment.

The assays for both compounds were found to be linear over the ranges encompassed by the aqueous standards (0—7.4 μg TBZ per ml; 0—132 μg 5OHTBZ per ml; 0—88 μg 5OHTBZ per ml using the 470-nm cutoff filter). In order to present this wide range of peak heights, the range setting on the fluorometer was often varied within a single chromatogram. Peak heights were then multiplied by the range setting to normalize all heights to the same range setting. Table I presents the slope and intercept data obtained using all the standards. 2-Methylindole solutions were slightly unstable so that over a period of weeks, the IS peak would diminish, causing an elevated slope in the standard curve. However, this slow degradation of IS never presented a problem during a single day's run.

TABLE I

LINEARITY DATA FOR AQUEOUS STANDARDS

Analyte	Cutoff filter (nm)	Intercept (*a*)	Slope (*b*) (ml/μg)	*r*	*n*	IS—acetonitrile concn. (μg/ml)
TBZ	370	0.0199	0.197	0.991	8	50
5OHTBZ	370	0.0879	0.0584	0.998	8	50
5OHTBZ	470	−0.0288	0.194	0.997	7	2000

Recovery from a serum base after protein precipitation was determined by spiking pooled human sera volumetrically to concentrations equal to those of the aqueous standards using the stock TBZ and 5OHTBZ solutions. Absolute recovery was determined by comparing peak heights for identical injection volumes of aqueous standards and serum specimens having the same concentration. These recoveries (%R = 100 × serum peak height/aqueous standard peak height, 7 concentrations) were found to average 103 ± 13% (S.D.) for 5OHTBZ and 108 ± 5% for TBZ. Relative recoveries were determined by measuring concentrations from the standard curve. These recoveries (%R = 100 × measured serum concentration/volumetrically spiked concentration, 7 concentrations) were found to average 91 ± 12% (S.D.) and 104 ± 18% for TBZ and 5OHTBZ, respectively. Thus, if the serum base materials were used as standards, similar slope and intercept parameters would be obtained (TBZ: $Y = 0.026 + 0.20X$, $r = 0.986$; 5OHTBZ: $Y = -0.014 + 0.056X$, $r = 0.996$: 370 nm cutoff filter; compare with parameters in Table I). Therefore, aqueous standards could be used throughout the study.

Table II presents precision data obtained for within-run and between-run studies. All the precision data were judged to be acceptable for clinical utility. The two instances of high coefficients of variation (43% and 19% for 5OHTBZ at 6 μg/ml) are of no great concern clinically because of the low 5OHTBZ concentration: precision is significantly better at the more important higher levels where toxicity may result. The low and high controls were also taken through the enzymatic conversion steps, but precision for 5OHTBZ was not significantly affected by these steps. We obtained coefficients of variation of 11.7% and 4.6% (n = 5) for the low and high 5OHTBZ controls, respectively.

Table III presents those drugs which were tested and found not to interfere with these assays. All were checked at concentrations greater than expected to

TABLE II

ANALYTICAL PRECISION PARAMETERS FOR SERUM CONTROL MATERIALS

Control	Analyte	$\overline{X}$ (μg/ml)	S.D. (μg/ml)	C.V. (%)	n	Cutoff filter (nm)
Within-run precision						
Low	TBZ	1.0	0.027	2.7	20	370
	5OHTBZ	4.5	0.14	3.1	20	370
High	TBZ	4.1	0.077	1.9	21	370
	5OHTBZ	53	1.2	2.2	21	370
Between-run precision						
Low	TBZ	1.1	0.083	7.3	14	370
	5OHTBZ	5.9	2.6	43	14	370
	5OHTBZ*	5.6	1.1	19	13	470
High	TBZ	4.9	0.55	11	16	370
	5OHTBZ	57	4.5	7.9	14	370
	5OHTBZ*	57	2.7	4.7	13	470

*These samples were not taken through the enzymatic conversion steps.

TABLE III

SUBSTANCES CHECKED FOR INTERFERENCES

Propranolol	Lidocaine
Quinidine	Guanethidine
Procainamide	Disopyramide
N-Acetylprocainamide	Diazepam
Prazosin	Benztropine
Minoxidil	Caffeine
Phenytoin	Chlorothiazide
Phenobarbital	Acetaminophen
Carbamazepine	Theophylline
α-Methyldopa	Nordiazepam
Furosemide	Propoxyphene
Hydralazine	Salicylic acid

be observed in clinical specimens. This does not rule out the possibility that metabolites of these drugs may interfere with these assays.

In summary, we have devised a method for the simultaneous quantitation of TBZ and 5OHTBZ which is simple and precise enough for clinical monitoring of TBZ therapy. A micro method for the enzymatic conversion of the glucuronide and sulfate esters of 5OHTBZ was developed to obtain estimates of the various metabolite fractions. Since monitoring would be most appropriate for patients unable to eliminate the potentially toxic metabolites, we also have devised a modified method which should eliminate interferences from endogenous fluorescent substances which can be expected to be present in the sera of uremic patients.

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CHROMBIO. 1238

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF INDALPINE, A NEW NON-TRICYCLIC ANTIDEPRESSANT, IN HUMAN PLASMA

IDENTIFICATION AND SIMULTANEOUS MEASUREMENT OF ITS MAJOR PLASMA METABOLITE

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SUMMARY

Indalpine or 4-[2-(3-indolyl)ethyl]piperidine, a selective inhibitor of 5-hydroxytryptamine uptake in central monoamine neurons, has proved to be an effective agent in the treatment of chronically ill depressed patients. We have developed a rapid high-performance liquid chromatographic method for the simultaneous determination of indalpine and its major metabolite in human plasma. Isolation by high-performance liquid chromatography and identification of this metabolite by mass spectrometry are also described.

INTRODUCTION

Indalpine (LM 5008), or 4-[2-(3-indolyl)ethyl]piperidine, is a potent and specific inhibitor of 5-hydroxytryptamine uptake, active orally as an antidepressant agent [1—4]. In order to correlate human doses with pharmacokinetic parameters a method for plasma determination has been improved, based on the fact that indalpine can be detected with suitable sensitivity by fluorescence. In the course of preliminary investigation on plasma from volunteers receiving a single dose of indalpine, a metabolite was detected in relatively high quantities. Thus, a procedure for isolation and identification of this metabolite was imperative in order to measure simultaneously its plasma concentrations and to compare its activity to that of indalpine.

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EXPERIMENTAL

Materials

Chromatography was performed on a component system consisting of a Waters Assoc. Model 6000A delivery system, Model U6K injector, Model 440 dual channel absorbance detection operated at 254 nm, and a Schoeffel FS 970 fluorescence LC detector. The outputs of the detectors were connected to a 10-mV linear recorder B 5000 Omniscribe (Houston Instruments). A μBondapak C_{18} (10 μm particle size) column (30 cm × 3.9 mm I.D.) was used with a Bondapak C_{18}/Corasil guard column (2 cm × 3 mm I.D.) in series (Waters Assoc.).

Thin-layer chromatography (TLC) was carried out on HP-KF Whatman precoated plates (200 μm) prewashed with methanol. Radioactive compounds were located using a TLC scanner. Low-resolution electron impact/chemical ionization (EI/CI) mass spectra were obtained using a VG Micromass Model 70-70F double focusing mass spectrometer operating at 70 eV. Both direct insertion probe and combined gas chromatography—mass spectrometry (GC—MS) were used for identifying the metabolite. A Hewlett-Packard gas chromatograph (Model 5710A) was equipped with an OV-101 coated glass capillary column (20 m × 2.5 mm I.D.). High-resolution data were obtained by the peak-matching method using the same instrument operating at a resolving power of 10,000 with heptacosaperfluorotributylamine as the reference compound. The elemental composition of the molecular ion for which accurate mass measurement was obtained was determined using computer program.

Reagents and chemicals

Methanol and methylene chloride were fluorometry grade (Merck, Darmstadt, G.F.R.). Acetic acid, dipotassium hydrogen phosphate, sodium hydroxide, and ammonia were analytical grade (Merck). Reagents were used without further purification; water was doubly distilled in glass. (Indole-2-^{14}C]-indalpine (specific activity 91.3 μCi/mg) and [^{3}H]indalpine (15.35 mCi/mg) were prepared at the Radiochemical Centre, Amersham, Great Britain. Indalpine, PK 10157 or 4[2-(3-indolyl)ethyl]-2-piperidinone, and derivatives were synthesized in our chemical department.

Internal standard

Another indole-4-piperidine derivative, 4[(5-methoxy-3-indolyl)methyl]-piperidine (PK 26042), structurally related to indalpine, was used as internal standard. Stock solution of PK 26042 (10 mg/l) was prepared in methanol and stored in glass at 4°C. Dilutions were made to bring the final volume added to the sample to 100 ng per 100 μl.

Extraction procedure

To a 15-ml glass stoppered centrifuge tube were added 2 ml of plasma, 100 μl of the internal standard solution and 0.2 ml of 5 *N* sodium hydroxide. The mixture was vortexed for 1 min and 4 ml of dichloromethane were added. The tube was sealed and shaken for 15 min. After centrifugation (3000 *g*, 10 min) the organic layer was transferred to a second tube and the extraction

was repeated under the same conditions.

The organic layers were pooled and evaporated to dryness at 38°C with a nitrogen stream. The residue was dissolved in 50 μl of mobile phase, Vortex mixed and centrifuged at 3000 *g* for 5 min.

Chromatography analysis

Ten microlitres of the extract were chromatographed (Fig. 1) on a μBondapak C_{18} column. Components were eluted isocratically at a flow-rate of 1 ml/min with a mobile phase consisting of methanol—0.01 *M* aqueous K_2HPO_4—acetic acid (50:50:1, v/v). The solution was degasified before use in an ultrasonic bath. Detection was carried out with a Schoeffel FS 970 fluorescence LC detector with the excitation monochromator set at 220 nm and emission filter cut-off at 370 nm.

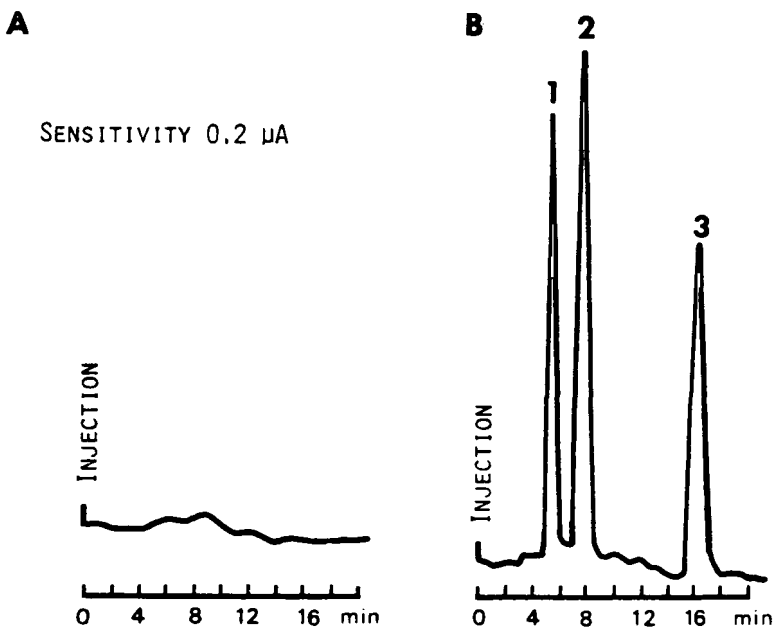

Fig. 1. (A) Chromatogram of extract of blank human plasma sample (2 ml). (B) Chromatogram of plasma sample extract 2 h after a single oral dose of 25 mg of indalpine. 1 = Internal standard; 2 = indalpine; 3 = metabolite. Chromatographic conditions are as given in the text.

RESULTS

Calibration curve

Known quantities of indalpine and PK 10157 (dissolved in 50 μl of methanol) ranging from 5 to 200 ng were added to 2 ml of blank plasma containing internal standard. The samples were extracted and chromatographed as outlined above. Calibration curves were constructed by calculating the ratio of the peak height of each compound to that of the internal standard and plotting the ratio

against the amount of compound added to the sample. The curves were linear for both indalpine, $Y_1 = 0.012X_1 + 0.023$ ($r = 0.998$), and its metabolite $Y_2 = 0.112X_2 - 0.008$ ($r = 0.998$).

Recovery

To estimate the recovery of indalpine a stock solution in methanol (65 pCi/μl) of [^{3}H]indalpine obtained by reduction with tritium gas of 4[2-(3-indolyl)-ethyl]pyridine was prepared. Ten microlitres of this solution, which correspond to 5 ng of indalpine, were added to 2 ml of plasma and extracted with dichloromethane in the same way as described above: 80.4 ± 3.6% (mean ± S.D., $n = 5$) of the radioactivity was recovered.

Analytical recovery of the compounds was also measured by comparing the peak heights of analyzed samples containing known amounts of indalpine, its metabolite and the internal standard to the respective peak height obtained by injecting equal amounts directly into the chromatograph. The recovery of all compounds from plasma was 75—80% when approximately 90% of the dichloromethane phase was available for evaporation. The recovery from plasma samples spiked with these compounds in the concentration range 25—100 ng was 75 ± 3.7% for internal standard, 79.5 ± 3.5% for indalpine and 80.2 ± 3.2% for PK 10157 (mean ± S.D., $n = 5$).

Accuracy

An accuracy study of plasma samples spiked with 5, 25 and 50 ng/ml indalpine and PK 10157 is reported in Table I. The coefficient of variation (C.V.) ranged from 9.9 to 2.7% for indalpine and 9.8 to 2.3% for PK 10157.

TABLE I

ACCURACY AND PRECISION TEST

Five assays in each case.

Indalpine			PK 10157		
Added (ng/ml)	Recovered (ng/ml)	C.V.(%)	Added (ng/ml)	Recovered (ng/ml)	C.V.(%)
5	4.5	9.9	5	4.7	9.8
25	24.7	3.5	25	24.4	3.7
50	49.8	2.7	50	49.9	2.3

Application of the method

Ten healthy volunteers received a single oral dose of 25 mg of indalpine. Venous blood samples were collected in heparinized bottles at 15, 30, 60, 120, 180 and 360 min after administration. The blood was centrifuged immediately and plasma was stored at −20°C until analysis. Plasma concentration—time curves for indalpine and its metabolite determined by the above procedure are shown in Fig. 2.

Indalpine and PK 10157 concentrations increased progressively in all subjects during the first hour following tablet ingestion, with a maximum value between 90 and 120 min. Mean peak concentration at 120 min was 44.5 ±

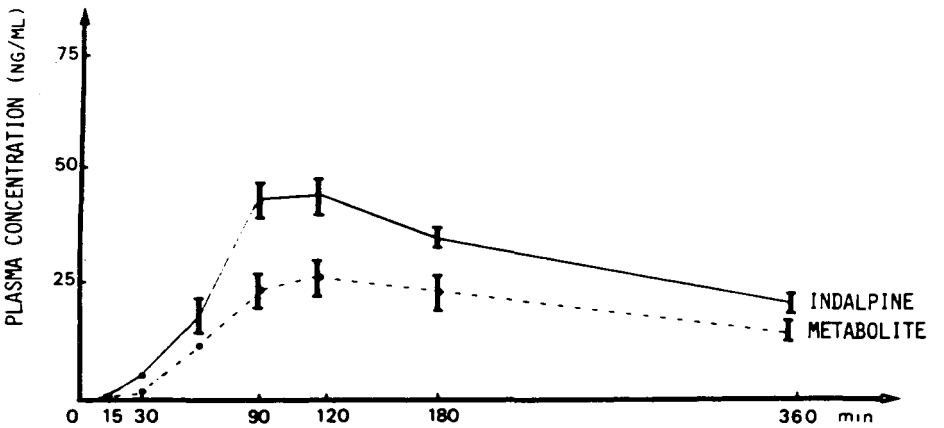

Fig. 2. Plasma concentrations of indalpine and its metabolite after oral administration of 25 mg of indalpine to ten subjects (mean ± S.E.M.).

3.0 (S.E.M.) ng/ml for indalpine and 23.3 ± 3.8 (S.E.M.) ng/ml for its metabolite. Six hours after ingestion mean plasma levels were 21.8 ± 2.4 (S.E.M.) ng/ml for indalpine and 14.6 ± 1.7 (S.E.M.) ng/ml for PK 10157. These results suggest that the clearance time is slower than the absorption time for indalpine and show similar plasma profile curves for indalpine and its metabolite.

Isolation and purification of the metabolite

Ninety millilitres of selected plasma samples were adjusted to pH 13 with 5 *N* sodium hydroxide and extracted with 360 ml of dichloromethane. The organic phase was evaporated to dryness and reconstituted in methanol (100 µl). This extract was injected into a µBondapak C_{18} column (60 cm × 3.9 mm I.D.) (Fig. 3) under the chromatographic conditions described above. The fractions isolated by HPLC were re-extracted with dichloromethane from the alkalinized mobile phase, concentrated in methanol and spotted on a silica gel TLC plate. The mobile phase used was chloroform—methanol—ammonia (75:23:2, v/v). Localization of the metabolite was made by examining the plate under UV light at 254 nm and by reference with the R_F of an identical ^{14}C-labelled metabolite obtained from a plasma extract of a monkey fed with [^{14}C]indalpine. The area of the silica gel TLC plate corresponding to the metabolite was then scraped off and extracted with methanol; a second purification using HPLC achieved good purification of the metabolite (Fig. 4).

Identification of the metabolite

EI 70-eV low-resolution mass spectra of the purified metabolite obtained by direct insertion probe or GC—MS show an abundant molecular ion at *m/z* 242 and a characteristic quinolinium fragment ion at *m/z* 130 (Fig. 5A).

CI low-resolution mass spectra obtained using ammonia or isobutane as reagent gas confirm the value determined for the molecular mass. Precise mass measurement of molecular ion by EI high-resolution mass spectrometry gives an elemental composition of $C_{15}H_{18}N_2O$, which shows an oxidation process of indalpine.

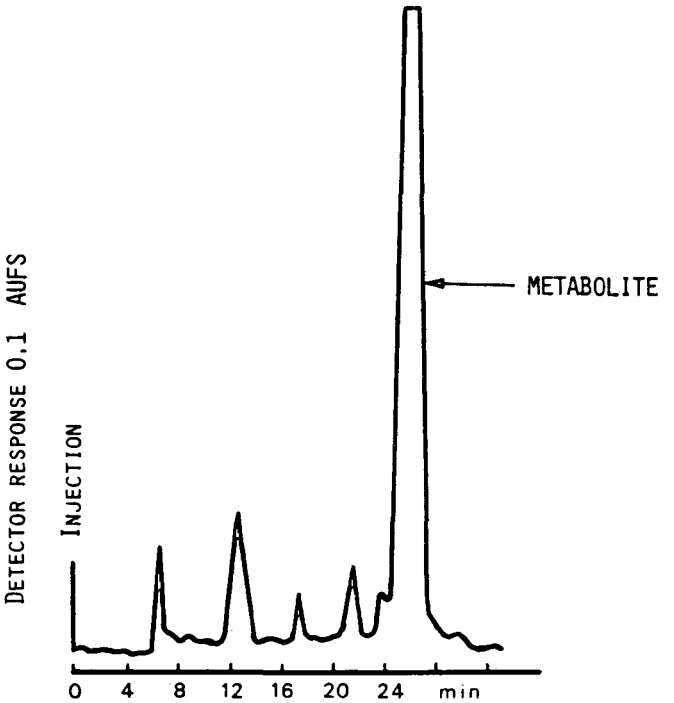

Fig. 3. Chromatogram of a reversed-phase separation of 90 ml of plasma extract using a UV detector at 254 nm.

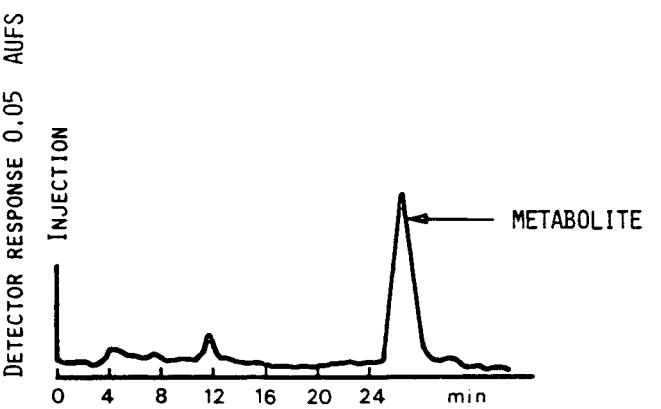

Fig. 4. Chromatogram of the purified metabolite.

Determination of the structure can be made by comparison of these results with EI mass spectra of indalpine, 4[2-(1-methyl-3-indolyl)ethyl] piperidine and 4[2-(5-methoxy-3-indolyl)ethyl] piperidine reproduced in Figs. 6—8. These spectra show abundant characteristic ions at *m/z* 85, 84 and 82, which arise from piperidine ring loss of these fragments in the metabolite spectra, suggesting oxidation of piperidine to lactam, which agrees with the observed physical properties and mass spectra results.

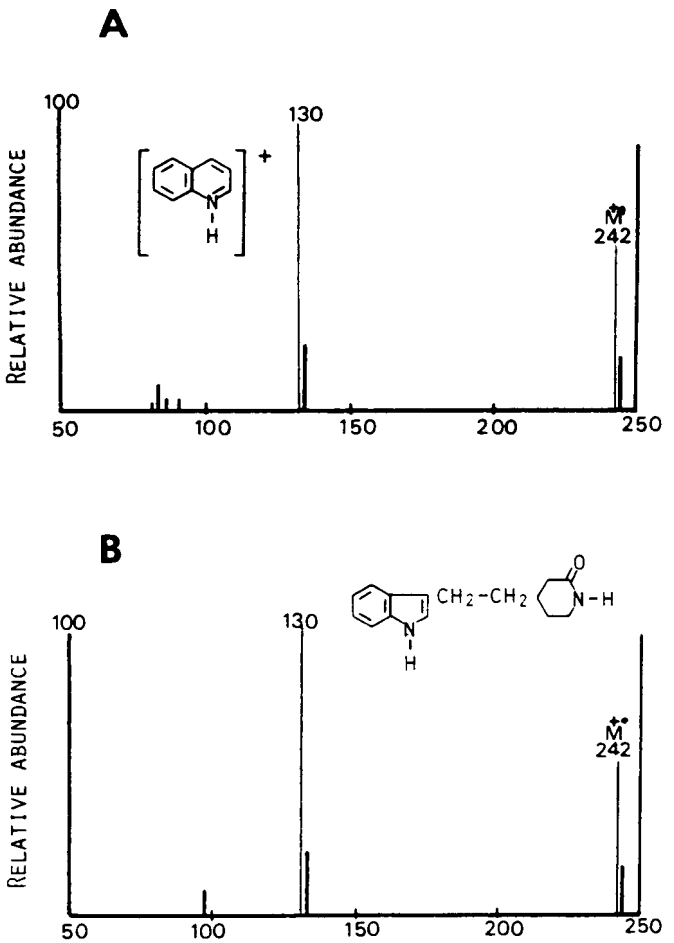

Fig. 5. (A) EI mass spectrum of metabolite isolated from plasma with chromatographic inlet. (B) EI mass spectrum of 4[2-(3-indolyl)ethyl]-2-piperidinone.

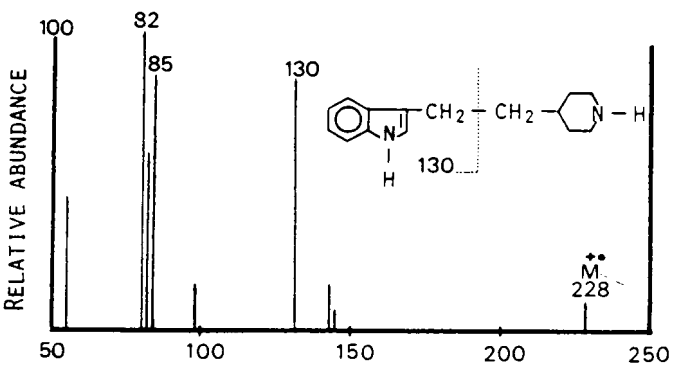

Fig. 6. EI mass spectrum of indalpine.

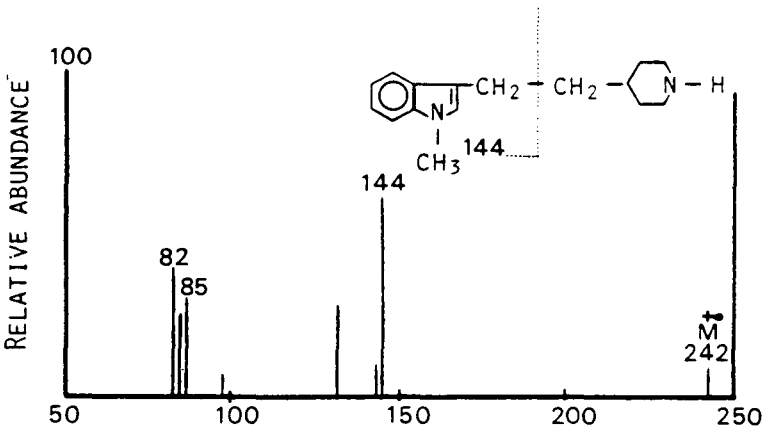

Fig. 7. EI mass spectrum of 4-[2-(1-methyl-3-indolyl)ethyl]piperidine.

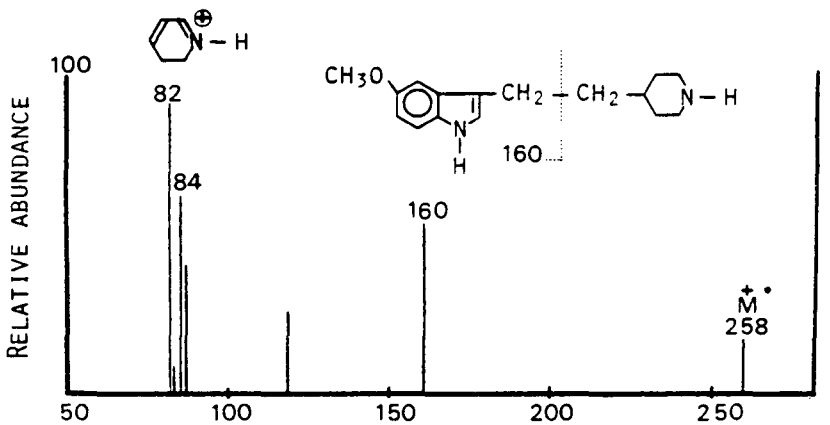

Fig. 8. EI mass spectrum of 4-[2-(5-methoxy-3-indolyl)ethyl]piperidine.

Verification

Corroborative evidence for the proposed structure was provided by synthesis of the lactam. Results obtained with HPLC and TLC using the chromatographic conditions described above show identical retention times for the metabolite and 4[2-(3-indolyl)ethyl]-2-piperidinone (PK 10157) chemically synthesized. The mass spectra were identical (Fig. 5A and B).

DISCUSSION

The extraction procedure described yields a clean extract (Fig. 1A) allowing measurement of indalpine and PK 10157 at the highest detector sensitivity. Under these conditions the lower limit of indalpine detection in plasma is about 2 ng/ml. This sensitivity fulfils the requirement for a pharmacokinetic study of indalpine and its metabolite at lower therapeutic doses (25 mg).

Lactam metabolite detected in human plasma was also present in plasma of other species such as rat, rabbit, guinea pig and monkey. Thus the biotransformation of indalpine into 4[2-(3-indolyl)ethyl]-2-piperidinone seems a general and important metabolic pathway for this drug. The biotransformation may be described by a two-step process [5], giving first the 2-hydroxypiperidine derivative, then the lactam derivative.

The kinetic curves observed for indalpine and its metabolite show that indalpine is rapidly metabolized into 4[2-(3-indolyl)ethyl]-2-piperidinone and that the metabolite level is a function of its parent drug level in the blood. This relationship led to investigations into the biochemical and pharmacological properties of PK 10157 which revealed that the lactam derivative is inactive in antidepressant tests.

ACKNOWLEDGEMENTS

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CHROMBIO. 1247

DETERMINATION OF MEXILETINE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of the antiarrhythmic drug mexiletine in human plasma, urine, and cerebrospinal fluid is described. Following extraction with diethyl ether, mexiletine and the internal standard 4-methylmexiletine were derivatized with 2,4-dinitrofluorobenzene. Analyses were performed using an alternating on-column enrichment technique on small Perisorb RP-2 30—40 μm pre-columns with pre-column backflushing for direct injection onto the analytical column of Spherisorb ODS 5 μm. Complete separation from endogenous constituents of plasma, urine or cerebrospinal fluid was achieved by isocratic reversed-phase ion-pair chromatography with 1-heptanesulfonic acid (0.005 *M*; PIC B7)—acetonitrile—tetrahydrofuran (42:48:10, v/v) as eluent. Interferences from other drugs were not observed. The limit of detection was 10 ng/ml (C.V. 6.5%). Day-to-day coefficients of variation were below 9%. The applicability of this rapid method for pharmacokinetic studies and clinical routine is demonstrated.

INTRODUCTION

Mexiletine (Mexitil^{R}) is an antiarrhythmic drug recently introduced for therapy and prophylaxis of ventricular arrhythmias [1—5]. Due to its oral efficacy and long plasma half-life (9—16 h) mexiletine compares favorably with currently available antiarrhythmic drugs such as lidocaine [6]. Intravenous or oral administration of mexiletine is followed by slow but extensive biotransformation to inactive metabolites [7, 8]. Therapeutic plasma concentrations of mexiletine have been reported to be 0.5—2.0 μg/ml, whereas toxicity becomes common above 3.0 μg/ml [9, 10]. Since the therapeutic to toxic effect ratio is low and the pharmacokinetics of mexiletine are highly dependent on the

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patient's condition, individualization of drug dosage is important [11, 12]. Therefore, routine monitoring of plasma levels of mexiletine will be helpful to obtain effective arrhythmia suppression without toxicity.

For the determination of mexiletine in biological fluids a number of gas chromatographic methods have been described which require flame ionisation detection [13—17], electron-capture detection [18—20], or nitrogen-selective detection [21—24]. However, only three of these methods [18, 19, 24] are sufficiently sensitive to quantitate mexiletine concentrations below 100 ng/ml. To our knowledge, there are no other published assays for the determination of mexiletine. We describe here a sensitive method for the rapid analysis of mexiletine by high-performance liquid chromatography (HPLC) following derivatization with dinitrofluorobenzene. Its applicability for drug monitoring and pharmacokinetic studies in plasma, urine, and cerebrospinal fluid is demonstrated.

EXPERIMENTAL

Materials

Mexiletine [1-(2,6-dimethylphenoxy)-2-aminopropane], the metabolites 4-hydroxymexiletine [1-(2,6-dimethyl-4-hydroxyphenoxy)-2-aminopropane] and 2-hydroxymethylmexiletine [1-(2-hydroxymethyl-6-methylphenoxy)-2-aminopropane], and the internal standard 4-methylmexiletine [1-(2,4,6-trimethylphenoxy)-2-aminopropane] were obtained from Boehringer Ingelheim (Ingelheim, G.F.R.). 2,4-Dinitrofluorobenzene (purity approx. 98%; Sigma, St. Louis, MO, U.S.A.) was used without further purification. Tests for interfering peaks were performed with plasma samples spiked with 10 μg/ml of the following drugs: lidocaine (Xylocain^R Astra), bretylium tosylate (Bretylol^R American Critical Care), phenytoin (Phenhydan^R Desitin), disopyramide (Rythmodul^R Albert-Roussel), propafenone (Rytmonorm^R Knoll), aprindine (Amidonal^R Madaus), chinidine (Chinidin-Duriles^R Astra), ajmalin (Giluryt-mal^R Giulini-Pharma), verapamil (Isoptin^R Knoll), propranolol (Dociton^R ICI-Pharma), acebutolol (Prent^R Bayer), metoprolol (Beloc^R Astra), pindolol (Visken^R Sandoz), dihydralazine (Nepresol^R Lappe), methyldopa (Presinol^R Bayer), dopamine (Cardiosteril^R Fresenius), dobutamine (Dobutrex^R Lilly), furosemide (Lasix^R Hoechst), nitroglycerin (Trinitrosan^R Merck), aminophyllin (Euphyllin^R Byk Gulden), morphine (Amphiole^R Merck), meperidine (Dolantin^R Hoechst), pentazocine (Fortral^R Winthrop), diazepam (Diazepam-ratiopharm^R Ratiopharm), and cimetidine (Tagamet^R SK Dauelsberg). All other substances were of analytical-reagent grade and were used without further purification.

Sample preparation

For routine monitoring samples of 0.5—1 ml blood were collected in tubes containing 50 units of heparin (10 μl of Thrombophob^R Nordmark, Hamburg, G.F.R.). After centrifugation at 8000 *g* for 5 min (Microfuge 5412; Eppendorf, Hamburg, G.F.R.), plasma samples of 0.2—0.4 ml were transferred to 15-ml capacity PTFE-lined screw-cap liquid scintillation vials (Greiner, Nürtingen, G.F.R.), followed by the addition of 1.2 or 1.0 ml of water, 0.2 ml of internal

standard (2 μg of 4-methylmexiletine), 0.5 ml of 1 *N* sodium hydroxide, and 5 ml of diethyl ether. Each sample was shaken for 1 min and dispersed for 3 min in an ultrasound bath. The organic and aqueous phases were separated by freezing in a dry-ice—acetone bath. The upper organic phase was poured into a second liquid scintillation vial, and evaporated at 60°C. Ether extraction was repeated two times. For derivatization 2.5 ml of 2.5% disodium tetraborate and 0.15 ml of 4% (w/v) dinitrofluorobenzene in dioxane were added to the vials, which were then sealed, shaken for 15 sec and held at 60°C for 20 min. The samples were found to be stable at room temperature for at least 1 h. Within this time 2 ml of each sample were injected directly into the chromatograph without further pre-clean-up procedures.

For pharmacokinetic studies at mexiletine concentrations expected to be below 50 ng/ml, ether extraction was performed with plasma volumes greater than 2 ml.

Urine samples (undiluted) and cerebrospinal fluid were treated in the same way. All native samples and evaporated ether extracts that were not analyzed immediately, were sealed and stored frozen at −16°C.

Chromatography

For enrichment of dinitrobenzene-mexiletine and dinitrobenzene-methylmexiletine an alternating pre-column switching technique was used similar to a method reported recently [25]. By the use of a column switching module Gynkotek SE-2 (Gynkotek, Munich, G.F.R.) two pre-columns were alternatingly connected to a Model 7125 sample injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 2-ml sample loop, and an HPLC pump (Model FR-4S,

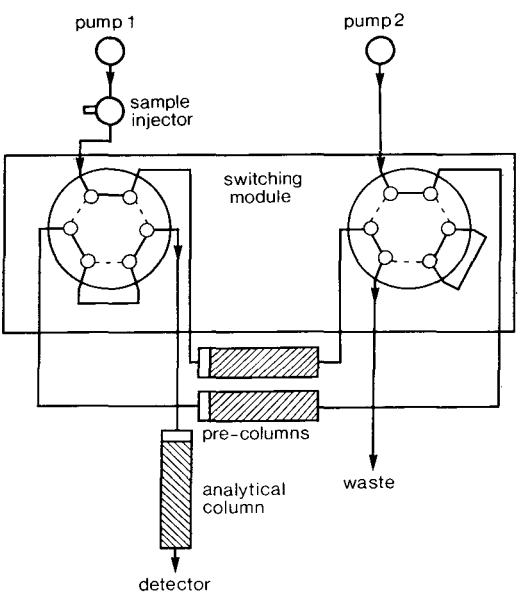

Fig. 1. Scheme of alternating pre-column switching technique for sample enrichment on the pre-column(s) and backflush elution to the analytical column. Further details are described in the text.

Spectra-Physics, Darmstadt, G.F.R.). As illustrated in Fig. 1, sample enrichment on the first pre-column and backflush elution from the second pre-column to the analytical column occur simultaneously. For backflush elution and chromatography on the analytical column a second HPLC pump (Model 600/200, Gynkotek) and a Uvikon 720 LC variable-wavelength ultraviolet detector (Kontron, Eching, G.F.R.) were used.

The pre-columns (5 cm × 4 mm I.D.) were dry-packed with Perisorb^R RP-2 30—40 μm (Merck, Darmstadt, G.F.R.). The analytical column (30 cm × 4 mm I.D.) was packed by forcing a slurry of 4 g of Spherisorb^R ODS 5 μm suspended in 40 ml of carbon tetrachloride into the stainless-steel tube under a pressure of 400 bars by means of a type MS 80/8 high-pressure membrane pump (Orlita, Giessen, G.F.R.). The pre-columns were routinely discarded after 200 injections of 2-ml samples.

The sample enrichment on the pre-column was operated by pump 1 (Fig. 1) with water as mobile phase at a flow-rate of 2 ml/min and a pressure of 20 bars at room temperature. The water-soluble co-products of the samples were eluted for 6 min into the waste. Then the pre-column with the enriched sample was switched to the eluent stream of pump 2, and the sample was directly administered onto the analytical column by the backflush mode. Parallel to this process, the other pre-column was switched to the water stream of pump 1, and 3 min later the next 2-ml sample was injected into the chromatographic system. For chromatography on the analytical column a ternary system of 1-heptanesulfonic acid (0.005 *M*; PIC B7, Waters, Königstein/Ts, G.F.R.)—acetonitrile—tetrahydrofuran (42:48:10, v/v) was used as eluent. The flow-rate was 2.4 ml/min at a back pressure of 230 bars at room temperature. Detection was set at 0.015 a.u.f.s. and at 352 nm, the absorption maximum of dinitrobenzene-mexiletine in the eluent.

For quantitation the areas under the curves were computed by an SP4100 computing integrator (Spectra-Physics). Calibration was performed by the method of internal standardization.

RESULTS AND DISCUSSION

Ether extraction from plasma at a concentration of 0.2—5 μg yielded a cumulative recovery of 46—69% for mexiletine, and of 49—74% for 4-methylmexiletine.

Since the molar extinction coefficient of mexiletine at λ_{max} (260 nm) was only 255 in 0.01 *N* HCl (d = 1 cm), mexiletine was not detectable in our chromatographic system at concentrations below 50 μg/ml. Therefore, the primary amino groups of mexiletine and 4-methylmexiletine were coupled with the chromophore 2,4-dinitrofluorobenzene, similar to methods described for the analysis of cyclohexylamine in cyclamates [26, 27]. Derivatization of mexiletine and 4-methylmexiletine was optimal and sufficiently reproducible at 60°C using dinitrofluorobenzene in a final concentration of 0.24%. The reaction was completed within 20 min. The dinitrobenzene derivatives were found to be stable for 60 min when stored at room temperature.

Dinitrobenzene-mexiletine and dinitrobenzene-4-methylmexiletine were selectively enriched from 2-ml reaction samples on RP-2 pre-columns using

water for elution of the excess of dinitrofluorobenzene and of other water-soluble co-products. The purge phase with simultaneous sample enrichment on the top of the pre-column was terminated at 6 min by automatic switching of this pre-column to the solvent stream of the second pump, thus delivering the ternary eluent, necessary for separation and chromatography, in the backflush mode from this pre-column to the analytical column. Parallel to this process, the other pre-column is switched to the water eluent of the first pump. After an equilibration phase of 3 min this pre-column was loaded with a further 2-ml sample. Sample injection was done manually, but automatic sample injection is possible.

Optimal resolution of dinitrobenzene-mexiletine and dinitrobenzene-4-methylmexiletine from endogenous constituents of plasma, urine, and cerebrospinal fluid was obtained by ion-pair chromatography with 1-heptanesulfonic acid using acetonitrile and tetrahydrofuran as organic modifier for the reversed-phase mode of partition. The addition of tetrahydrofuran improved solvent selectivity and provided sharp, symmetrical and well-defined peaks of derivatized mexiletine and internal standard. Of the several available reversed-phase materials, Spherisorb ODS, 5 μm, was the best stationary phase with respect to efficiency, permeability and stability. Therefore, chromatography was performed on a Spherisorb ODS column (30 cm $\times$ 4 mm I.D.) with 1-heptanesulfonic acid (0.005 *M*; PIC B7, Waters)—acetonitrile—tetrahydrofuran (42:48:10, v/v) as eluent (2.4 ml/min). In this chromatographic system dinitrobenzene-mexiletine was eluted at 7.5 min and the internal standard (1 μg/ml) at 10 min. The dinitrobenzene derivative of the mexiletine metabolite 4-hydroxymexiletine was eluted at 3 min. Its detection was usually disturbed by endogenous constituents of plasma. The dinitrobenzene derivative of the other mexiletine metabolite 2-hydroxymethylmexiletine, however, was eluted later than 20 min, and was not regarded for analysis. Typical chromatograms of dinitrobenzene-mexiletine and internal standard in plasma, urine, and cerebrospinal fluid are shown in Fig. 2. Constituents of plasma (Fig. 2A—C), urine (Fig. 2D and E) or cerebrospinal fluid (Fig. 2F and G) did not interfere with the resolution of either compound.

Separation was not disturbed by 25 other drugs (see Materials) tested for possible interference. No interfering peaks were found in plasma of patients who were treated with the following drugs: lidocaine, acebutolol, dihydralazine, dopamine, dobutamine, hydrochlorothiazide, triamteren, furosemide, nitroglycerin, isosorbide dinitrate, aminophyllin, pentazocine, diazepam, cimetidine, digoxin, heparin, acetyl salicylic acid, dipyridamole, sulfinpyrazone, allopurinol, and bezafibrate. Twenty-five drug-free blood samples obtained from healthy volunteers provided no evidence that normal components of plasma interfered with the determination of mexiletine and 4-methylmexiletine.

Identification of the mexiletine peak was made by its retention time relative to the retention time of the internal standard. The variation was less than 1%, as demonstrated from day to day with plasma standards.

Quantitation was performed using the method of internal standardization.

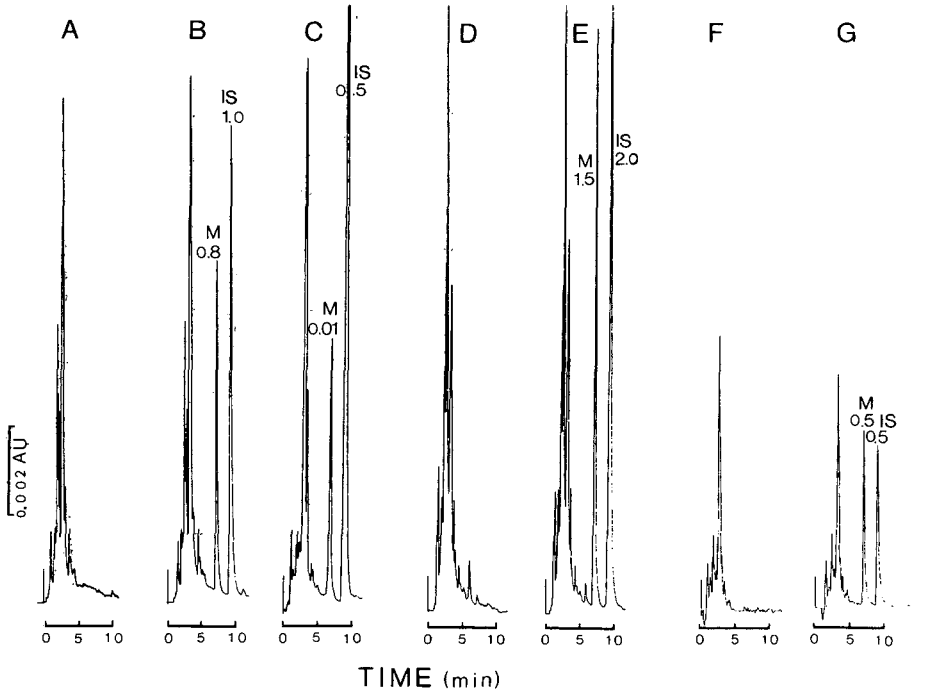

Fig. 2. High-performance liquid chromatograms of mexiletine (M) and the internal standard 4-methylmexiletine (IS) in plasma (B—C), urine (E), and cerebrospinal fluid (G). Traces A, D and F represent the corresponding blanks. The concentrations of mexiletine and of the internal standard are given in μg/ml. The samples were applied onto the Spherisorb ODS 5 μm column (30 cm × 4 mm I.D.) by backflush elution. The eluent, 1-heptanesulfonic acid (0.005 *M*, PIC B7)—acetonitrile—tetrahydrofuran (42:48:10, v/v), was forced at 2.4 ml/min through the analytical column. The back-pressure was 230 bars; room temperature. Detection was at 352 nm.

For mexiletine a linear relationship between peak area and concentration was obtained for the range 0.01—25 μg/ml of plasma. Regression analysis revealed a correlation coefficient that was better than 0.999.

Within-run precision was established in pooled drug-free plasma, which contained 4-methylmexiletine (1 μg/ml) and mexiletine at concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 1, 5 and 25 μg/ml. For each concentration the within-run precision of six consecutive runs was determined with a coefficient of variation of less than 7%. The day-to-day precision, as determined on six consecutive days for frozen samples of plasma containing 1 μg/ml mexiletine, was found to be 8.6%.

The sensitivity of the proposed HPLC assay allowed quantitation of mexiletine at 50 ng/ml in a 4-ml blood sample and at 10 ng/ml in a 20-ml blood sample with a precision of better than 7%.

The accuracy of the HPLC assay was tested by comparison with a gas chromatographic method ([24]; Boehringer Ingelheim). The correlation between the results obtained by the gas chromatographic method and the HPLC assay is illustrated by Fig. 3. At a *p*-level of 0.05 the mean value of the results obtained by HPLC ($\bar{x}$ = 2.14 μg/ml) was not significantly different from

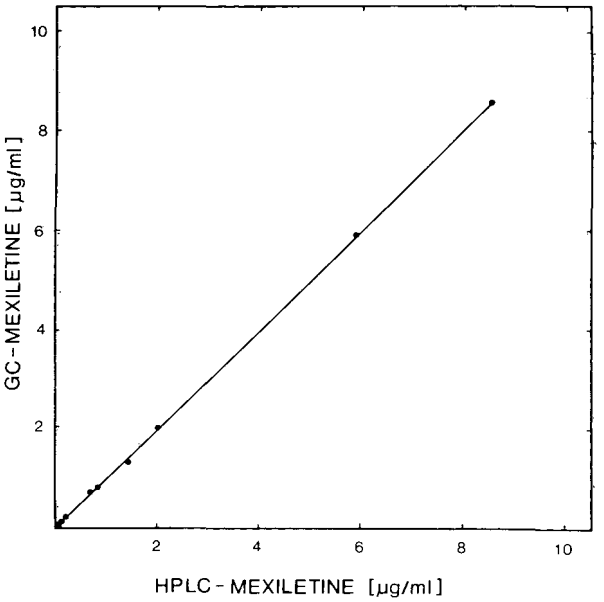

Fig. 3. Mexiletine concentrations in plasma as measured by gas chromatography (GC) and high-performance liquid chromatography (HPLC). Ten plasma samples were spiked with mexiletine in the range 0.05—10 µg/ml. The line was calculated by linear regression analysis.

the value obtained by gas chromatography ($\bar{y}$ = 2.28 µg/ml). The correlation between the results was good (r = 0.998). The slope of the regression line was 1.014, and the intercept was at 0.0068 µg/ml (HPLC values on the abscissa).

The total time for a single analysis is 50 min: 20 min for ether extraction, 20 min for derivatization, and 10 min for chromatography (with sample loading and backflush elution). However, for analysis of multiple samples the speed of analysis is considerably shortened: within 1 h up to five samples can be analyzed.

The stability of the chromatographic system is extremely high. In a series of 200 analyses of 2-ml samples of derivatized mexiletine and internal standard derived from plasma, there was a 30% decline of the efficiency from 28,000 to 19,600 theoretical plates per metre. Replacement of the pre-columns was followed by restoration of the primary efficiency.

Memory effects from the pre-column and/or the sample injector (with the 2-ml loop) were not observed when therapeutic concentrations of mexiletine (0.5—2.0 µg/ml) were injected. However, injection of blank solutions after a run with high amounts of mexiletine (10 µg/ml) revealed a memory effect of about 0.1%.

The proposed HPLC method with alternating pre-column sample enrichment was used for the determination of mexiletine in the plasma of a patient who had received mexiletine for several days. The concentration—time curve of mexiletine is shown in Fig. 4. Adverse symptoms attributable to the drug occurred at plasma concentrations of 3 µg/ml. Apparent side-effects observed in this patient were nausea, vomiting, and nystagmus. Disappearance from

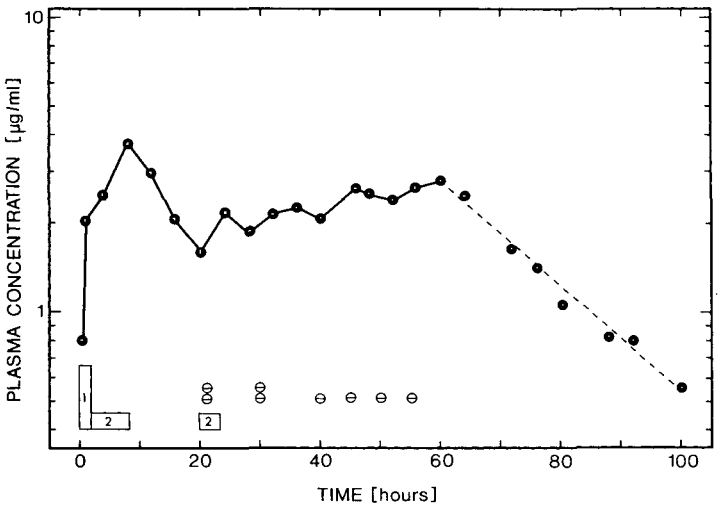

Fig. 4. Time course of mexiletine (•) in a patient's plasma during and following intravenous and oral administration of mexiletine. (1 and 2), infusion of mexiletine with, respectively, 230 mg/h and 60 mg/h (75 kg body weight). (⊖), oral application of mexiletine capsules (Mexitil^{R}, 200 mg). Broken line: monoexponential disappearance of mexiletine as calculated from measured values (r^2 = 0.985). Because of the occurrence of toxic symptoms at plasma levels of about 3 µg/ml, infusion of mexiletine was stoppped for several hours.

plasma was monoexponential with an elimination half-life of 17 h, which is comparable to the half-life values of about 9—16 h reported by others [9, 10].

In summary, a rapid and simple HPLC assay for the analysis of the antiarrhythmic drug mexiletine in plasma, urine, and cerebrospinal fluid is described. Following ether extraction and derivatization to a dinitrobenzene derivative, mexiletine was analyzed using an alternating on-column enrichment technique on small pre-columns in addition to pre-column backflushing for direct injection onto the analytical column. A programmable autosampler can easily be integrated into the chromatographic system thus allowing full automation of the whole analytical procedure. The method is sufficiently sensitive to determine mexiletine at therapeutic concentrations, which are reported to be 0.5—2 µg/ml [9, 10]. The limit of precise determination, 10 ng/ml, allows this HPLC assay to be applied to clinical research and pharmacokinetic studies. Moreover, this method is useful in clinical routine. Rapid monitoring of mexiletine levels in plasma has been shown to be a valuable means of avoiding subtherapeutic or toxic concentrations during antiarrhythmic therapy with this drug.

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DETERMINATION OF PYRIMETHAMINE IN HUMAN PLASMA AFTER ADMINISTRATION OF FANSIDAR OR FANSIDAR—MEFLOQUINE BY MEANS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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(Received December 30th, 1981)

SUMMARY

A sensitive, rapid and selective high-performance liquid chromatographic (HPLC) method has been developed to measure plasma levels of pyrimethamine in human subjects dosed with the antimalarials Fansidar or Fansidar and mefloquine. The drug was extracted from plasma at basic pH with *n*-butyl chloride—dichloromethane (96:4, v/v) and quantified on a normal-phase HPLC column with fluorescence detection (excitation 290 nm, emission 345 nm).

Pyrimethamine was almost quantitatively extracted from plasma in the concentration range 20—200 ng/ml. The sensitivity limit was about 10 ng/ml of plasma, using a 0.5-ml specimen. The method was shown to be specific with respect to the other two components in the antimalarial combinations, namely sulfadoxine and mefloquine, and their metabolites.

The assay was applied to pharmacokinetic studies of pyrimethamine in man following the oral administration of Fansidar or Fansidar and mefloquine.

INTRODUCTION

The emergence of plasmodial resistance to many currently applied antimalarials is becoming a severe problem all over the world. In South East Asia and Latin America especially, the efficacy of chloroquine-related drugs is much less than in previous years. The development of plasmodial resistance can be delayed by the administration of combinations rather than single drugs.

Fansidar, which is used in this study, is an effective antimalarial combination of pyrimethamine and sulfadoxine (weight ratio 1:20). These two drugs inhibit two different enzymes in the plasmodial folate biosynthesis pathway (dihydrofolate reductase and dihydropteroate synthetase). The synergistic

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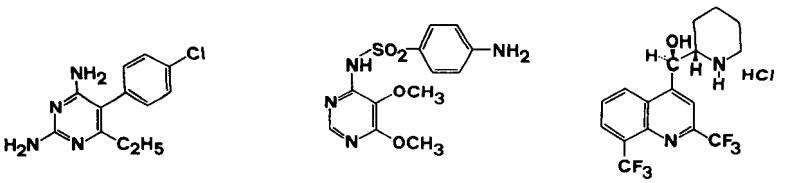

Fig. 1. Chemical structure of pyrimethamine, sulfadoxine and mefloquine.

antiplasmodial effect of pyrimethamine and sulfadoxine can be enhanced by the additive effect of mefloquine, which is a quinoline methanol derivative (for structures see Fig. 1). The use of this triple combination results in a marked delay of resistance development and, because lower doses may be given, in better tolerance and fewer side-effects.

For pharmacokinetic studies, a rapid, specific, and sensitive method was required for the determination of plasma concentrations of pyrimethamine. However, the simultaneous occurrence of the antimalarial components and their respective metabolites in the plasma makes the analytical determination of pyrimethamine difficult, especially as the three components appear in plasma concentrations which differ by a factor of several magnitudes.

A number of methods for the determination of pyrimethamine and related diaminopyrimidines in body fluids and tissues are available, such as microbiology [1], photometry [2], thin-layer chromatography [3—5], gas chromatography [6—8] and liquid column chromatography (with UV detection) [9, 10].

In this paper, we describe a new, normal-phase high-performance liquid chromatographic (HPLC) method for the quantitation of pyrimethamine in plasma without interference from sulfadoxine, mefloquine, or their respective metabolites, combining a rapid single-step extraction with selective and sensitive fluorescence detection.

EXPERIMENTAL

Reagents and solvents

Sodium hydroxide (p.a.), dichloromethane (for pesticide residue analysis), methanol (for fluorescence spectroscopy), diisopropyl ether (p.a.), aqueous ammonia solution (25%, p.a.) and 5-μm LiChrosorb Si 60 were obtained from E. Merck, Darmstadt, G.F.R. *n*-Butyl chloride (p.a.) was purchased from Fluka, Buchs, Switzerland. Acetonitrile was HPLC-grade S (Rathburn, Walkerburn, Great Britain). Pyrimethamine was of pharmaceutical grade (F. Hoffmann-La Roche & Co., Basle, Switzerland).

Diisopropyl ether, *n*-butyl chloride and acetonitrile were redistilled prior to use. The redistilled diisopropyl ether and the aqueous ammonia solution were stored in the refrigerator.

For preparation of plasma standards, sodium citrated human blood was received from a blood bank (Blutspendezentrum SRK, Basle, Switzerland) and centrifuged at approx. 1000 *g* for 20 min. The blank plasma obtained was tested for the absence of endogenous components interfering with the pyrimethamine and stored at −20°C. In pharmacokinetic studies volunteer blood

samples (10 ml) were collected using vacutainers containing potassium ammonium oxalate as anticoagulant. The plasma was prepared and stored as described above.

Plasma standards

Pyrimethamine (10 mg) was dissolved in 10 ml of methanol by ultrasonication to yield the stock solution. The working solutions a—f containing 3200, 1600, 800, 400, 200 and 100 ng of pyrimethamine per 0.1 ml were obtained by diluting aliquots of the stock solution with methanol. The plasma standards A—F containing 320, 160, 80, 40, 20 and 10 ng of pyrimethamine per ml were prepared by diluting 0.1 ml of the corresponding working solution with human blank plasma to 10 ml.

A batch of quality control samples containing 200 ng of pyrimethamine per ml was prepared by diluting 0.5 ml of a methanolic working solution (20 μg/ml) with human blank plasma to 50 ml.

To obtain optimum control of the assay, plasma standards and quality control samples were prepared by different persons using different stock solutions.

The stock solution could be stored in a refrigerator for about four weeks. The working solutions were prepared prior to use. The plasma standards and quality control samples were divided into aliquots of 2.5 ml and stored deep-frozen (−20° C) until required for analysis.

Chromatographic system

A modular HPLC system was used, consisting of an Altex pump 110 A (Altex Scientific, Berkeley, CA, U.S.A.), a Rheodyne injector 7125 with a 50-μl loop (Rheodyne, Berkeley, CA, U.S.A.), a Labotron spectrofluorometer SFM 22 (Kontron, Zürich, Switzerland), excitation wavelength 290 nm, emission wavelength 345 nm, a recorder W+W Model 1100 (Kontron) range 50—100 mV, chart speed 0.5 cm/min, and a stainless-steel column, 25 cm × 3.2 mm I.D., containing 5-μm LiChrosorb Si 60. The isocratic mobile phase used was a mixture of methanol—acetonitrile—aqueous ammonia solution (25%)—diisopropyl ether (6:25:0.1:71, v/v). Except for the aqueous ammonia solution, all solvents were degassed before mixing by ultrasonication for about 5 min.

Procedure

Extraction. An aliquot of 0.5 ml of sample* was added to a ground-glass stoppered centrifugation tube and mixed with 0.5 ml of bidistilled water and 0.2 ml of 2 *N* sodium hydroxide solution. After the addition of 7 ml of *n*-butyl chloride—dichloromethane (96:4, v/v), the sample was extracted by shaking for 15 min at 15 r.p.m. on a rotating shaker (Heidolph) and centrifuged (1700 *g*) at 10° C for 10 min. An aliquot (6 ml) of the organic phase was transferred to a conical glass tube and evaporated to dryness at 40° C by means of a

*Either plasma standard (calibration), control sample (quality control), biological sample (analyses), drug-free plasma (plasma blank), or bidistilled water (reagent blank).

gentle stream of pure nitrogen. The tube containing the remaining extraction residue was stored in the refrigerator until required for analysis.

Chromatography. The residue of the extraction was reconstituted with 100 μl of the mobile phase and an aliquot (50 μl) of the clear solution was injected for HPLC analysis.

The flow-rate of the mobile phase was adjusted to 0.7 ml/min, effecting a back pressure of approximately 70 bar. The column was reconditioned overnight with a purge solvent mixture consisting of methanol—acetonitrile—diisopropyl ether (6:25:71, v/v), filtered through a 0.5-μm filter (Millipore) prior to use. The flow-rate of the purge solvent was decreased to 0.2 ml/min.

Calibration and quality control. Along with the biological samples, four specimens of standard plasma A—F in the expected concentration range were analysed daily. An external standard curve was obtained by a least-squares regression of the peak heights measured versus the concentrations of pyrimethamine added to the plasma. This calibration curve was then used to interpolate concentrations of pyrimethamine in biological samples from peak height measurements.

Each day a quality control sample containing 200 ng of pyrimethamine per ml was carried through the procedure. Results from the biological samples were accepted provided the calculated concentrations of the quality control samples lay within the range 180—220 ng of pyrimethamine per ml.

RESULTS

Separation

When operating under the chromatographic conditions described above, the retention time of pyrimethamine was approximately 4.6 min.

Sensitivity and limit of detection

The detector parameters were optimized to obtain 80% full-scale deflection (50 mV recorder input) for a solution containing 50 ng of pyrimethamine per 50 μl of eluent.

The limit of detection for the method was about 10 ng of pyrimethamine per ml of plasma, using a 0.5-ml specimen. For this minimum detectable concentration a signal-to-noise ratio of ca. 3:1 was observed (Fig. 2b).

Selectivity

Several blank plasma samples from different human subjects were tested for the absence of interfering endogenous plasma components. Fig. 2a shows a typical chromatogram of a blank plasma extract.

The selectivity of the assay with respect to sulfadoxine, mefloquine and their metabolites was investigated by administration of a single oral dose of 500 mg of sulfadoxine or 140 mg of mefloquine, respectively, to two healthy male volunteers. Blood samples were taken up to 48 and 264 h, respectively, after administration and analysed as described. In both cases no peak with a retention time similar to that of pyrimethamine was detectable.

In a third experiment a potential interference of the assay by pyrimethamine metabolites was studied. Following a single oral dose of a Fansidar tablet

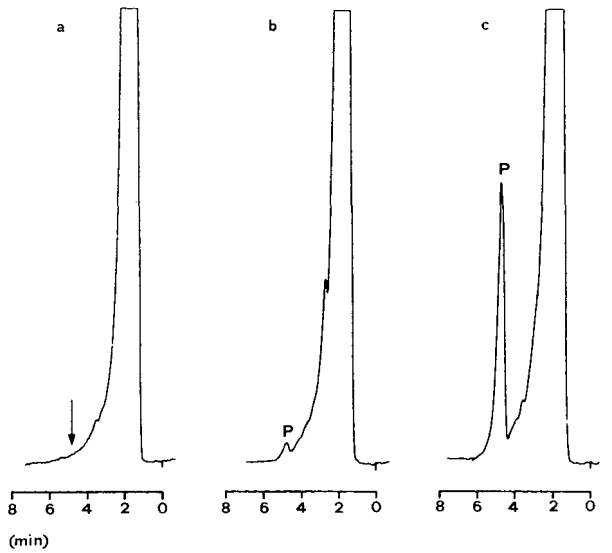

Fig. 2. (a) Chromatogram of a human blank plasma sample. The arrow indicates the retention time of pyrimethamine. (b) Chromatogram of a human blank plasma sample spiked with 10 ng of pyrimethamine (P) per ml. (c) Chromatogram of a human volunteer plasma sample, collected 24 h after a single dose of 40 mg of pyrimethamine (P), 800 mg of sulfadoxine and 280 mg of mefloquine; calculated pyrimethamine concentration 195 ng/ml.

containing 25 mg of pyrimethamine and 500 mg of sulfadoxine, human plasma samples were collected at various times and analysed. No peaks corresponding to possible pyrimethamine metabolites were detectable.

Linearity

A linear correlation between peak height and concentration of pyrimethamine was found in the concentration range 10—320 ng/ml of plasma. The coefficient of determination (r^2) was generally better than 0.9990 and the intercept did not differ significantly from zero.

Recovery

The recovery (extraction yield) was determined by adding known amounts of pyrimethamine to human blank plasma (20 and 200 ng/ml) and analysing each sample in quadruplicate according to the described procedure. Compared to a series of unextracted reference standards the recovery varied between 90 and 98%.

Reproducibility

The reproducibility of the external standard method was evaluated over a concentration range of 30.3—303 ng of pyrimethamine per ml. For each concentration a set of five specimens was analysed as described in one day (intra-assay reproducibility).

The inter-assay reproducibility was obtained by analysing one specimen from each concentration on five different days. Additional quality control samples were analysed together with the biological samples on 29 days covering a period of four months.

TABLE I

REPRODUCIBILITY

Concentration added (ng/ml)	Concentration found (ng/ml)	C.V.* (%)	No. of replicates	Difference between found and added concentration (%)
Inter-assay reproducibility				
30.3	29.4	4.7	5	−3.0
151.5	151.9	3.2	5	+0.3
303.0	300.0	2.4	5	−1.0
200**	196.0	4.1	29	−2.0
Intra-assay reproducibility				
30.3	30.0	4.3	5	−1.0
151.5	158.0	1.4	5	+4.1
303.0	304.2	2.2	5	+0.4

*Coefficient of variation.
**Quality control sample.

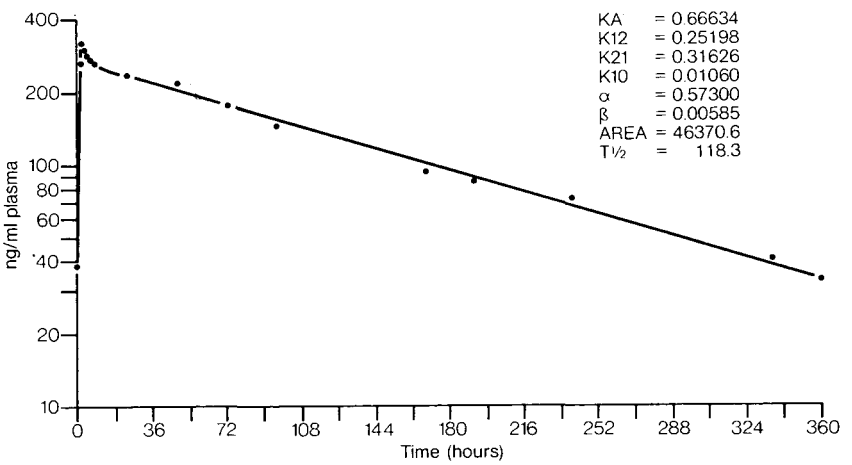

Fig. 3. Pyrimethamine plasma concentrations of volunteer U.T. after administration of two tablets of Ro 13-5112 containing 40 mg of pyrimethamine, 800 mg of sulfadoxine and 280 mg of mefloquine. The calculation of the curve is based on the assumption of a two-compartment model with first-order absorption. Pharmacokinetic data are shown in the inset.

The data presented in Table I demonstrate that the precision (relative standard deviation of replicate analyses) and accuracy (difference between added and found concentration) were acceptable over the concentration range investigated.

Stability of pyrimethamine in plasma

Plasma samples containing 320 ng of pyrimethamine per ml were stored for 24 h at ambient temperature and for four months at −20° C. In both cases no significant decomposition of the drug was observed.

Application of the method to biological samples

The method has been successfully applied to the analysis of 300 plasma samples from four clinical studies. Fig. 2c shows a typical chromatogram from these studies, demonstrating the validity of the new assay.

A complete plasma profile of one volunteer (U.T.) is given in Fig. 3. Pyrimethamine clearly exhibits two-compartment pharmacokinetics and the plasma concentration—time curve is best fitted by non-linear regression analysis according to $C_{pl} = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-K_a t}$. The evaluation of the pharmacokinetic data is based on computer programs as described in ref. 11. In the present case we obtained a terminal elimination half-life of 118 h.

DISCUSSION

The recently published gas chromatographic method of Jones et al. [7] has a sensitivity similar to the method described in this report. However, the analysis time is long (26 min) as a result of long-retained endogenous components. Jones et al. state in their paper that HPLC with fluorescence detection cannot be applied to pyrimethamine on account of its poor fluorescence. We also found that the fluorescent response of pyrimethamine was not as strong as its ultraviolet absorption. However, in our hands, working with a large number of different plasma samples we found fluorescence to be far more specific than ultraviolet absorption. In addition, the sensitivity for this method is as good as that quoted for the methods referred to (10 ng/ml, taking a 0.5-ml sample).

The extraction solvent was varied to obtain a sufficiently high recovery of pyrimethamine without interference from coextracted plasma components. Chloroform, dichloromethane, diethyl ether, benzene and benzene—dichloromethane (9:1, v/v) in some cases gave rise to extraction of endogenous plasma compounds which interfered with pyrimethamine. Less-polar solvents such as heptane and hexane extracted the drug with low efficiency.

An alternative to these "classical extraction solvents" is *n*-butyl chloride, which is being used increasingly for the extraction of basic drugs from biological fluids, especially when using gas—liquid chromatography with nitrogen detection for quantitation [12—15]. We used a mixture of *n*-butyl chloride and dichloromethane (96:4, v/v) and obtained both high recovery of pyrimethamine and clean blank plasma extracts.

Haefelfinger [16] showed recently that the reproducibility of HPLC assays cannot always be improved by means of an internal standard. We investigated two different pyrimethamine analogues as potential internal standards. These substances did not increase the precision of the assay, and for this reason an external standard method was established which showed good reproducibility.

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Note

Separation of six major prostacyclin metabolites by high-performance liquid chromatography

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Prostacyclin, (5-*Z*)-9-deoxy-6,9α-epoxy-Δ5-prostaglandin $F_{1\alpha}$ (PGI_2), a bicyclic ether derivative of arachidonic acid, is produced by blood vessels and endothelial cells [1, 2]. In addition to its antiplatelet aggregatory activity [1], PGI_2 relaxes vascular smooth muscles [3] and lowers systemic blood pressure [4]. PGI_2 has a relatively short half-life and its biological activity in aqueous solution disappears within a few minutes during the course of its transformation to a more stable product, 6-keto-$PGF_{1\alpha}$ [5]. Although PGI_2 is not metabolized during one passage across the lung [6], it is metabolized by 15-hydroxyprostaglandin dehydrogenase and 13,14-dihydroreductase in blood vessels and kidney to yield 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ [7, 8]. Recently, we demonstrated that, in the liver and platelets, PGI_2 is converted to a biologically active metabolite, 6-keto-PGE_1 [8], via 9-hydroxyprostaglandin dehydrogenase, and is also converted to pentanor-$PGF_{1\alpha}$ (γ-lactone) by β-oxidation followed by oxidative decarboxylation [9]. Although high-performance liquid chromatographic (HPLC) separation of PGI_2 [10, 11] and other classical prostaglandins [12, 13] has been described, there has been no method available for the separation of the various metabolites of PGI_2. This report describes a simple and rapid reversed-phase HPLC (RP-HPLC) method for the separation of up to six major metabolites of PGI_2. Concomitantly, the antiplatelet aggregatory activity of 6-keto-PGE_1, before and after RP-HPLC, is monitored, which offers a useful method for the detection of this biologically active metabolite of PGI_2 in the nanogram range in biological fluid.

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EXPERIMENTAL

Apparatus

HPLC was performed on a system incorporating dual pumps (Waters Assoc. Model 6000A, Milford, MA, U.S.A.) and Ultrasphere C_{18}-ODS (octadecylsilyl silica, 25 cm × 4.6 mm I.D., particle size 5 μm, Beckman, Palo Alto, CA, U.S.A.), a WISP 710B automatic injection system (Waters), and a Model SF770 Spectroflow monitor coupled with a Model GM770 variable-wavelength UV monochrometer (Kratos, Schoeffel Instruments, Westwood, NJ, U.S.A.), operated at 192 nm. Chromatograms were recorded on a Waters Data Module operated at 0.5 cm/min. The ODS reversed-phase column was guarded with a pre-column packed with Bondapak C_{18} Corasil (2.2 cm × 4 mm I.D., Waters). All procedures were executed through a Waters System Controller, which is connected by an Inter-link system with the WISP pumps, detector and data module. Fractions of 0.4 ml were collected at room temperature with an on-line fraction collector (Gilson 800A), and simultaneously monitored for biological activity (e.g. antiplatelet aggregatory activity).

Reagents

HPLC grade water was obtained through a Milli-Q Water Purification System (Millipore, Milford, MA, U.S.A.). Phosphoric acid (H_3PO_4), acetonitrile and methanol (HPLC grade) were obtained from Fisher (Fairlawn, NJ, U.S.A.). Diluted phosphoric acid (pH 2.95) and acetonitrile were filtered through a Millipore filter (aqueous 0.45 μm and organic 0.5 μm, respectively), followed by sonication for 3—5 min to ensure the solvents were free of gas.

Procedure

A solution containing a microgram mixture of 6-keto-$PGF_{1\alpha}$, 6-keto-PGE_1, pentanor-$PGF_{1\alpha}$ (γ-lactone), 6,15-diketo-$PGF_{1\alpha}$, 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ and 6,15-diketo-PGE_1 as free acids (Upjohn, Kalamazoo, MI, U.S.A.) in acetonitrile, was injected and eluted isocratically with a solvent system of diluted phosphoric acid (pH 2.95)—acetonitrile (70:30, v/v). The flow-rate was set at 0.4 ml/min under a pressure of approximately 50 bar, and the eluent was monitored with a UV detector at 192 nm (0.1 a.u.f.s.). Subsequently, individual prostaglandins were injected and eluted under essentially identical situations (0.02 a.u.f.s.) to analyze and plot detection reproducibility in the nanogram range. Peak areas of each metabolite were recorded and identified by their retention time.

RESULTS AND DISCUSSION

A reversed-phase column was chosen over a silicic acid column to avoid co-chromatography of various prostaglandins as reported by Whorton et al. [12]. The chromatographic peaks for 6-keto-$PGF_{1\alpha}$, 6-keto-PGE_1, pentanor-$PGF_{1\alpha}$ (γ-lactone), 6,15-diketo-$PGF_{1\alpha}$, 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ and 6,15-diketo-PGE_1 were separated isocratically with retention times of 17.97 ± 0.35, 21.84 ± 0.41, 23.37 ± 0.30, 24.52 ± 0.53, 28.82 ± 0.87 and 31.20 ± 0.78 min (mean ± S.D.), respectively (average of 18 experiments). These experiments

were performed over the course of one month, and it appeared that the latter three metabolites (i.e. 6,15-diketo-$PGF_{1\alpha}$, 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ and 6,15-diketo-PGE_1) were more likely than the former three to be eluted at altered retention times in response to slight changes in diluted phosphoric acid pH (ca. 2.93—2.97) A typical HPLC run is presented in Fig. 1. Under our chromatographic conditions, the UV monitoring wavelength of 192 nm appeared to offer the best compromise between efficiency of the detector and maximum sensitivity for each prostaglandin, while avoiding interferences from the solvents. This HPLC method should prove invaluable in analyzing prostacyclin metabolites in in vitro biological samples (Fig. 2). The recovery of over 90% of antiplatelet aggregatory activity of 6-keto-PGE_1 after RP-HPLC indicated that there was no substantial loss of biological activity of 6-keto-PGE_1 during the HPLC separation (Fig. 3). In addition, 6,15-diketo-PGE_1, the metabolite of 6-keto-PGE_1 by the action of 15-hydroxyprostaglandin dehydrogenase in the kidney [14], was well separated by our chromatographic conditions. Nanogram levels of various metabolites can also be detected under this condition at 0.04 a.u.f.s. The possible interference by other polar prostaglandins (e.g. TXB_2 and $PGF_{2\alpha}$), which also absorb spectrophotometrically at 192 nm, was also investigated. Under identical conditions TXB_2 and $PGF_{2\alpha}$ were well separated from all six prostacyclin metabolites with retention times of 35.4 and 41.3 min, respectively. Since the data module stores the retention

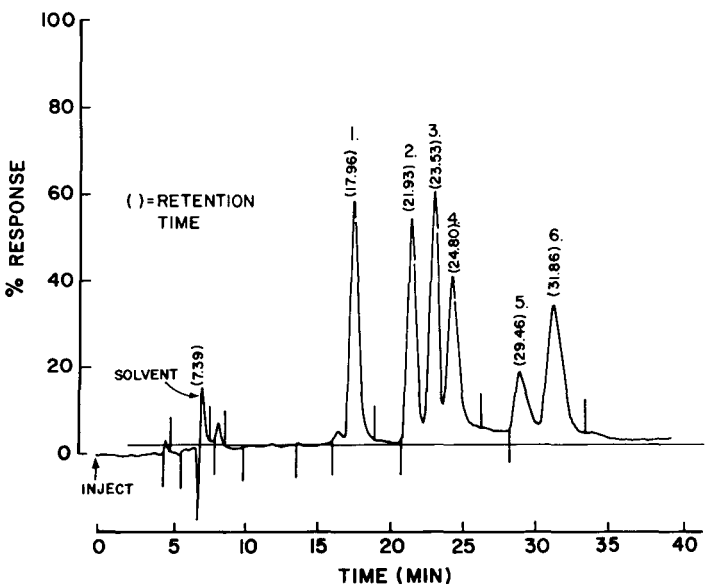

Fig. 1. HPLC separation of prostacyclin metabolites. Five μl of acetonitrile containing the following mixture: (1) 6-keto-$PGF_{1\alpha}$ (1.25 μg); (2) 6-keto-PGE_1 (0.625 μg); (3) pentanor-$PGF_{1\alpha}$ (γ-lactone 1.25 μg); (4) 6,15-diketo-$PGF_{1\alpha}$ (3.75 μg); (5) 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ (12.5 μg) and (6) 6,15-diketo-PGE_1 (5 μg) were injected into an Ultrasphere-ODS column (25 cm × 4.6 mm I.D.) by the use of an automatic injector (Waters Assoc. WISP 710B). Separation was performed isocratically at room temperature at a flow-rate of 0.4 ml/min using acetonitrile—water (pH 2.95) (30:70, v/v); UV detector at 192 nm at 0.1 a.u.f.s.; recorder chart speed 0.5 cm/min. Retention times (min) in parentheses.

time, peak area, peak height and response factor (RF) of all sample peaks formed during every run, it is possible to recall the previously stored peak area and RF values from the data module memory system when calculation of an unknown compound is needed [17].

Since thin-layer chromatography cannot resolve and separate all five metabolites from 6-keto-$PGF_{1\alpha}$ [9], this HPLC method is useful and convenient for the separation of large numbers of major PGI_2 metabolites, and it retains the biological activity (e.g. 6-keto-PGE_1). The latter inhibits platelet aggregation similar to PGI_2 [15], and was found to be four- to five-fold more potent than PGI_2 in the stimulation of renin release [16]. The biological activity from

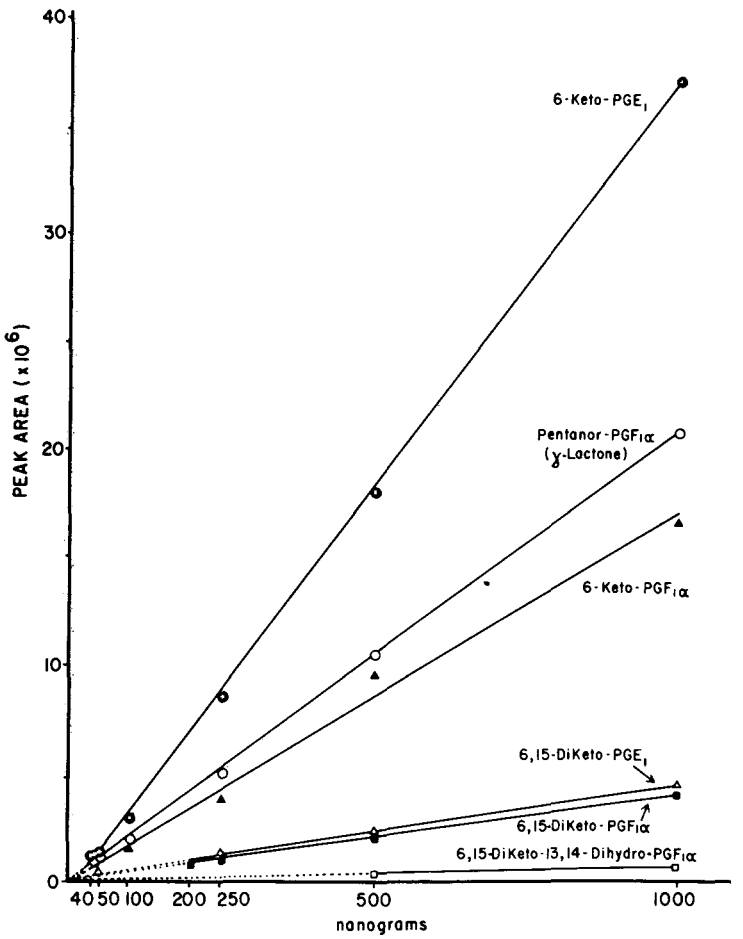

Fig. 2. Relationship of peak area and amount of various prostacyclin metabolites. Samples were dissolved in acetonitrile and injected into an Ultrasphere-ODS column (25 cm × 4.6 mm I.D.) by the use of an automatic injector (Waters Assoc. WISP 710B). Separation was performed isocratically at room temperature at 0.4 ml/min using acetonitrile—water (pH 2.95) (30:70, v/v); UV detector at 192 nm at 0.02 a.u.f.s.; peak area response equivalent to μm^2.

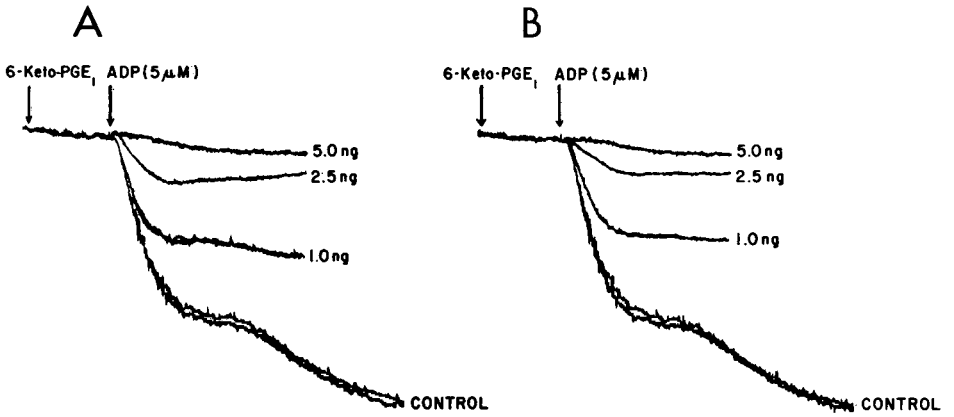

Fig. 3. Biological activity of 6-keto-PGE_1 as monitored by the inhibition of platelet aggregation. Nanogram amount of 6-keto-PGE_1 was injected into the Ultrasphere-ODS column and reported from other prostacyclin metabolites as described in Fig. 2. Fractions of 0.4 ml were collected with an on-line fraction collector (Gilson 800A). Fractions corresponding to 6-keto-PGE_1 were pooled and dried with nitrogen, and the residue was resuspended in Tris buffer (50 m*M*, pH 8.4) and tested for its anti-aggregatory activity on human platelets induced by ADP as described by Wong et al. [15]. (A) Anti-aggregatory activity before injection; (B) anti-aggregatory activity after HPLC separation.

the 6-keto-PGE_1 fractions collected by an on-line fraction collector will be useful for the quantitative in vitro assays for various PGI_2 metabolites isolated from biological samples.

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Note

Determination of γ-aminobutyric acid by reversed-phase high-performance liquid chromatography and pre-column labeling for fluorescence detection

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The role of γ-aminobutyric acid (GABA) in biological processes has received much attention since its discovery in the brain nearly 30 years ago [1, 2]. It plays an important role in polyamine metabolism [3—6]. In the central nervous system it acts as a major inhibitory neurotransmitter and may have a role in various neurological and mental disorders [7—9].

The quantitation of GABA has been accomplished by a variety of methods [10] which usually require chromatographic separation combined with derivatization [11—17]. The application of high-performance liquid chromatography (HPLC) for the analysis of dansylated GABA has not been demonstrated. This report describes a method of GABA analysis involving pre-column derivatization with dansyl chloride and separation by reversed-phase HPLC.

MATERIALS AND METHODS

Chromatographic equipment

A Series-3B high-performance liquid chromatograph equipped with a Rheodyne 7125 injection valve was used in combination with a Model LC-1000 fluorescence detector (Perkin-Elmer, Norwalk, CT, U.S.A.). The analyses were performed on a 25 × 0.46 cm column packed with 5-μm C-8 reversed-phase packing, LiChrosorb RP-8 (E. Merck, Darmstadt, G.F.R.). Chromatograms were recorded on a 10 in. recorder (Beckman Instruments, Fullerton, CA, U.S.A.) set at 1 mV. Sample injections were made using a Microliter S701 syringe (Hamilton, Reno, NV, U.S.A.).

Reagents and standards

GABA (Calbiochem-Behring, LaJolla, CA, U.S.A.), glutamic acid, guanidino-

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acetic acid (Sigma, St. Louis, MO, U.S.A.), γ-guanidinobutyric acid and β-hydroxy-γ-aminobutyric acid (Aldrich, Milwaukee, WI, U.S.A.) were used as supplied without further purification. Spectrophotometric grade acetone, toluene (Aldrich) and acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were used. All other chemicals were analytical reagent grade. 4-Amino-*n*-[U-^{14}C] butyric acid (226 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.).

Assay

The dansylation procedure is similar to that described by Seiler [10]. All samples were prepared in 0.4 *M* perchloric acid, centrifuged and the supernatant saved for dansylation. Three times the sample volume of a solution of dansylchloride in acetone (20 mg/ml) was added to the sample and the solution saturated with solid Na_2CO_3. The tubes were agitated at room temperature overnight. Excess dansylchloride was removed by addition of 5 mg of proline dissolved in 20 μl water. The dansyl-amides were extracted with 6 ml toluene. The toluene and aqueous phase were separated by centrifugation. The extracted toluene phase was washed with a 6-ml solution of saturated $NaHCO_3$ to eliminate dansyl-amino acids. The toluene phase was separated and evaporated to dryness. The residue was dissolved in 1 ml acetone and 0.5 ml of 2 *M* potassium hydroxide was added. After 30 min the reaction mixture was saturated with $NaHCO_3$ and extracted twice with 6 ml toluene. The toluene phase was removed and discarded while 1 ml of acetone plus 0.1 ml acetic anhydride were added to the aqueous phase. After 90 min the samples were extracted with 6 ml toluene. The toluene was evaporated and samples redissolved in acetone.

Chromatography

All analyses were performed using a mobile phase consisting of 55% acetonitrile in water with a flow-rate of 60 ml/h. All chromatography was carried out at room temperature (ca. 23°C).

RESULTS

The adjusted retention time for GABA, β-hydroxy-γ-aminobutyric acid and γ-guanidinobutyric acid (Table I) indicated good separation of γ-amino acids with this procedure. Glutamic acid and guanidinoacetic acid were absent in the final extraction solutions since no fluorescent peaks were observed. Dansylated

TABLE I

ADJUSTED RETENTION TIMES OF γ-AMINO ACIDS AFTER REACTION WITH EXCESS DANSYL CHLORIDE

γ-Amino acid	t'_R (min)
γ-Aminobutyric acid	6.5
β-Hydroxy-γ-aminobutyric acid	3.2
Glutamic acid	—
γ-Guanidinobutyric acid	5.8
Guanidinoacetic acid	—

γ-amino acids were also assayed by thin-layer chromatography (TLC) [10]. This technique was used to confirm the results obtained with HPLC.

The extraction efficiency was determined using ^{14}C-radiolabeled GABA. Standards were prepared with a known amount of radioactive GABA and the assay carried out. The radioactivity was determined in an aliquot of the final extraction solution. Recovery of GABA was 87 ± 5%. This compares to previously reported values [10].

Fig. 1 represents chromatograms of a standard, blank and a rabbit brain tissue sample obtained with this assay procedure. GABA was well resolved from other peaks, which may be due to contaminants present in the GABA standard or derivatization reagent. Peak height plotted against concentration was linear over a range from 20 pmole to 2 nmole. The detection limit was determined by injecting a 100-pmole GABA standard. It was calculated that 20 pmole of dansylated GABA could be detected at twice the signal-to-noise ratio. Therefore, this method is sensitive enough to detect GABA present in 10—15 μg of rabbit cerebrum.

The accuracy of this method was determined by comparing the GABA concentration found in rabbit cerebrum with established techniques. The results obtained with the present method differed by + 1.0% when compared to TLC [10] and −5.5% when compared to that of an amino acid analyzer [11]. The precision was calculated to be ± 4.8% after analyzing eight separate samples of rabbit cerebrum.

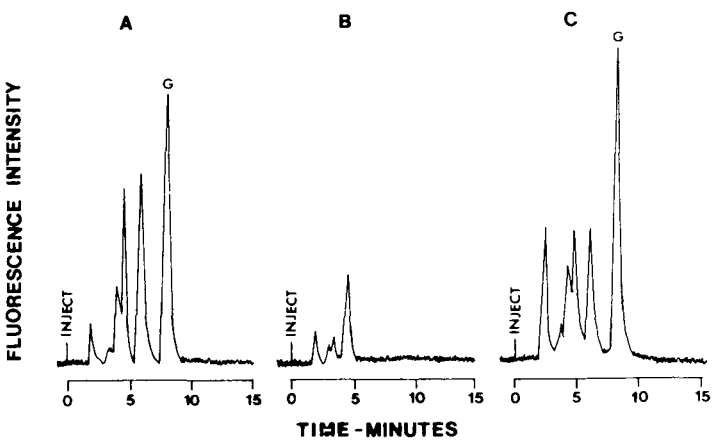

Fig. 1. Chromatograms obtained from (A) a 333 pmole dansylated GABA standard, (B) reagent blank and (C) homogenized rabbit cerebrum (ca. 200 μg). Peak G corresponds to the dansylated GABA product.

DISCUSSION

GABA is frequently assayed by using ion-exchange HPLC coupled with post-column derivatization and fluorescence detection [11—16]. This involves minimal sample preparation with detection limits ranging from 1—50 pmole. The assay time varies from 15 min to 2 h and requires a dedicated instrument and special buffers. On the other hand, reversed-phase HPLC which is applica-

ble to a wide spectrum of methods, requires minimal effort to change assay conditions.

The separation and assay of dansylated polyamines has been reported by Seiler using reversed-phase HPLC [18]. In this report we describe a reversed-phase HPLC method which allows quantitation of the GABA derivative, dansyl-oxopyrrolidine. The procedure allows rapid separation (less than 15 min) with no column equilibration necessary since an isocratic mobile phase is employed.

HPLC is a favorable technique when compared to TLC since it combines the sensitivity of the dansylation procedure with a technique suitable for automation. Since separation and quantitation are completed in one step the total analysis time is shortened. The overall resolution is greatly improved due to the development of high efficiency columns and general instrumentation technology. Radioactive measurements can easily be made on collected fractions.

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Note

High-performance liquid chromatographic determination of urocanic acid isomers in biological samples

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(First received December 10th, 1981; revised manuscript received January 26th, 1982)

It is known that urocanic acid is formed from L-histidine by an enzymatic reaction due to the enzyme histidine ammonia-lyase [1—5]. In the epidermis, where the enzymes of the catabolic pathway are absent, urocanic acid accumulates, forming as much as 0.5% of the dry weight of the epidermis [6—10]. The role of urocanic acid has still to be established, although it is believed that it acts as a sunscreen [11—14].

High-performance liquid chromatographic (HPLC) methods have been reported, but they suffer from the following drawbacks: a failure to resolve the two geometrical isomers of urocanic acid [15—18] and lack of sensitivity [19]. In our hands, the method of Morrison et al. [20] was unreliable since the elution conditions used resulted in a rapid deterioration of the stationary phases. Accordingly, we have developed and report here a simple, sensitive and reliable method for the quantitative determination of the two isomers of urocanic acid (Fig. 1) in biological samples.

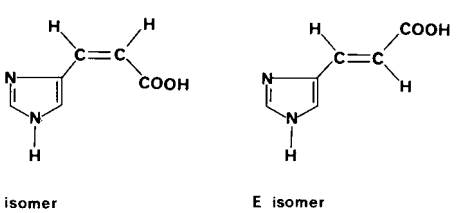

Fig. 1. Chemical structures of (*Z*)- and (*E*)-urocanic acid.

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EXPERIMENTAL

Instrumentation

HPLC was performed either on a Hewlett-Packard 1084 B chromatograph fitted with a variable-wavelength detector, or a system consisting of a Waters 6000 A pump, a Valco 7000 p.s.i. valve and a Waters M440 UV—visible dual-wavelength detector fixed at 254 and 280 nm.

The column used, LiChrosorb-NH_2 Hibar 250 RT-4 (25 cm × 4.6 mm I.D., 10 μm particle size) was purchased from Merck (Darmstadt, G.F.R.) and fitted with a 3 cm long precolumn filled with LiChrosorb-RP-18 (10 μm particle size).

The UV—visible spectrophotometer was a UVIKON 820 (Kontron) and the gas chromatography—mass spectrometry (GC—MS) apparatus was a 1010 C from Nermag.

Acetonitrile was chromatographic grade from Merck; KH_2PO_4, KOH and $HClO_4$ were analytical grade from Merck. Urocanic acid was supplied by Sigma (St. Louis, MO, U.S.A.). Water was purified, after deionizing, on a Milli Q system from Millipore.

The chromatographic eluent was filtered under vacuum on a Millipore filter (FHULP 0.45 μm).

To obtain (*Z*)-urocanic acid, we irradiated a solution of 1 mg/ml (*E*)-urocanic acid in the chromatographic eluent, with a 100 W Hanovia Xenon lamp filtered at 280 nm for 1 h. Under these conditions, we obtained a mixture containing 60% of (*Z*)-urocanic acid which was identified by its UV—visible spectrum and its mass spectrum, which were identical to the literature data [21].

We have confirmed in the same way the identity of the urocanic isomers in a healthy plantar callus extract, obtained by semi-preparative chromatography (same packing and eluent). In the routine procedure, the urocanic acid isomers are identified by the ratio of the absorbances at 254 and 280 nm.

Extraction procedure

After homogenization of the biological sample (50 mg) in 2 ml of 0.4 *M* perchloric acid with a Polytron homogenizer at 4° C (speed 8), the homogenate was centrifuged for 5 min at 6000 *g*, 4° C. A 1.5-ml portion of the supernatant was neutralized with KOH, diluted with 2 ml of acetonitrile, mixed and centrifuged for 5 min at 4500 *g* to remove the precipitated $KClO_4$ and other acetonitrile-insoluble material. An aliquot of the supernatant (50 μl) was then subjected to HPLC analysis.

RESULTS AND DISCUSSION

The effect of both the proportion of organic modifier and the ionic strength of the buffer used on the capacity factor k' of the *E*-isomer of urocanic acid is shown in Fig. 2. The results suggest that the separation on the amino group bonded phase is due to a mixture of partitioning and ion exchange. In fact, with up to 40% of acetonitrile there is practically no change in the capacity

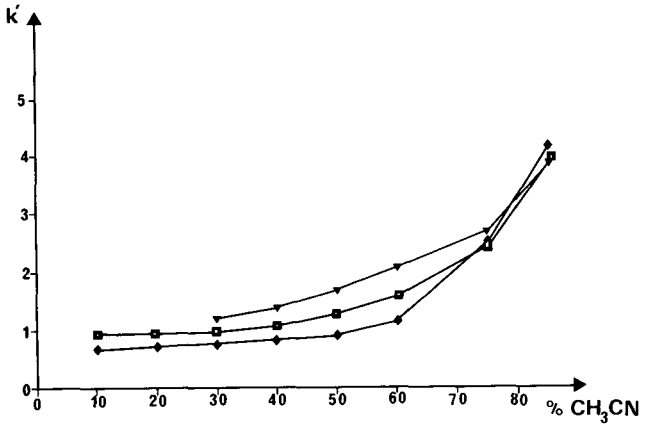

Fig. 2. Variation of the capacity factor, k', with the elution conditions: 0.1 M KH_2PO_4 (▼); 0.05 M KH_2PO_4 (■); 0.01 M KH_2PO_4 (♦).

factor on increasing the proportion of organic solvent, thus indicating that the column is working mainly in the ion-exchange mode, as shown by the variation of k' with ionic strength.

If we further increase the concentration of acetonitrile, the partition mode of separation becomes dominant, and the variation of the capacity factor with the proportion of organic solvent is important. The effect of the ionic strength thus becomes negligible for proportions of acetonitrile greater than 75%.

For the *Z*-isomer, the variation of the capacity factor is negligible up to 75% of acetonitrile. In this case, there is no effect at all of the ionic strength of the eluent on k', and the mechanism of separation seems to be only that of a partition effect.

In view of these results, we chose to perform the analysis under conditions giving the separation of a pure mixture of the isomers of urocanic acid shown in Fig. 3.

Under these conditions, the detection limit for the *E*-isomer of urocanic acid is 0.2 ng injected and 0.3 ng for the *Z*-isomer. This has to be compared to the respective limits of detection of 2 and 2.4 ng previously reported [19]. The response of the compounds is linear in the range of concentration studied (1—100 ng injected). By virtue of the symmetrical nature of the peaks, either peak area (HP 1084B) or peak height (Waters system) has been used for quantitation with the same accuracy. The reproducibility of five repeated injections of the same sample was better than 1%.

Our experiments on the extraction of urocanic acid from skin samples showed us the importance of the duration of the homogenization step. In fact, (*E*)-urocanic acid is stable at 4°C in 0.4 M perchloric acid, but when we perform the homogenization the recovery of urocanic acid is directly related to the time of treatment. At the homogenization speed used, as much as 40% of urocanic acid is decomposed in 8 min, mainly through a thermal process.

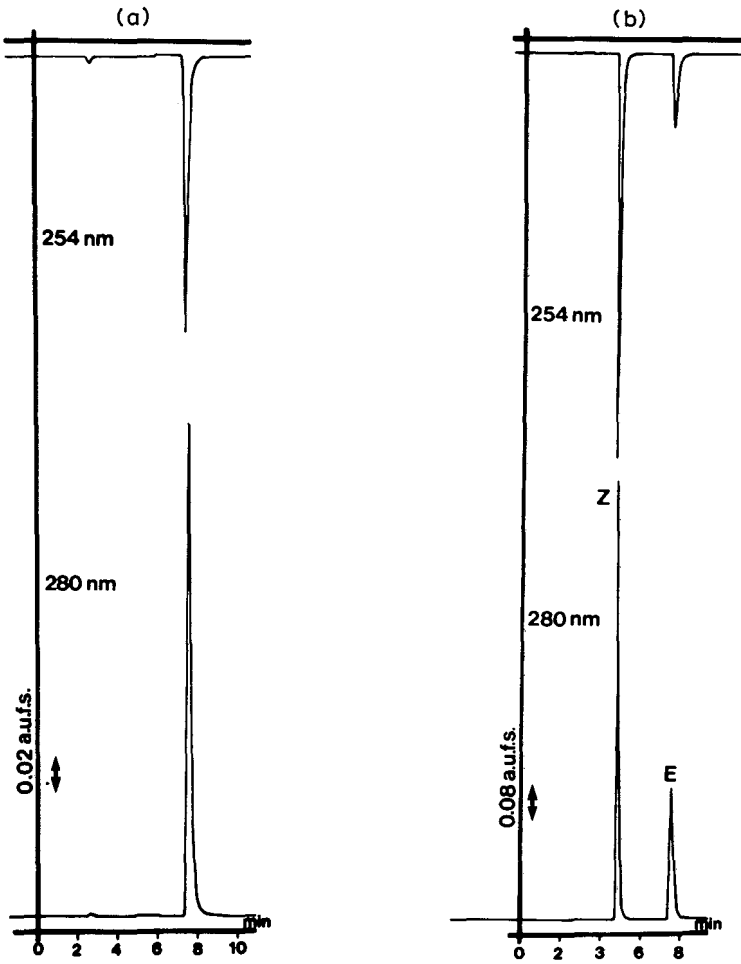

Fig. 3. Chromatograms of: (a) pure (*E*)-urocanic acid (0.5 μg); (b) a mixture of pure *Z*- and *E*-isomers (1.5 and 0.5 μg, respectively). Eluent: KH_2PO_4 (0.05 *M*)—acetonitrile (1:1, v/v), pH 7. Flow-rate: 1 ml/min. Pressure: 80—100 bars.

Addition of 0.1 *M* EDTA increased the total recovery of about 15%, but the decomposition is still significant.

The best conditions were found to be a 4-min homogenization in steps of 1 min, with a 1-min pause between each step to allow sufficient cooling of the Polytron shaft. Under these conditions, we obtained a 90% recovery of (*E*)-urocanic acid after addition of a known amount of urocanic acid to healthy plantar callus.

The reproducibility of the complete analytical procedure was found to be better than 5%.

Fig. 4 shows the chromatogram obtained with an extract of plantar callus of a healthy subject. We can see only minor interferences with (*E*)-urocanic acid. Dual-wavelength detection (254 and 280 nm) was usually used to identify the compounds by the ratio of the signals at these two wavelengths which, for a given compound, is constant.

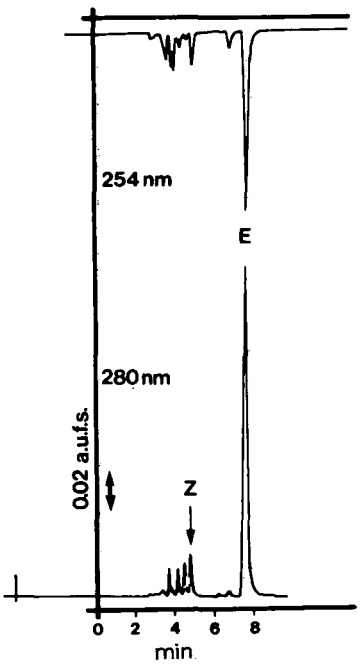

Fig. 4. Chromatogram of a healthy plantar callus extract. Conditions are the same as in Fig. 3.

The concentration of (*E*)-urocanic acid found in plantar callus is in good agreement with the literature values [14], ranging from 0.5 to 0.3% of dry weight, depending on the subject under study.

We are currently studying the urocanic acid content of the skin by this improved analytical procedure in patients with dermatological diseases.

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Note

Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography

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(First received October 7th, 1981; revised manuscript received January 18th, 1982)

ATP is one of the most important substances for the proper functioning of the heart [1]. Creatine phosphate (CrP) serves as an "energy buffer", and could also play a role in the rapid transport of energy between cell compartments [2]. Separations by high-performance liquid chromatography (HPLC) of nucleotides on an ion-exchange column with detection at 254 nm have been carried out [3, 4]. Recently, determinations of nucleotides on reversed-phase columns [5], with or without ion-pairing [6, 7], have also been made. CrP, however, cannot be detected at 254 nm. Juengling and Kammermeier [6] detected CrP at 210 nm, but their HPLC system could not separate adenine nucleotides. Heldt et al. [8] used a phosphate analyzer as a detector and were able to detect CrP in one run with nucleotide quantitation. We modified the separation and detection conditions of Edelson et al. [9]. Analysis time was decreased by a factor of two and detection at 210 nm was introduced. We obtained sufficient resolution to quantitate CrP and adenine nucleotides within 30 min.

EXPERIMENTAL

Reagents

Hexokinase (EC 2.7.1.1, 140 U/ml), creatine kinase (EC 2.7.3.2, 25 U/ml), and adenylate kinase (EC 2.7.4.3, 360 U/ml) were purchased from Boehringer, Mannheim, G.F.R.; AMP-deaminase (EC 3.5.4.6, 30—60 U/mg) was obtained from Sigma (St. Louis, MO, U.S.A.). KH_2PO_4, H_3PO_4, KOH and $HClO_4$ were from Merck (Darmstadt, G.F.R.), the nucleotides and CrP were from Boehringer and creatine was from Technicon (Tarrytown, NY, U.S.A.). Water

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was purified with the Milli-Ro4/Milli-Q system (Millipore, Bedford, MA, U.S.A.).

High-performance liquid chromatography

A Varian 8520 HPLC system (Varian, Palo Alto, CA, U.S.A.) was used, which consisted of two positive displacement pumps, a variable-wavelength UV detector (Varichrom) set at 210 nm, a pneumatic sampling device, and a chromatographic data system (CDS-111) connected with a chart recorder. Buffers were prepared on the day of use and filtered through a 0.45-μm filter (Millipore). Buffer A consisted of 0.01 *M* H_3PO_4, adjusted to pH 2.85 with KOH; buffer B consisted of 0.75 *M* KH_2PO_4, pH 4.40. The column (Partisil-10-SAX, 0.4 × 25 cm, particle size 10 μm; Whatman, Maidstone, Great Britain) was eluted with buffer A at a flow-rate of 2.0 ml/min. Five minutes after injection, a gradient started with an increase of 4% B per minute until 100% B.

Rat heart perfusions and sample treatment

Isolated hearts from male rats (Wistar strain, 300—400 g) were perfused retrogradely for 30 min and quickly frozen as described before [10]. After weighing, cardiac tissue was ground in a mortar, precooled with liquid nitrogen. One half of the sample (about 0.5 g) was freeze-dried for determination of percentage dry weight; the other half was extracted with 3.0 ml of 0.8 *M* $HClO_4$. After thawing and centrifugation, 2.0 ml of the supernatant fluid were neutralized at 0°C with about 200 μl of 6 *N* KOH and the $KClO_4$ was spun down at 4°C. A 20-μl volume of this supernatant was applied to the HPLC column.

RESULTS AND DISCUSSION

Chromatograms

Fig. 1 shows the separation of a standard mixture of thirteen nucleotides and creatine compounds. Fig. 2A gives the separation of the adenine nucleotides and CrP in an extract of an oxygenated rat heart.

Linearity, recovery and sensitivity

The lower detection limit with a 20-μl sample loop varies between 20 pmol for AMP and 100 pmol for CrP, and the determination is linear up to 400 nmol for all compounds indicated. Recoveries, determined in a model system (high-energy phosphates added to a 1 g/ml albumin solution), after deproteinization and neutralization, exceeded 95%, with a standard deviation of <3% for the adenine nucleotides and <6% for CrP (n = 5).

Peak identification

Fig. 2A gives the chromatogram of a rat heart extract. The main peaks in the extract are creatine, NAD, CrP, ADP and ATP. Peak identification was made by comparing retention times with standards and by enzymic peak shifts [3]. Fig. 2B—E shows chromatograms after subsequent incubation with hexokinase (B), creatine kinase (C), adenylate kinase (D) and AMP-deaminase (E). Complete removal of ATP, CrP and AMP was observed, with a concomitant increase of the conversion products ADP, creatine and IMP. ADP in the chromatogram

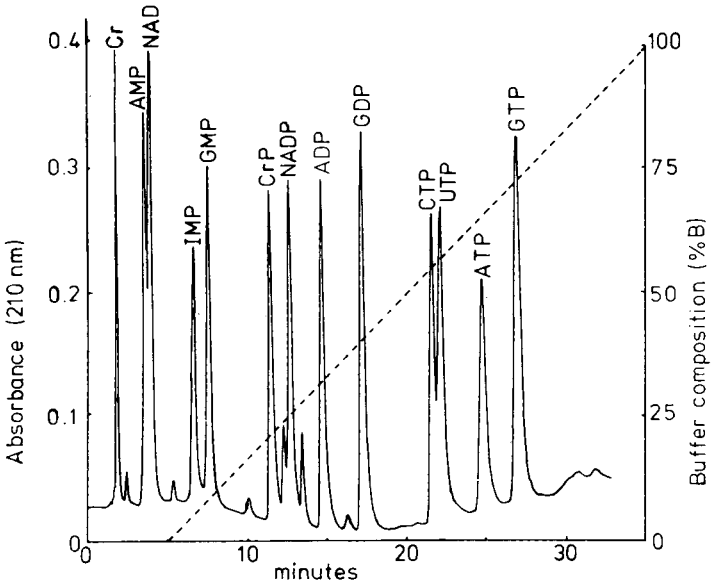


Fig. 1. Separation of standard nucleotides and creatine (Cr) compounds (0.2—0.5 mmol/l). Injection volume, 20 µl; flow-rate, 2.0 ml/min; eluents, A = 0.01 *M* H_3PO_4, adjusted to pH 2.85 with KOH, B = 0.75 *M* KH_2PO_4 (pH 4.40); column, Partisil-10-SAX.

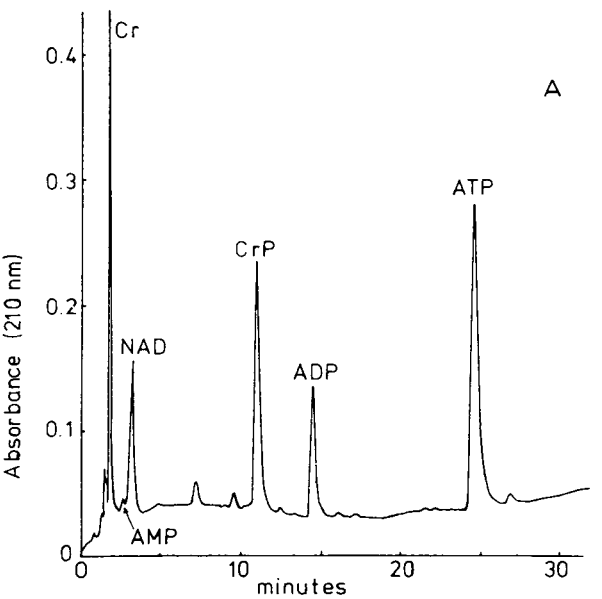
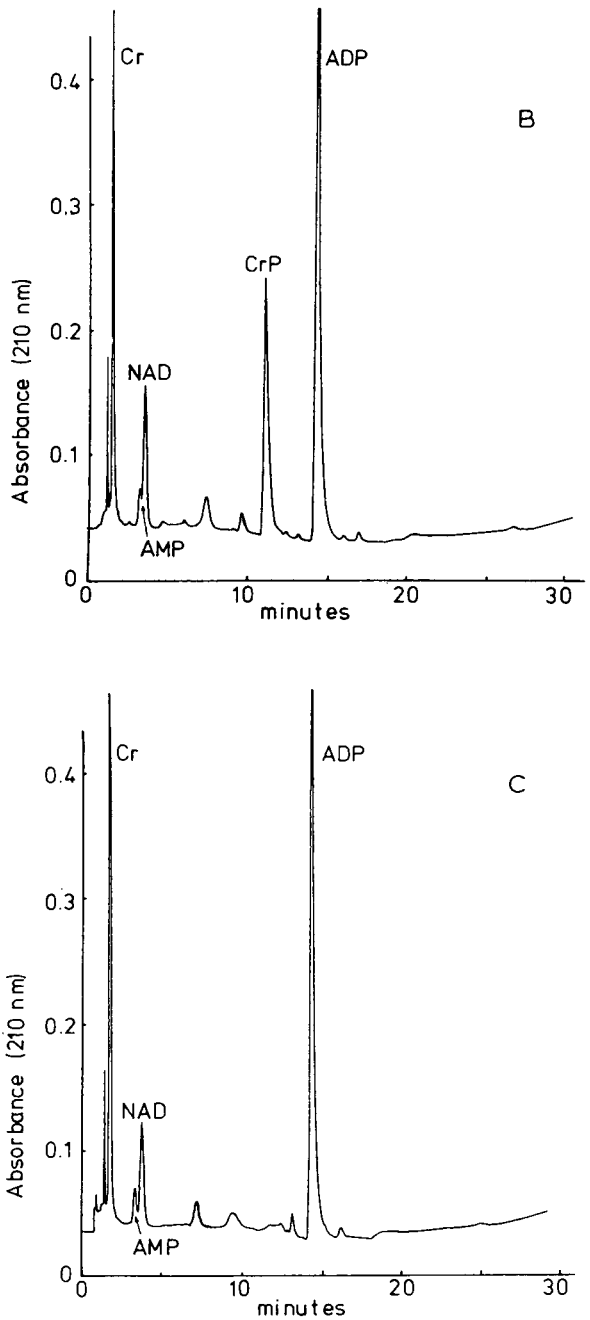


Fig. 2.

(Continued on p. 134)


Cr
ADP
B
CrP
NAD
AMP
Absorbance (210 nm)
0.4
0.3
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Cr
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Fig. 2.

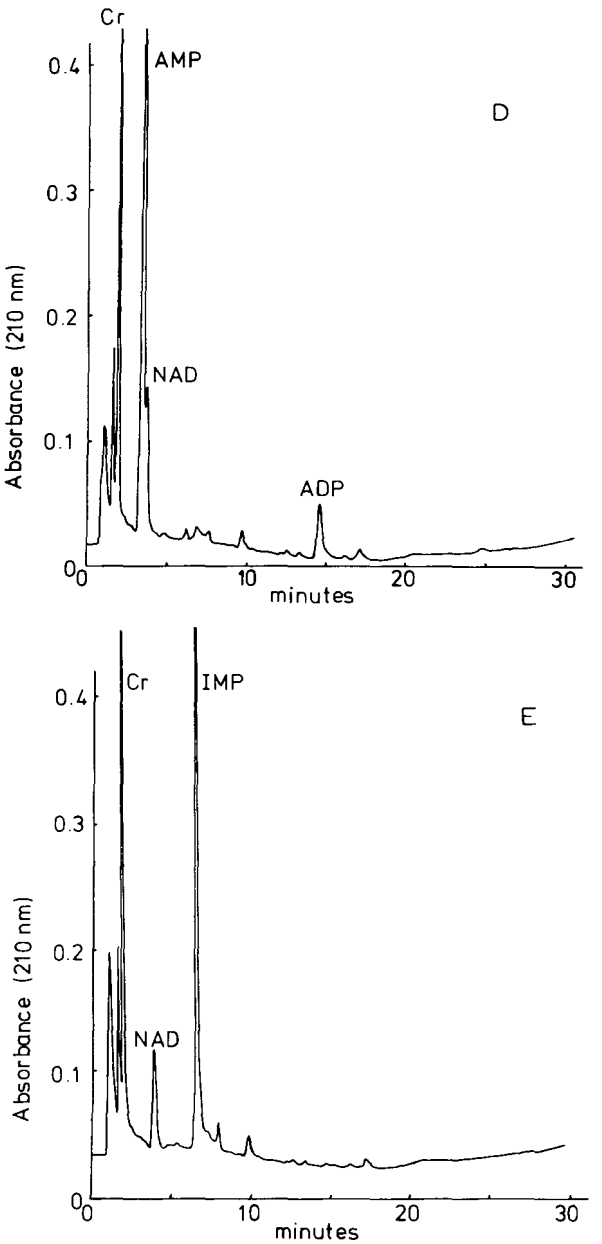

Fig. 2. Chromatogram of a rat heart extract and peak identification by enzymic conversions. HPLC was carried out with a 20-μl extract, prepared as described in the text. For HPLC conditions, see Fig. 1. Panel A gives the chromatogram of untreated extract. Enzymic peak shifts were carried out according to the method of Brown [3]. An aliquot of the extract was incubated for 30 min at 37°C with 0.7 mU hexokinase and D-glucose to convert ATP: ATP + glucose → ADP + glucose-6-phosphate (panel B). Subsequently CrP was broken down by addition to the extract of 0.1 mU of creatine kinase and $MgCl_2$. Under the influence of creatine kinase and hexokinase, the overall reaction is: CrP + glucose → creatine + glucose-6-phosphate (panel C). In a similar way ADP was converted with 1.8 mU of adenylate kinase: 2ADP + glucose → 2AMP + glucose-6-phosphate (panel D). Finally, the AMP peak was shifted with 0.4 mU of AMP-deaminase: AMP → IMP + NH_3 (panel E).

shown in Fig. 2D is not completely removed, presumably because of an unfavorable equilibrium of the adenylate kinase reaction. After addition of AMP-deaminase, which removes AMP, all ADP is converted (Fig. 2E).

Rat heart concentrations

In Table I concentrations are given for adenine nucleotides and CrP, measured in normal and anoxic rat hearts. These values are in the same range as found in the literature [1, 11, 12].

TABLE I

CONCENTRATION OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE IN RAT HEART

Hearts were perfused retrogradely (Langendorff perfusion) for 30 min with a modified Tyrode solution, equilibrated with 95% O_2—5% CO_2 (normoxia), or for 15 min perfused with 95% O_2—5% CO_2 and 15 min with 95% N_2—5% CO_2 (anoxia). Heart extracts were prepared as described in the text. For HPLC conditions and peak identification, see Figs. 1 and 2.

Condition	μmol/g dry weight (± S.E.M.)			
	ATP	ADP	AMP	CrP
Normoxia	20.9 ± 0.5	7.3 ± 0.4	0.2 ± 0.1	29.3 ± 2.8
Anoxia	9.9 ± 1.9*	9.5 ± 2.2	5.1 ± 1.6*	7.4 ± 1.8*

*$p < 0.005$ vs. normoxia, $n = 5$—7.

Conclusion

We conclude that the method presented here is a quick and accurate way to determine myocardial high-energy phosphates. Within 30 min the concentration, energy charge [13] and ATP/CrP ratio can be read from the chromatogram.

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CHROMBIO. 1241

Note

Chromatofocusing of human hemoglobins

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Chromatofocusing is a relatively new technique in which a pH gradient is produced on an ion exchanger by utilizing the buffering action of the charged groups on the ion exchanger [1, 2]. This article describes separation of the commonly occurring human hemoglobins A_2, E, C, S, A_0, F_0, and F_1 by this chromatographic technique.

EXPERIMENTAL

Reagents

Polybuffer 96, polybuffer exchanger PBE 94, and columns were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). All other chemicals were commercial analytical grade products.

Hemoglobin preparations

Human red blood cells were collected from whole blood in EDTA anticoagulant by centrifugation and washed three times with 5 volumes of 0.15 *M* sodium chloride. Cord blood cells were the source for fetal hemoglobin. The washed red cells were lysed with 2 volumes of deionized water.

Hemoglobin electrophoresis

Hemoglobin electrophoresis was performed on cellulose acetate at pH 8.4 and on citrate agar at pH 6.2 with an apparatus from Helena Laboratories (Beaumont, TX, U.S.A.) [3].

Chromatofocusing

Chromatofocusing was performed with 0.9 cm diameter columns containing

21—54 cm of anion-exchanger gel PBE 94. The eluates were continuously monitored (percent transmission) at 540 nm with a Model 100-10 spectrophotometer and flow-through cuvette (Hitachi, Tokyo, Japan) that was interfaced with a strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.).

The anion exchanger was equilibrated with starting buffer, 0.025 *M* imidazole · HCl, pH 7.4 or pH 8.0, by passing several column bed volumes of buffer through the column. The eluting ampholyte polybuffer, PBE 96, was prepared by diluting the stock with 10 volumes of deionized water, adjusting the pH to 6.0 with 1 *M* hydrochloric acid, and adding water to a final dilution of 1:13. All buffer solutions were degassed under vacuum (water aspirator) immediately prior to use.

A mixture of hemoglobin variants was prepared by combining erythrocyte lysates that contained hemoglobins E, C, A_2, S, A_0, F_0, and F_1. One hundred microliters of this mixture containing 4—5 g/dl hemoglobin were applied to the column. Elution with polybuffer 96 was performed by gravity flow at a rate of 14—24 ml/h. Electrophoretic analysis of the fractions was performed after concentrating them in an A25 Amicon macrosolute concentrator (Amicon, Lexington, MA, U.S.A.).

RESULTS

With a 54 × 0.9 cm column excellent separations of hemoglobins E plus A_2, S, A_0, F_0, F_1 were achieved within 18 h, but hemoglobins A_2 and E failed to separate (Fig. 1). Hemoglobin C was not available for this separation. The hemoglobin type in each peak was confirmed by electrophoresis, although neither chromatofocusing nor electrophoresis resolved hemoglobins E and A_2. Hemoglobins F_0 and F_1 were further identified by their resistance to denaturation in alkali [5, 6].

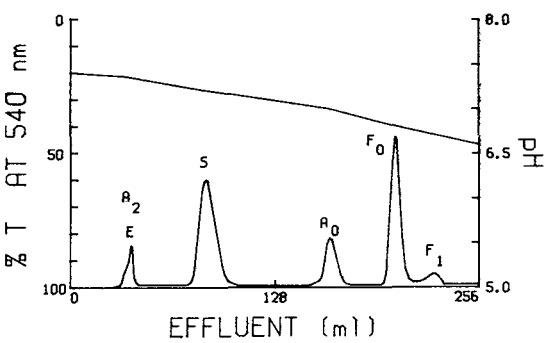

Fig. 1. Chromatofocusing of human hemoglobin variants A_2, E, S, A_0, F_0, and F_1 on a long column. A mixture of oxyhemoglobins (A_2, E, S, A_0, F_0, F_1) containing a total concentration of 4.7 mg hemoglobin was chromatographed on a PBE gel column, 54 × 0.9 cm, starting at pH 7.4, 0.025 *M* imidazole · HCl and eluting by gravity with pH 6.0 polybuffer 96—HCl at a flow-rate of 14.3 ml/h.

A similar pattern (Fig. 2) with high resolution can be achieved in about 6 h using a shorter column (26 × 0.9 cm). Hemoglobins S, A_0, F_0 and F_1 were clearly resolved from each other and from a heterogeneous peak that consisted

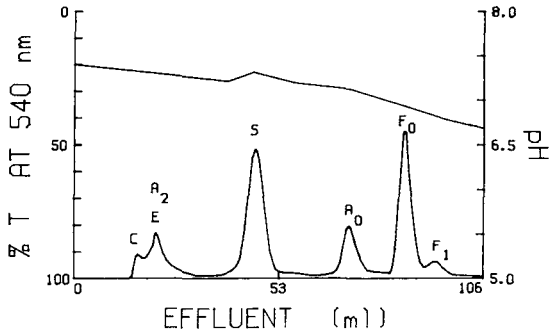

Fig. 2. Chromatofocussing of human hemaglobin variants C, A_2, E, S, A_0, F_0 and F_1 on a short column (pH 7.4 to 6.0). Conditions were the same as in Fig. 1 except that the column dimensions were 26 × 0.9 cm with a flow-rate of 17.5 ml/h and hemoglobin C was added to the mixture.

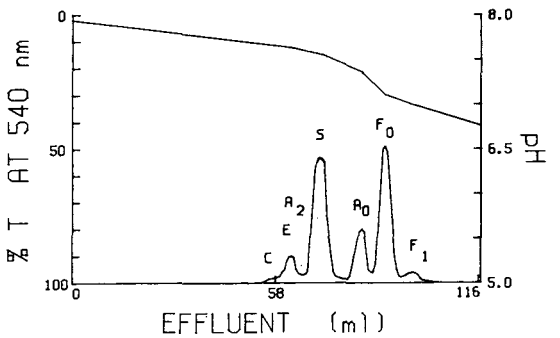

Fig. 3. Chromatofocusing of human hemoglobin variants C, A_2, E, S, A_0, F_0, and F_1 on a short column (pH 8.1 to 6.0). Conditions were the same as in Fig. 1 except that the column dimensions were 21 × 0.9 cm with a flow-rate of 23.6 ml/h and the starting buffer was at pH 8.1.

of hemoglobins A_2, E and C, although C migrated slightly faster than A_2 and E.

When the elution was performed with pH 8.1 rather than pH 7.5 polybuffer, resolution of the hemoglobin variants was less satisfactory and elution of the peaks occurred later (Fig. 3).

A comparison of the isoelectric points of the hemoglobin variants and the pH at which they chromatofocused showed that the hemeproteins emerged at a pH close to their p*I*, and in the order of their isolelectric points, except that hemoglobin F_0 emerged after A_0 even though F_0 has a more basic p*I* (Table I). Moreover, we observed that the pH at which the hemoglobins focused varied with the pH of the starting buffer. When the starting pH was at 8.0 the more basic hemoglobins A_2, C, E, S and A_0 eluted slightly above their p*I* values, while F_0 and F_1 eluted near their p*I* values, whereas with a starting buffer at pH 7.4, hemoglobins A_2, E, C, S, F_0 and F_1 eluted near their p*I* values, while A_0 emerged from the column at a pH above its p*I*.

Attempts to separate hemoglobins C, A_2 and E by starting at pH 7.8 and eluting with either pH 7.2 or pH 6.8 failed to resolve these hemeprotein variants.

TABLE I

ISOELECTRIC POINTS OF HEMOGLOBIN VARIANTS COMPARED WITH ELUTION pH IN CHROMATOFOCUSING

Hemoglobin species	p*I**	Elution pH**	
		Gradient pH 7.4 to 6.0	Gradient pH 8.1 to 6.0
C	7.40	7.36	7.63
E	7.40	7.34	7.63
A_2	7.40	7.34	7.63
S	7.25	7.26	7.55
A_0	6.95	7.06	7.36
F_0	7.15	6.86	7.11
F_1	6.90	6.74	6.98

*Obtained by isoelectric focusing in polyacrylamide gel [4] except for hemoglobin E which was estimated from our data.
**From Figs. 1—3.

DISCUSSION

Protein purification by chromatofocusing is dependent on eluting a given protein at, or near, its p*I* [1, 2], and thus, the choice of a narrow pH interval from 7.4 to 6.0 was sufficient to separate most of the commonly occurring human hemoglobins as sharp peaks with 0.05 pH units at half width. These hemeproteins emerged from an anion-exchange column in order of their isoelectric points, with the exception of fetal hemoglobin, which behaved as if it has a more acidic p*I*, a phenomenon that was also observed with pH-gradient chromatography on DEAE-Sephadex [7].

By using a pH interval of 8.1 to 6.0 for chromatofocusing, the hemoglobins emerged from the column at pH values somewhat higher than their p*I* (except for hemoglobin F_0). This was probably due to some separation by ampholyte displacement because the hemeproteins were eluted after 4 to 8 column bed volumes of eluents had passed through the column (Fig. 3) when ambient buffer concentration increased and ampholyte displacement was more pronounced [8].

Others have utilized ampholyte displacement chromatography to separate human hemoglobin variants [9]. Mixtures of A_2 and A_0, A_2, S and A_0, and C, S, and F were resolved but no experiments with a complete mixture of all the variants were reported. Although ampholyte displacement chromatography is an effective procedure for separating some hemoglobin variants, its major limitation is the high cost of the carrier amphoteric buffers which are used in many-fold greater concentrations than in chromatofocusing [8, 9].

The method described in this report is especially useful for preparative isolation of hemoglobin species. But its application as a routine analytical procedure would not appear to be as convenient as gel electrophoresis which is faster and less expensive than chromatofocusing.

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Note

Mass spectrometric identification of isoputreanine, a metabolite of spermidine and/or spermine, in human urine

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Polyamines are functional components of living cells which, most probably by their association with nucleic acids, are indissolubly united with growth processes [1—5]. During the last decade much attention has been given to their role as general markers for neoplastic diseases. In this respect the use of determinations of polyamines in biological fluids for the early diagnosis of cancer has been disappointing. On the other hand, their value as markers for tumor cell kinetics before, during and after chemotherapy is now widely accepted. The concept of Russell [6], linking extracellular putrescine concentrations to the tumor growth fraction and those of spermidine to cell turnover, has contributed much to this picture.

Although the regulation of polyamine synthesis during growth processes has been studied in detail [1—5], in man relatively little attention has been paid to the deactivating mechanisms of these potent growth factors. From the two most frequently mentioned deactivating routes, i.e. N-conjugation with acetic acid, propionic acid, glutamic acid, peptides and pyridoxal phosphate and (amino) oxidation, possibly followed by intermediate aldehyde oxidation, only fragmentary knowledge is available about the importance of the latter route in man [7].

0378-4347/82/0000—0000/$02.75

In this study the mass spectrometric identification of isoputreanine, a metabolite of spermidine and/or spermine, is described. The possible significance of this compound in the diagnosis and follow-up of cancer is discussed.

MATERIALS AND METHODS

Standards and reagents

1,3-Diaminopropane , putrescine, cadaverine, spermidine, spermine, 1,6-diaminohexane and 1,7-diaminoheptane were from Sigma Chemical Co. (St. Louis MO, U.S.A.), bis-(3-aminopropyl)amine and 1,2-bis-(3-aminopropylamino)ethane were from Fluka (Buchs, Switzerland) and N-(3-aminopropyl)-2-pyrrolidone (the γ-lactam form of isoputreanine) was from Aldrich Europe, (Beerse, Belgium). Sep-Pak silica cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.); heptafluorobutyric anhydride was from Pierce Chemical Co. (Rockford, IL, U.S.A.); all other reagents were from Merck (Darmstadt, G.F.R.).

Samples

Urine samples (24 h) from normal persons and patients were collected, acidified to pH 1—2 with hydrochloric acid and stored at —20° C until analysis.

Equipment

Gas chromatography with flame ionization detection was performed with a Hewlett-Packard Model 5880 gas chromatograph equipped with a Model 7672 A automated sampler and a 30 m × 0.32 mm I.D., CP-Sil-5 coated (0.2 μm film thickness), fused silica capillary column (Chrompack, Middelburg, The Netherlands). Gas flow-rate (helium) was 1.0 ml/min, split ratio 1:12, detector temperature 300° C, and injector temperature 280° C. The oven temperature program was 110° C, 7° C/min to 240° C, 15 min at 240° C.

Gas chromatography—mass spectrometry was performed using a Varian Aerograph 1400 gas chromatograph directly coupled to a Varian MAT 112 mass spectrometer equipped with a similar capillary column. Ionization energy was 70 eV; mass spectra were taken at a scanning rate of 100 a.m.u. per sec near the top of the gas chromatographic peak. No corrections were made for background or changes in total ion current during scanning.

PROCEDURES

Acid hydrolysis

To five millilitres of acidified urine were added 400 μl of an internal standard cocktail containing 50 nmol each of 1,6-diaminohexane, 1,7-diaminoheptane, bis-(3-aminopropyl)amine and 1,2-bis-(3-aminopropylamino)ethane, dissolved in a 1:1 (v/v) mixture of methanol and 1 mol/l hydrochloric acid. The urine was evaporated to almost dryness at 120° C under a stream of air and hydrolyzed at 120° C overnight in 2 ml of 6 mol/l hydrochloric acid.

Clean-up

The hydrolysate was evaporated to dryness at 120° C under a stream of air

and the residue resuspended in 2 ml of 0.01 mol/l hydrochloric acid. The suspension was centrifuged and the supernatant transferred to a plastic tube containing 8 ml of 0.05 mol/l borax buffer pH 9.0. The solution was adjusted to pH 9.0 by the addition of a few drops of 4 mol/l sodium hydroxide solution, centrifuged and passed over a Sep-Pak silica column, previously washed with 0.1 mol/l hydrochloric acid in methanol (12 mol/l hydrochloric acid in water, diluted to 0.1 mol/l with methanol) and water. The Sep-Pak column was washed with 25 ml of water and eluted with 11 ml of 0.1 mol/l hydrochloric acid in methanol. Regeneration was performed by washing with 10 ml of 0.1 mol/l hydrochloric acid in methanol and 30 ml of water.

Derivatization

The eluate (11 ml) was evaporated to dryness at 80° C under a stream of air. To the residue were added 400 μl of a 5:1 (v/v) mixture of acetonitrile and heptafluorobutyric anhydride. The tube was sonicated for 5 min and stored at room temperature overnight.

Isolation of derivatives

The solutions were evaporated to dryness at room temperature under a stream of air and the residue dissolved in 1 ml of 0.5 mol/l phosphate buffer pH 7.0. The derivatives were extracted into 3 ml of dichloromethane. After drying the dichloromethane layer over a small amount of anhydrous sodium sulfate, the solution was evaporated to dryness at room temperature under a gentle stream of air. The residue was dissolved in 200 μl of ethyl acetate, and 2-μl aliquots were injected into the gas chromatograph.

RESULTS AND DISCUSSION

During the development of a new gas chromatographic method with flame ionization detection for the profiling in urine of the five naturally occurring polyamines — 1,3-diaminopropane, putrescine, cadaverine, spermidine, and spermine — we observed three relatively large peaks in the gas chromatograms of urines of a patient with a non-African, Burkitt-type, non-Hodgkin lymphoma. The elucidation of the structures and possible clinical importance of the three corresponding chemical compounds is the subject of this paper. More details about the methodology, quality control, normal values and values during various types of cancer will be described elsewhere.

Fig. 1A shows the gas chromatogram of a urine sample of the patient described above, in which three large peaks, assigned to 1,3-diaminopropane and two other substances named X_1 and X_2, can be seen. Their mass spectra are shown in Fig. 2. The mass spectrum of urinary 1,3-diaminopropane was found to be similar to that of the derivatized authentic compound.

Quantitative data, obtained from the determination of polyamines and the estimation of X_1 and X_2 in 56 24-h urines during a three-month follow-up of the patient, were collected. These data clearly show that the excretion of 1,3-diaminopropane, X_1 and X_2 run almost parallel to that of spermidine and spermine.

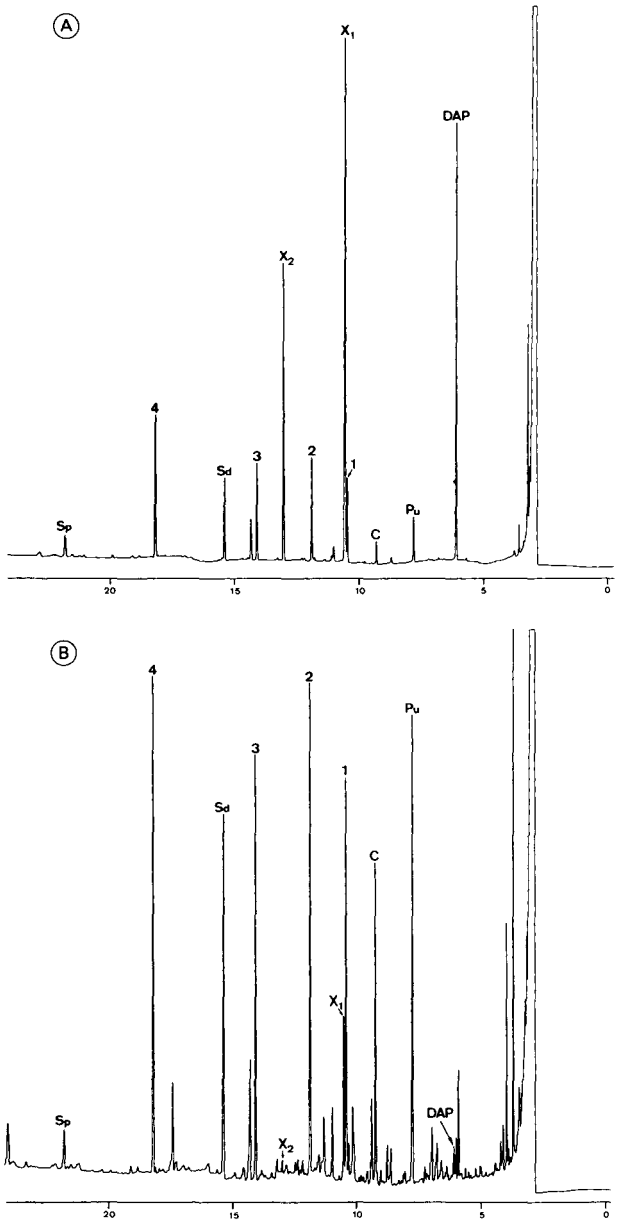

Fig. 1. Capillary gas chromatograms of the heptafluorobutyryl derivatives of polyamines extracted from the urine of a six-year-old patient with a non-African, non-Hodgkin, Burkitt-type lymphoma (A), and of a normal, healthy, 31-year-old adult (B). DAP = 1,3-diaminopropane; Pu = putrescine; C = cadaverine; 1 = 1,6-diaminohexane; X_1 = N-heptafluorobutyryl-γ-lactam form of isoputreanine; 2 = 1,7-diaminoheptane; X_2 = N,N'-diheptafluorobutyrylmethylester of isoputreanine; 3 = bis-(3-aminopropyl)amine; Sd = spermidine; 4 = 1,2-bis-(3-aminopropylamino)ethane; Sp = spermine; 1—4 are added internal standards. Time axis in minutes.

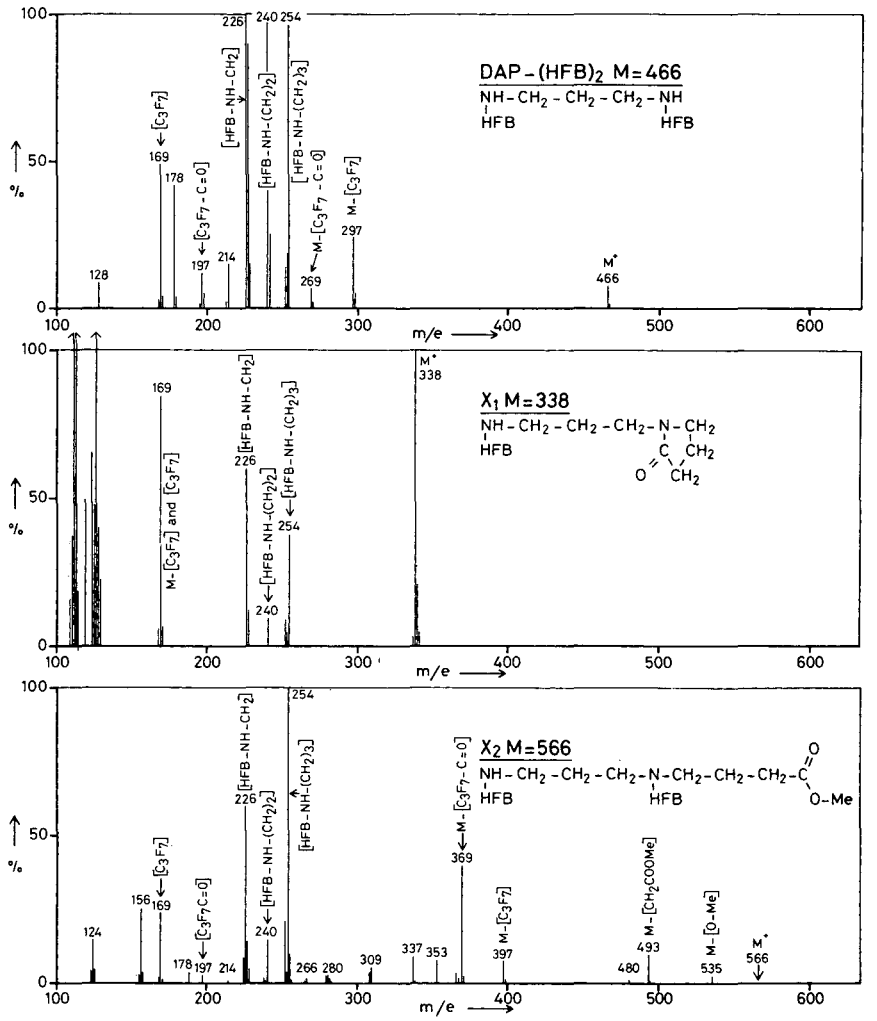

Fig. 2. Mass spectra of 1,3-diaminopropane (DAP), X_1 and X_2, obtained from a derivatized urinary extract of a patient with a Burkitt-type lymphoma. For gas chromatogram and abbreviations used see Fig. 1A. HFB, heptafluorobutyryl; Me, methyl.

The excretion of putrescine and of cadaverine was not related to spermidine, nor were they related to each other. This suggested that 1,3-diaminopropane, X_1 and X_2 are metabolically linked to spermidine and/or spermine and reflect cell turnover according to the concept of Russell [6].

On the basis of these findings, the mass spectral data and retention times, we were able to identify X_2 as the methylester—N,N'-diheptafluorobutyryl derivative of isoputreanine and X_1 as its N-monoheptafluorobutyryl-γ-lactam derivative (for structures see Fig. 2). The methylation of part of the isoputreanine was found to be an artefact introduced by evaporating the Sep-Pak eluate at 80° C, whereas γ-lactamization of isoputreanine occurs readily by dehydration during derivatization with heptafluorobutyric anhydride. In urine,

isoputreanine is most probably predominantly present as a conjugate since the analysis of unhydrolyzed urines did not show peaks with the correct retention times.

Isoputreanine has previously been identified in rat urine [8] and normal human urine [9]. On the basis of tracer experiments in rats, Asatoor [9] considered N^8-amino oxidation of (N^1-conjugated) spermidine, followed by oxidation of the intermediate aldehyde, to be a possible explanation for the formation of isoputreanine. Another possibility may be that, analogous to the spermidine dehydrogenase found in *Serratia marcescens* [7], spermidine and spermine are degraded by dehydrogenases, giving rise to 1,3-diaminopropane/Δ^1-pyrroline and 1,3-diaminopropane/isoputreanine, respectively. However, until now, neither enzymatic pathway leading to the formation of isoputreanine has been shown to exist in man.

Isoputreanine was excreted in relatively low amounts by normal persons (Fig. 1B). In the urine of an adult patient with non-Hodgkin lymphoma we observed a highly significant increase of isoputreanine during successful chemotherapy but not of spermidine. These data, together with the (in comparison to spermidine) much higher excretion of isoputreanine and 1,3-diaminopropane in the urine of the patient with Burkitt-type lymphoma, suggest that at least some cases of cancer are characterized by an increased degradative metabolic pressure on polyamines. The impact of this phenomenon on the diagnosis and follow-up of such patients using measurements of polyamines and/or their acetyl conjugates remains to be established. However, the ignorance of the polyamine degradative routes in health and disease may have considerably contributed to the scepticism concerning the use of determinations of polyamines as general markers for neoplastic diseases.

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Note

Determination of tulobuterol in human serum by electron-capture gas—liquid chromatography

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Tulobuterol—HCl {α-[(*tert.*-butylamino)methyl]-*o*-chlorobenzyl alcohol hydrochloride} (Fig. 1) is a new bronchodilator which has been shown, in animals and in early clinical studies, to possess an intensive and selective β_2 profile [1, 2].

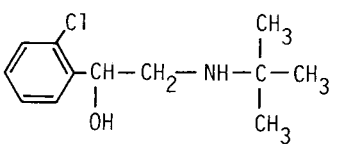

Fig. 1. Structure of tulobuterol.

Determination of the pharmacokinetic profile of a new therapeutic agent may be important for gaining a better understanding of its mechanism of action and for ensuring more efficient therapeutic application. Because of the low therapeutic dose of tulobuterol—HCl (1—3 mg/day), a sensitive analytical method is needed for its determination in human serum after oral administration.

In order to conduct a clinical pharmacokinetic study, we developed a selective and sensitive electron-capture gas chromatographic assay for the determination of tulobuterol in human serum.

EXPERIMENTAL

Reagents and materials

The tulobuterol—HCl used was synthesized [3] in our laboratory. 1,1-Bis-(4-fluorophenyl)-2,2-dichloroethane (internal standard) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and trifluoroacetic anhydride (TFAA) was from Nakarai Chemical (Kyoto, Japan). Other reagents and solvents were of analytical grade and were used without further purification.

Drug administration and sample collection

The healthy volunteers received an oral dose of 2 mg of tulobuterol—HCl in tablet form (2 × 1-mg tablet). Blood samples of about 8 ml were withdrawn before the administration and at 1, 2, 3, 4, 5, 6, 8 and 10 h. After the samples had been left to stand for 30—60 min, the serum was separated by centrifugation (2000 *g*, 10 min) and stored frozen until analysis.

Gas chromatographic conditions

A Shimadzu Model 5A gas chromatograph, equipped with a ^{63}Ni electron-capture detector operated at a pulse interval of 8 μsec, was used. A glass column, 2 m and 3 mm I.D., packed with 2% OV-1 on Chromosorb W AW DMCS, 60—80 mesh (Gasukuro Kogyo, Tokyo, Japan), was operated at a temperature of 130°C. The injection port and detector temperatures were 230°C and 250°C, respectively, and the carrier gas (nitrogen) flow-rate was 60 ml/min.

Extraction procedure and derivatization

One millilitre of serum was placed in a 15-ml glass centrifuge tube and 0.5 ml of 1.0 *N* sodium hydroxide was added. The tube was stoppered and extracted with 9 ml of hexane by shaking for 10 min. After centrifugation (2000 *g*, 5 min), 8 ml of the hexane layer were transferred to a second tube containing 2 ml of 1 m*M* hydrochloric acid (adjusted to pH 3.0), and the tube was shaken and centrifuged. After complete removal of the hexane layer, 0.5 ml of 1.0 *N* sodium hydroxide was added to the aqueous phase and extracted with 6 ml of hexane. Five millilitres of the hexane layer were transferred to a third tube and evaporated to dryness under reduced pressure in a water-bath at 30—35°C.

The extract was dissolved in 4 ml of ethyl acetate containing TFAA (1%, v/v) and the mixture was heated at 70°C for 45 min. After evaporation of the reaction mixture, 5 ml of distilled water were added to the residue which was finally extracted with 1 ml of hexane containing internal standard (35 ng). The tube was shaken, centrifuged, and part of the hexane phase (6 μl) was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Fig. 2 shows a gas chromatogram of a sample extracted from human serum

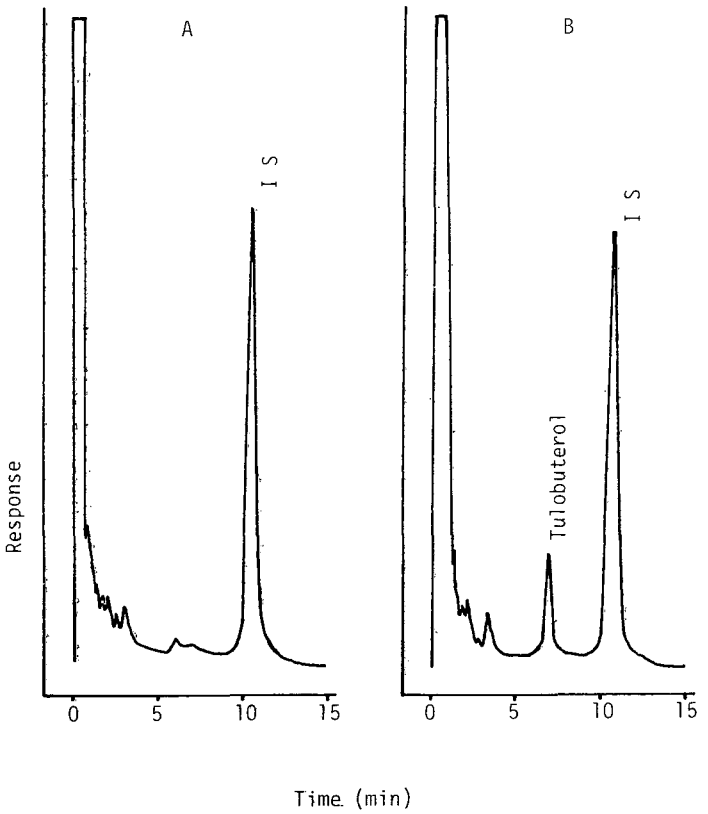

Fig. 2. Gas chromatograms of a serum extract. (A) Serum free from drug; (B) serum to which known amounts of tulobuterol—HCl (4 ng) and internal standard (IS) (35 ng) were added.

to which was added tulobuterol—HCl (4 ng/ml) and internal standard (35 ng/ml). The technique successfully separated the compound of interest from the endogenous materials in the serum.

The ratio of the peak height of tulobuterol to that of the internal standard varied linearly with the serum tulobuterol concentration in the range studied (up to 10 ng/ml). The stastical regression line can be presented as $Y = 0.130X + 0.006$ ($r = 0.9995$). The minimum detectable level of tulobuterol was 0.5 ng/ml.

Table I demonstrates the precision and accuracy of the present method. The recovery of our method was 95.5—102.1%. Over the therapeutic tulobuterol concentration range of below 10 ng/ml, the assay precision was satisfactory.

Electron-capture gas chromatographic analysis was employed for the determination of serum samples from an absorption study using ten subjects. Fig. 3 gives typical chromatograms for serum after oral administration of 2 mg of tulobuterol—HCl; no characteristic peaks other than that of unchanged tulobuterol were seen in the chromatogram.

Our previous study by mass fragmentography [4] quantitated the formation of some metabolites in human urine after a therapeutic dose of tulobuterol—

TABLE I

PRECISION IN THE DETERMINATION OF TULOBUTEROL IN SPIKED HUMAN SERUM SAMPLES

Concentration of tulobuterol—HCl (ng/ml)	Mean concentration found (ng/ml, ± S.D.)	Coefficient of variation (%) (n = 6)
2.00	1.96 ± 0.07	3.6
4.00	3.82 ± 0.18	4.7
7.00	7.15 ± 0.19	2.7

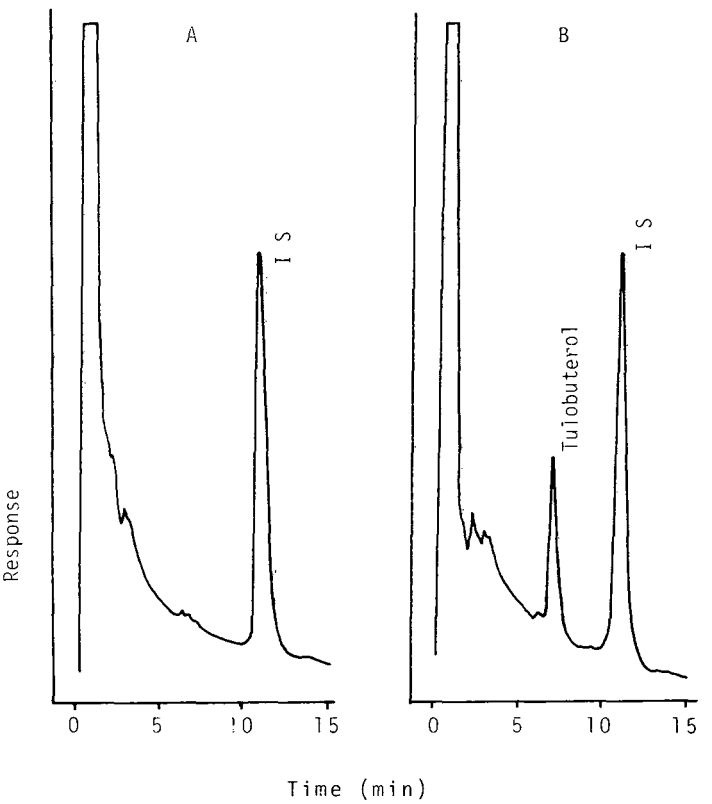

Fig. 3. Typical chromatograms of a serum extract (subject I). (A) Pre-dose; (B) 3 h after oral administration of 2 mg of tulobuterol—HCl.

HCl. So we also adapted this derivatization method with TFA to the ring-hydroxylated metabolites of tulobuterol, but did not succeed because of the instability of the resulting derivatives. However, bronchodilators which possess a hydroxy group on the benzene ring, such as sulbutamol [5] and terbutaline [6, 7], have been determined as their silylated derivatives, but no report about TFA derivatization of these drugs has been presented. From these facts, it

seems that the hydroxy group on the benzene ring would probably be responsible for the instability of this type of derivative.

The mean serum concentration—time curve of tulobuterol is shown in Fig. 4. A peak serum tulobuterol concentration of 6 ng/ml was reached at 3 h after dosing. The mean values for the elimination half-life and area under the concentration—time curve were 3.19 h and 30.5 ng h ml^{-1}, respectively.

The sensitivity and selectivity of this method make it suitable for bioavailability and pharmacokinetic studies.

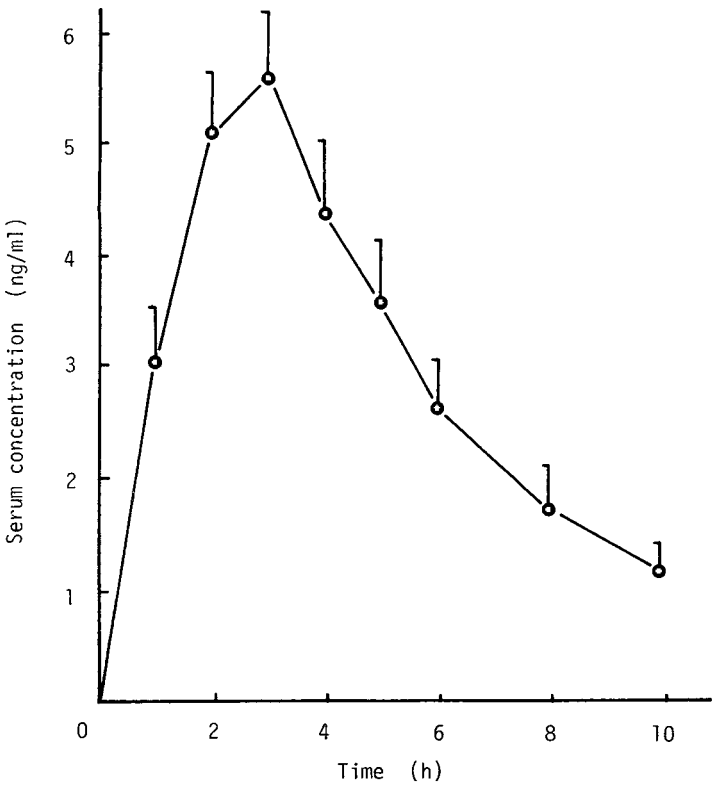

Fig. 4. Serum concentration versus time curve after oral administration of 2 mg of tulobuterol—HCl to human subjects (n = 10).

ACKNOWLEDGEMENTS

We are grateful to Mr. S. Kurata of our laboratory for supplying the reference sample of tulobuterol.

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Note

Gas chromatographic method for the determination of progabide (SL 76.002) in biological fluids

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(First received June 16th, 1981; revised manuscript received January 19th, 1982)

Progabide (Sl 76.002) is a novel γ-aminobutyric acid receptor agonist which easily crosses the blood—brain barrier and has shown a broad anticonvulsant spectrum in various animal models [1—4]. In man, progabide appears to possess an interesting therapeutic action in epilepsy and in spastic syndromes, as indicated by open pilot [5] and controlled clinical trials [6, 7].

The knowledge of the pharmacokinetic profile of any given drug is these days considered a necessary step for a better understanding of its pharmacodynamic profile, as well as for the rational definition of its therapeutic regimen. For these reasons, a rapid, sensitive and specific method for the determination of progabide in biological fluids has been developed using gas—liquid chromatography with electron-capture detection (GLC-ECD).

EXPERIMENTAL

Standards and reagents

Progabide, 4-{[(4-chlorophenyl-5-fluoro-2-hydroxyphenyl)methylene]amino}butanamide, and the internal chromatographic standard SL 78.050, 4-{[(4-chlorophenyl-5-chloro-2-hydroxyphenyl)methylene]amino}butanamide, were synthesised by Dr. Kaplan of the Department of Chemistry at L.E.R.S. [3]. Their structural formulae are shown in Fig. 1.

*Present address: Servier Laboratories Ltd., Horsenden Lane South, Greenford, Middlesex, Great Britain.

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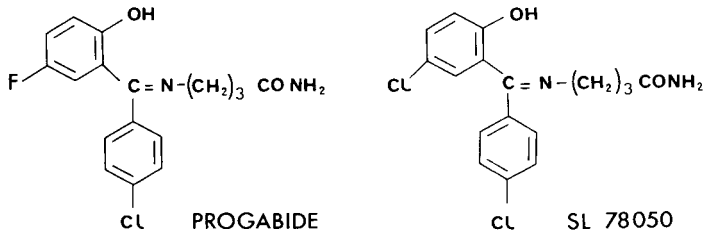

Fig. 1. Structural formulae of progabide and SL 78.050 (internal standard).

The solvents used were toluene, ethanol and *n*-hexane (analytical reagent grade, E. Merck, Darmstadt, G.F.R.); the derivatising reagent heptafluorobutyric anhydride (HFBA) was purchased from Fluka, Buchs, Switzerland.

Gas—liquid chromatographic conditions

Analyses were performed under isothermal conditions in a Perkin-Elmer Model 3920B gas chromatograph equipped with a ^{63}Ni linear electron-capture detector operating at −55 V (pulse current) with a 250-nsec width. The glass column (2 m × 2 mm I.D.) was packed with Gas-Chrom Q (80—100 mesh) coated with 3% OV-17 (Applied Science Labs., State College, PA, U.S.A.). The column was conditioned for 1 h at 270° C (nitrogen flow-rate 40 ml/min), for 4 h at 320° C (no nitrogen flow) and, finally, for 24 h at 280° C (nitrogen flow-rate 40 ml/min). The column temperature was 230° C, injection port and detector 275° C, and carrier gas (nitrogen) flow-rate 40 ml/min.

Mass spectrometric conditions

A Hewlett-Packard 5703 gas chromatograph (Avondale, PA, U.S.A.) coupled to a VG Micromass 70-70 mass spectrometer (Altringham, Great Britain) which was in turn connected to a VG 2050 series data system were used. The data system had a dynamic range of twelve bits (1:4095).

The GLC conditions were as described above. The mass spectrometer conditions were as follows: trap current 200 μA, source temperature 200° C, interface temperature 220° C, electron beam energy 70 eV.

Calibration graph and quantitation

Standard solutions of progabide (10 μg/μl) and of internal standard SL 78.050 (10 μg/μl) were prepared in ethanol. These solutions were stored at 4°C and under these conditions were stable for at least fifteen days. However, fresh solutions were prepared every week. Further dilutions were made to obtain lower concentrations for the standard graph. The standard graphs were prepared by adding 10, 30, 50, 100, 300, 500, 1000 ng of progabide and 500 ng of SL 78.050 to 1 ml of blank plasma. The samples were extracted according to the method described below and the extract derivatised by heating with HFBA at 60° C for 20 min. In order to remove excess reagent, the solution was evaporated to dryness under nitrogen at 60° C; 500 μl of hexane were added and 1 μl of this solution injected onto the column. The graphs were prepared by plotting the ratios of the peak height of progabide to the internal standard, against the known amounts of progabide. This curve was used to calculate the amount of progabide in unknown samples.

Extraction procedure

Spiked plasma samples for the calibration graph (see above) and plasma samples to be quantified were extracted at the same time and the procedure was as follows. SL 78.050 (500 ng) as internal standard, 100—500 μl of plasma (adjusted to 1 ml with the same acetate buffer), 1 or 2 ml of 0.2 *M* acetate buffer (pH 4.7) and 5 ml of toluene were added to a 10-ml stoppered tube. The tubes were gently agitated on a rotating mixer for 15 min. After centrifugation at 4°C for 10 min at 1000 *g*, 4.5 ml of the organic phase were transferred to a second series of test-tubes and then evaporated to dryness under nitrogen at 60°C. A solution of HFBA (10%, v/v) in ethyl acetate (200 μl) was then added to the dry residue and the derivatisation was carried out as described above.

An identical procedure was used for the analysis of urine.

RESULTS AND DISCUSSION

Representative GLC traces obtained from plasma samples of a dog which was given 300 mg/kg progabide orally, and a blank plasma spiked with the internal standard, are shown in Figs. 2A and B, respectively. The two peaks, one of derivatised progabide, retention time 2.4 min, and that of the derivatised internal standard SL 78.050, retention time 4.2 min, were not interfered with by peaks formed by any endogenous substances.

Calibration graphs were prepared as described above. A linear response was obtained up to 1000 ng with a regression coefficient of 0.998 (twelve points), a slope of 0.0043 ng^{-1}, and an intercept of 0.0026. The minimum concentration of progabide detectable in plasma was 1 ng/ml.

The reproducibility of the method was checked by repeating the analyses of plasma samples to which known amounts of progabide had been added (see Table I). A very small variation was observed (less than 2.5%).

GLC—mass spectrometric analysis of plasma samples confirmed the identity of the peaks with standards, and an investigation was made of the chemical identity of the HFB derivative of progabide. The mass spectrum of progabide derivatised with HFBA as above is given in Fig. 3. The molecular ion of *m/z* 512 is 18 a.m.u. less than expected for the monoacyl derivative.

There are several examples in the literature of the conversion of primary amides to nitrile upon treatment with either a perfluoroacyl anhydride or a silanising reagent [8, 9]. In this case, the formation of a nitrile would explain the observed molecular ion, and the unexpectedly good chromatographic properties. The derivatised authentic nitrile, analysed under exactly the same conditions as the derivatised progabide, gave the same spectrum with the same retention time (2.4 min). The principal high mass ions may be explained by a straightforward fragmentation: *m/z* 511, M—hydrogen; 493, M—fluorine; 477, M—chlorine; 458 (M—54), M—(CH_2CH_2CN).

Possible routes of the rearrangement ions *m/z* 125 (base peak) and *m/e* 138 are given in Fig. 4. These two assignments are supported by accurate mass determinations.

The described procedure has been applied to the analysis of several animal plasma and urine samples as well as of human plasma specimens from

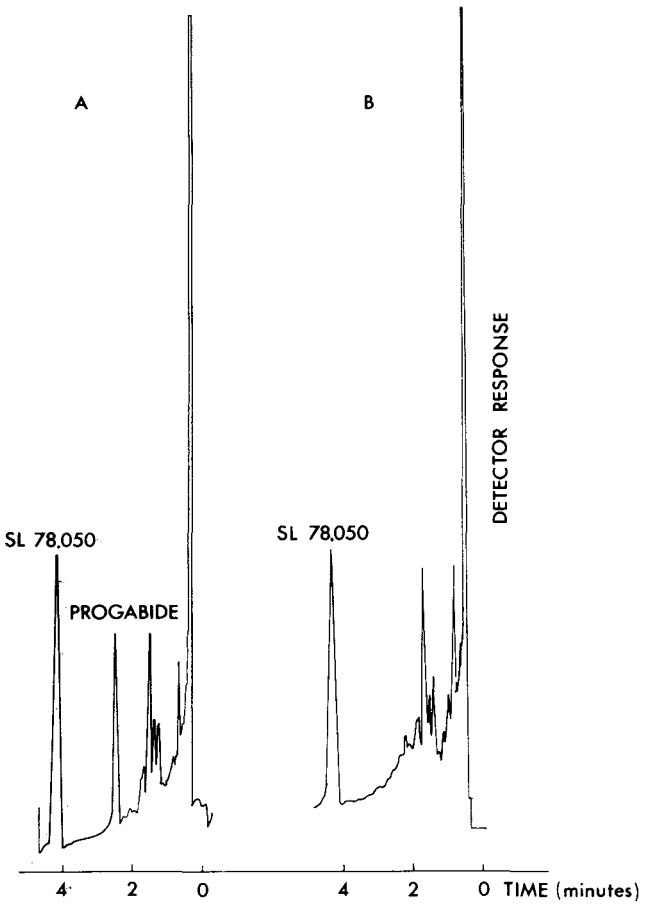

Fig. 2. Gas chromatograms obtained after extraction of plasma (200 μl) of a dog given 300 mg/kg progabide orally (A), and a blank dog plasma spiked with SL 78.050 (B). The tracings represent a concentration of 30 ng/ml progabide and 500 ng/ml internal standard.

TABLE I

CONCENTRATION OF PROGABIDE FOUND IN PLASMA FOR KNOWN AMOUNTS OF THE ADDED DRUG

Calculations were made on more than three determinations.

Amount added to plasma (ng/ml)	Amount recovered (ng/ml, mean ± S.D.)	Coefficient of variation* (%)
10	8.7 ± 0.34	3.9
30	27.6 ± 1.40	5.0
50	49.1 ± 3.40	6.9
100	104.0 ± 4.10	3.9
500	501.7 ± 15.50	3.1
1000	973.2 ± 41.30	4.2

*(S.D./mean) × 100.

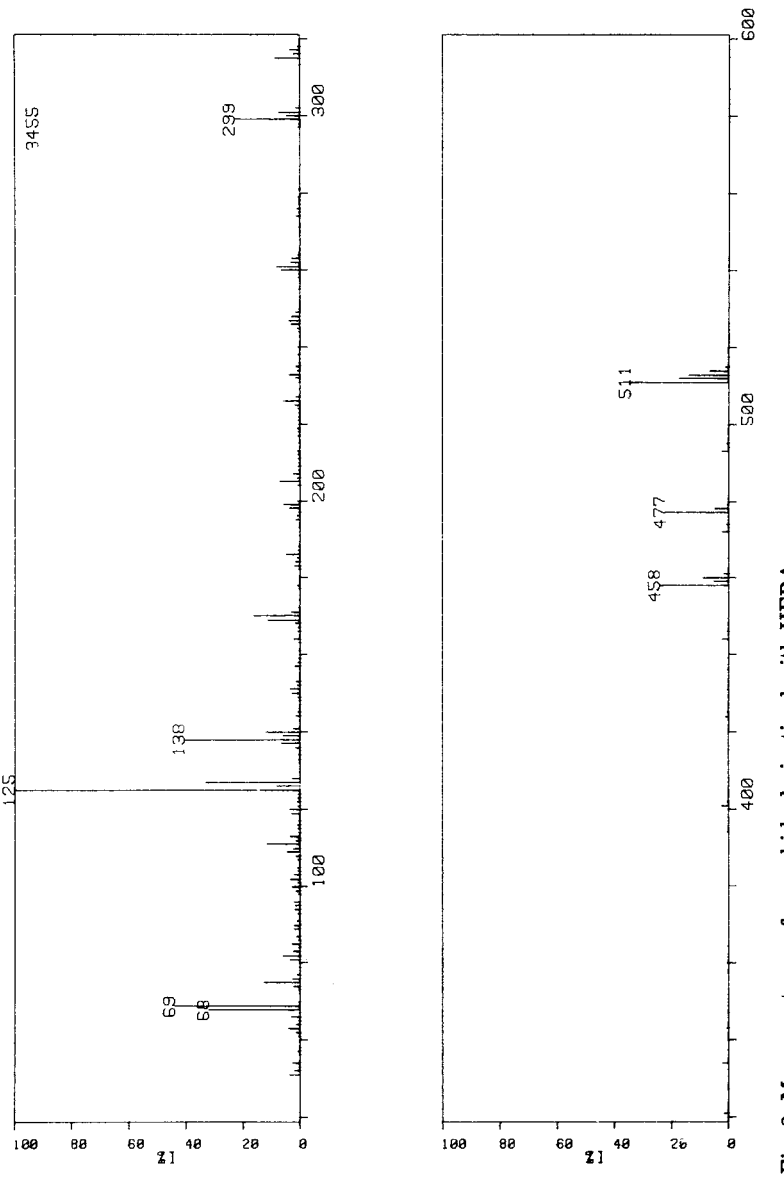

Fig. 3. Mass spectrum of progabide derivatised with HFBA.

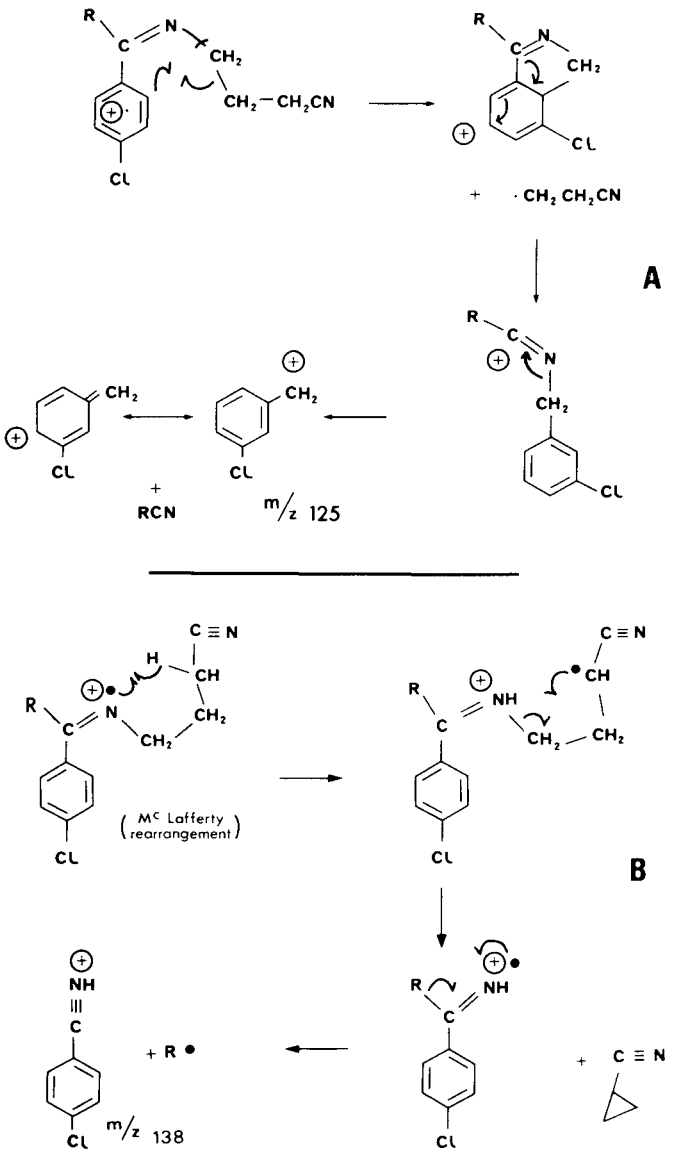

Fig. 4. Possible route of rearrangement of ions *m/z* 125 (A) and *m/z* 138 (B).

volunteers and patients. Representative plasma concentration curves over time following a single oral dose in rat, dog and man are reported in Fig. 5. Furthermore, endogenous substances and commonly used antiepileptic drugs were found not to interfere.

A skilled technician can run 35—40 samples a day.

Because of its simplicity, sensitivity and specificity, the described methodology has been found very suitable for both pharmacokinetic studies in experimental animals and man and for routine therapeutic drug monitoring during chronic treatment with progabide.

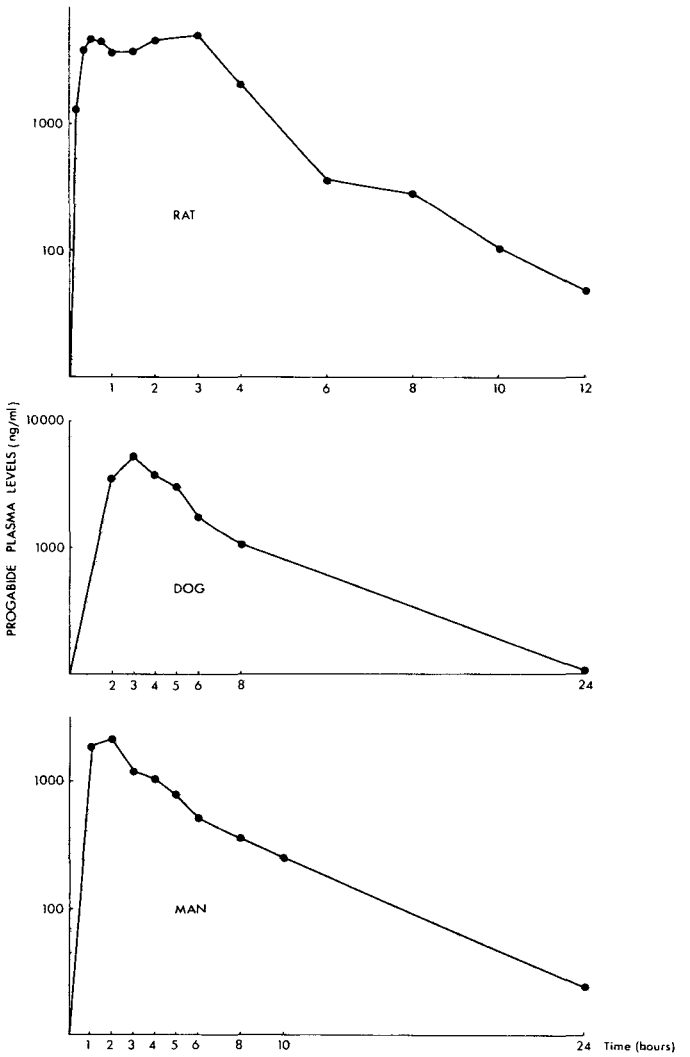

Fig. 5. Plasma concentration—time curves obtained after oral administration of progabide to rats (200 mg/kg), a dog (300 mg/kg) and a man (300 mg).

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Note

Determination of α-methyldopa and methyldopate in human breast milk and plasma by ion-exchange chromatography using electrochemical detection

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(First received October 23rd, 1981; revised manuscript received January 22nd, 1982)

Hypertension during pregnancy is not uncommon and may require drug therapy. α-Methyldopa and, if an injected form of the drug is required, methyldopate (methyldopa hydrochloride ethyl ester), are often prescribed. Since any drug regimen at this time may be dangerous the clinical need must outweigh the risk. The active treatment of hypertension during pregnancy however, has been shown to be associated with a significantly improved foetal outcome [1]. A disadvantage is that in the neo-natal period the child will carry a load of the drug transferred in utero [2, 3] and in addition may receive further doses from its mother's milk [2, 4]. At birth the child may have a sufficiently high plasma level of methyldopa to produce a clinically measurable effect [5]. Although the few results published so far [2, 4] suggest that there is little clinical significance in the small additional amounts transferred in the breastmilk, insufficient work has been done to confirm this and establish the amount excreted in the milk and its relation, both temporal and absolute, to methyldopa dosage.

Many assays for methyldopa in plasma and a few for methyldopate have been published. Most recently the methods are by high-performance liquid chromatography (HPLC) using a variety of strategies. Although some early work was reported using ion-exchange methods [6, 7], recent workers have favoured a reversed-phase column and an ion-pair technique [8—10]. This is a good method for the related catecholamines but less so for methyldopa. The use of 3,4-dihydroxybenzylamine (DHBA) [11] as an internal standard is becoming accepted for these assays as well as for those of the catecholamines. Detection by UV [7, 12] and also by fluorescence [2, 13] have been reported. However UV detection is not quite sensitive enough for the lowest level of drug found in clinical samples and, although more sensitive, fluorescence methods require a comparison with blank samples which may not always be available. Electrochemical detection is emerging as the method of choice [8—10]. This

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paper describes an assay using a strong cation-exchange material coupled with the sensitivity and specificity of electrochemical detection.

MATERIALS AND METHODS

Chemicals and reagents

2-Methyl-3-(3,4-dihydroxyphenyl)-L-alanine (L-α-methyldopa) and 3,4,-dihydroxybenzylamine were supplied by Sigma (St. Louis, MO, U.S.A.). α-Methyldopate was a gift from Merck Sharp and Dohme Research Laboratories (Hoddesdon, Great Britain). The internal standard (DHBA) was dissolved in the running solvent: 5 μl (equivalent to 2.5 μg DHBA) of a stock solution containing 500 μg/ml was added to each assay. The sulphatase preparation used was Helix pomatia juice (Industrie Biologique Française, Paris, France). All reagents and solvents were Analar grade.

High-performance liquid chromatography

Isocratic elution was performed with a Waters Assoc. (Milford, MA, U.S.A.) 6000A pump. Samples were applied through a Rheodyne 7125 injection valve with a 20-μl loop and detection made with a TL-8A transducer (glassy carbon electrodes) connected to an LC-4 electronic controller (Bioanalytical Systems, Lafayette, IN, U.S.A.). The column (24 cm × 4.6 mm I.D.) was packed with Partisil-10 SCX strong cation exchanger, particle size 10 μm (Whatman, Maidstone, Great Britain). The mobile phase was 33.8 m*M* perchloric acid (0.2% v/v), 50 m*M* sodium perchlorate and 0.2 m*M* EDTA in 20% (v/v) methanol in water at a flow-rate of 1.5 ml/min. Before starting all the stainless-steel surfaces to the top of the column were passivated with 2 *M* nitric acid. The column was then pre-washed with a solution of 250 m*M* sodium perchlorate in aqueous 0.2% (v/v) perchloric acid, followed by water (25 ml), then methanol (25 ml) before equilibrating with the eluting solvent. The column was run at ambient temperature. A hydrodynamic voltammogram for α-methyldopa with glassy carbon electrodes at a current of 100 nA was constant above an applied voltage of 0.75 V; the applied voltage used in these analyses was 0.80 V.

Extraction procedure and sample preparation

Methyldopa is present free and conjugated as the sulphate in both milk and plasma. Total methyldopa in milk was assayed after deconjugation of the sulphate with sulphatase. Milk (1 ml) containing DHBA (2.5 μg) was adjusted to pH 4.8 with 0.2 *M* acetate buffer, sulphatase (0.01 ml) added and the mixture incubated at 37°C for 48 h. Methyldopa was then assayed as below. Total methyldopa in plasma was assayed after acid hydrolysis, according to the method of Saavedra et al. [13]. The analytical method for the analysis of methyldopa in both milk and plasma was similar except that plasma samples were not submitted to a defatting step.

The milk—sulphatase—buffer mixture after deconjugation (see above), or for free methyldopa milk (1 ml) containing DHBA (2.5 μg), was cooled in ice and defatted by extraction with diethyl ether (3 × 1 ml). Separation of the layers by centrifugation was only found necessary for the final extraction volume. It is important at this stage to keep the mixture cold and avoid too vigorous

shaking, e.g. with the Whirlimixer, which produces an intractable gel. Residual ether was removed by bubbling nitrogen through the cooled sample. The defatted milk was deproteinised by first adding 4 *M* perchloric acid (0.1 ml per ml sample), Whirlimix for 15 sec, standing for 5 min then heating in a water bath at 90°C for 5 min. After cooling the mixture was centrifuged (at 2000 *g* for 10 min), the supernatant removed and the residue washed with water (1 ml), recentrifuged and the supernatants combined. Filtration through a 1.2-μm filter gave the sample for analysis.

Calibration curves and extraction yields

The validity of the method was established by adding known amounts of the compounds to drug-free samples of milk and plasma and assaying the mixture by the same extraction procedure described above; peak heights were plotted against the concentrations of the compound. Calibration curves were linear throughout the range studied as shown in Fig. 1 which also illustrates the greater response of the detector for the amine compared to the amino acid. Because of its particular clinical use methyldopate was only assayed in plasma.

The relative response of the detector to the analytes was determined using standard solutions of the compounds in the running solvent. Work-up of milk and plasma samples containing added known amounts of one compound with addition of another as standard at the end of the procedure enabled the efficiency of the method to be determined. Methyldopa and methyldopate are carried through the work-up of both milk and plasma without loss (95—106% recovered), however some DHBA is lost during the procedure (70—79% recovered).

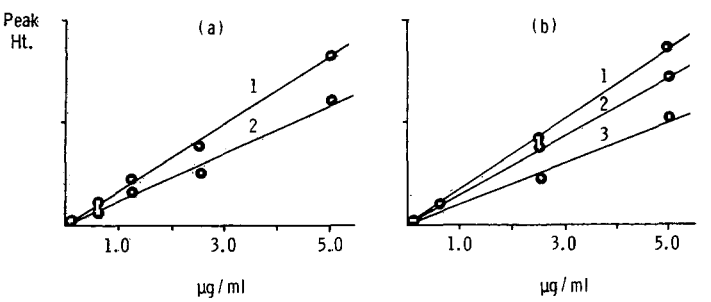

Fig. 1. Calibration curves of standard mixtures extracted from (a) milk and (b) plasma. 1, DHBA; 2, methyldopa; 3, methyldopate. The dots encompass the S.E.M. from replicate assays.

RESULTS AND DISCUSSION

Milk is well recognised by the analyst as an awkward matrix to work with, human milk presenting more problems than cow milk. An assay as described herein for non-lipid-soluble drugs requires the removal and discarding of the fat as a preliminary step. The lipid concentration of milk varies throughout a feed increasing to the end. For the assay of non-lipid-soluble materials it is preferable therefore to obtain a sample by expression from a full breast thus obtaining a sample containing less lipid. Mechanical removal of the fat after centrifu-

gation followed by cooling in ice [14] was not satisfactory in our hands due to low mechanical strength of the fat pellet in our samples. Extraction with diethyl ether proved superior if care was taken not to agitate the system excessively.

The removal of proteins from the sample is not a simple matter as human milk contains the soluble, or whey, proteins lactalbumin and lactoglobulin. Whey proteins are not coagulated by acid but they are coagulated by heat. Thus precipitation of casein and similar proteins by perchloric acid was followed by heating to precipitate the remaining soluble protein material. This work-up resulted in no loss of the analytes methyldopa and methyldopate but a consistent loss of some of the internal standard both from milk and plasma. Also, under these conditions no observable hydrolysis of the conjugated drug occurred.

The problems arising from the assay of the O-sulphate have not previously been discussed fully. It has been stated that deconjugation with sulphatase results "in substantial degradation of methyldopa" [15]. We have found that loss of methyldopa and the internal standard occurs upon incubation, with and without the enzyme present, in plasma samples. There is no similar loss in milk samples, it is not unreasonable to propose that the fat layer of the milk is protective possibly by the exclusion of oxygen. We explored the alternative acid hydrolysis for the plasma samples. Generally losses occurred or interfering compounds developed. Most previous assays have relied upon fluorescence measurements: loss of methyldopa, as measured by HPLC with electrochemical detection, may not be loss of a fluophore. It is almost certain that plasma levels of the O-sulphate conjugate have no pharmacological significance and therefore we have not exhaustively explored the possibilities of assaying plasma for this compound. Milk, like urine, represents an end compartment and the concentrations of conjugate therein are the result of active or passive transport from the blood. The pharmacological significance of the conjugate in the milk relies upon gut absorption and the sulphatase activity in those compartments of the infant where the drug conjugate appears.

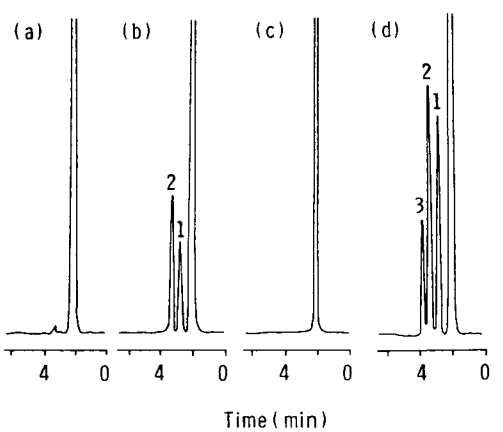

Fig. 2. Chromatograms showing the separation of (1) methyldopa, (2) DHBA and (3) methyldopate extracted from (b) milk and (d) plasma (2.5 μg/ml; 20 nA) compared to drug-free extracts of (a) milk and (c) plasma at the same sensitivity.

The chromatograms in Fig. 2 show the adequate separation and good peak symmetry obtained with this method. No significant background interferes with the assay over the concentration range studied but it was noted that an extension of the assay, looking for the drugs in erythrocytes, would be frustrated in the case of methyldopate by the presence of a background peak. The sulphatase preparation used did not introduce any interfering compound. Neither was there interference from other drugs prescribed: anti-anxiolytics, diuretics and anti-bacterials.

The use of an ion exchanger rather than an ion-pairing technique apparently gives superior results and eliminates the many problems associated with the latter method. No degradation of the column performance was noted over several months. When not in use the column and detector were stored with 20% methanol in water, or methanol when the period was a week or more, and reequilibration with the running solvent was quickly established.

The test of any assay method is its use for real samples. Unfortunately throughout the period of this study only samples containing methyldopa were received. Methyldopate is however hydrolysed in vivo to methyldopa [7, 13] and we have shown that the assay works for the mixture of the two compounds that is found clinically when this drug is prescribed [7, 13]. The results are shown in Table I. The concentration of methyldopa, free and conjugated, is less in milk than in plasma for samples collected at the same time as expected for a weakly acidic material. Milk (average pH 7.0) is more acid than plasma (average pH 7.4) and the distribution of a drug between the two, in the absence of an active transport system, may be calculated by the method of Rasmussen [16], using a modification of the Henderson–Hasselbach equation:

$$\frac{[\text{milk}]}{[\text{plasma}]} = \frac{1 + 10^{\text{pH}_{\text{milk}} - \text{p}K_a}}{1 + 10^{\text{pH}_{\text{plasma}} - \text{p}K_a}}$$

The pK (COOH) of methyldopa is 2.2, thus for plasma at pH 7.4 and milk pH 7.0 the distribution of the drug [milk]/[plasma] = 0.40. This compares well

TABLE I

PATIENTS TAKING METHYLDOPA (n = 8), 0.25—1.5 g PER DAY DIVIDED DOSE, WHILE BREASTFEEDING

As time of dose is different and variable from time of sampling there is no correlation between dose and drug levels.

Sample			Concentration of drug ± S.D. (μg/ml)
Milk	Free	(n = 14)*	0.17 ± 0.08
	Conjugated	(n = 17)*	0.26 ± 0.16
Plasma**	Free	(n = 8)	0.37 ± 0.26

*When only trace quantities (< 0.05 μg/ml) were detected these are not included.

**Consistent results for conjugated drug in plasma were not obtained. Inconsistencies were reproducible, differed between patients but not between samples from the same patient; a probable reason is polypharmacy.

with the figure of 0.46 calculated from the total number of samples examined and 0.41 when paired milk and plasma samples only are included.

ACKNOWLEDGEMENTS

The authors are grateful to Mrs D.C. Clark for some preliminary work, The National Childbirth Trust for a supply of drug-free milk and Mr G. Buddon for the clinical samples used in this study.

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Note

Determination of sodium flavodate in body fluids by high-performance liquid chromatography

Application to clinical pharmacokinetic studies

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Sodium flavodate (disodium-5-phenyl-5,7-dioxyacetate-benzo-γ-pyrone, Pericel®, LIRCA (Farmaceutici, Limito, Milan, Italy) is a synthetic hydrosoluble derivative of natural flavonoids (Fig. 1) [1]. Sodium flavodate (SF) has been shown to reduce capillary fragility and permeability [1—3].

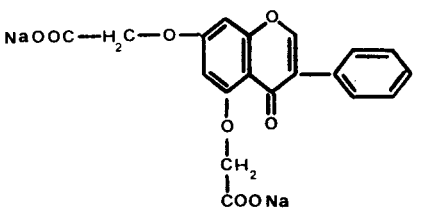

Fig. 1. Chemical structure of sodium flavodate.

Whereas gas and liquid chromatographic methods for the quantitation of flavonoids in natural products, such as quercitine in tea leaves and soy beans, have been described, no methods for the determination of these compounds in plasma and/or urine can be found in the literature [4—9].

In the present work we describe a high-performance liquid chromatographic (HPLC) method for the determination of SF in plasma and urine and its application to the study of SF pharmacokinetics in man.

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EXPERIMENTAL

Reagents and standard solutions

Sodium flavodate was supplied by LIRCA, and 1-naphthylacetic acid (NAA, internal standard) was obtained from Fluka (Buchs, Switzerland). Stock solutions at a concentration of 1 mg/ml were prepared in distilled water containing 1 mg/ml $NaHCO_3$. Working standard solutions, at a concentration of 100 μg/ml, were prepared by dilution in water.

Methanol, phosphoric acid, hydrochloric acid and diethyl ether were analytical grade reagents (Merck, Darmstadt, G.F.R.).

Apparatus and chromatographic conditions

A Perkin-Elmer (Norwalk, CT, U.S.A.) series 2/2 high-pressure liquid chromatograph equipped with a Perkin-Elmer LC-75 variable-wavelength UV detector and Autocontrol system was used.

The column was a LiChrosorb RP-18 (10 μm particle size, 25 cm × 4.0 mm I.D.) from Merck. The mobile phase was prepared by mixing 60 volumes of methanol with 40 volumes of 0.1% phosphoric acid for plasma and 57:43 for urines. A flow-rate of 1.3 ml/min and a wavelength of 268 nm were used. This wavelength was chosen as this gives the best signal-to-noise ratio on the UV spectrum recorded by the Autocontrol system.

Samples were introduced via a syringe into a Rheodyne 7105 (Berkeley, CA, U.S.A.) injection valve.

Procedure

Into a 12-ml tube were placed 1 ml of plasma or urine, 300 μl of hydrochloric acid (18.5%), 100 μl of the internal standard solution and 7 ml of diethyl ether, and the tubes were shaken on a reciprocal shaker for 10 min. After centrifugation at 2000 rpm for 15 min, 6 ml of the organic phase were transferred to other 12-ml tubes and evaporated to dryness under a gentle flow of nitrogen at room temperature. The residues were reconstituted with 100 μl of the mobile phase and 10—20 μl were injected into the chromatograph.

In addition to the unknown samples, plasma and urine calibration standards containing 0.1, 0.5, 1, 3, 5, and 10 μg of SF and 10 μg of NAA were prepared and processed as above. A calibration curve was constructed by plotting the SF concentrations versus the ratio of SF to the NAA peak heights for both plasma and urine.

Typical chromatograms of plasma and urine extracts are shown in Figs. 2 and 3.

Recovery

Percentage recovery was calculated by comparing the peak heights of SF standards prepared in mobile phase solutions with those obtained by injecting plasma and urine calibration standards at the same concentrations.

Subjects and pharmacokinetic measurements

Six healthy male volunteers (23—33 years, 59—90 kg) took two tablets containing 500 mg of sodium flavodate with 100 ml of water at 8.00 a.m. after 12

TABLE I

PLASMA CONCENTRATIONS (μg/ml) AFTER ORAL ADMINISTRATION OF 1 g OF SODIUM FLAVODATE

Subjects	Weight (kg)	Hours after administration							AUC 0—24 h (μg h ml^{-1})
		0.5	1	2	4	8	10	24	
RP	62	2.20	1.01	0.70	0.51	0.04	0.06	<0.05	6.01
MS	68	1.70	0.51	0.52	0.45	0.30	0.35	0.25	8.56
PV	90	0.45	0.50	0.55	0.55	0.20	0.20	<0.05	4.52
SQ	72	0.85	0.73	0.55	0.48	0.25	0.12	<0.05	4.60
LG	59	3.00	3.41	2.00	1.50	0.72	0.45	0.18	17.52
LZ	77	1.39	0.77	0.56	0.38	0.30	0.23	0.16	6.96
Mean ± S.E.	71.3 ± 4.5	1.60 ±0.40	1.15 ±0.46	0.81 ±0.24	0.64 ±0.17	0.36 ±0.08	0.23 ±0.06	0.12 ±0.03	8.04 ±1.994

h of fasting. Blood samples were drawn 0.5, 1, 2, 4, 6, 10 and 24 h later and 0—12 h and 12—24 h urines were collected. Blood samples were immediately centrifuged and the plasma transferred and stored at −30°C until analysis.

The following pharmacokinetic parameters were calculated from results in Table I. Elimination constant rate (K_e) and half-life were determined by linear regression analysis of the concentrations between 6 and 24 h. The experimental area under the curve ($AUC^{0\rightarrow n}$) was measured by the trapezoidal rule and $AUC^{0\rightarrow\infty}$ by adding the extrapolated term $C(n)/K_e$ to $AUC^{0\rightarrow n}$, where $C(n)$ is the last determined plasma concentration.

RESULTS

Chromatograms of blank plasma and urine extracts do not show interfering peaks and both SF and NAA peaks were well separated. Retention times of SF and NAA were 6.0 and 7.5 min, respectively, for plasma, and 7.5 and 9.5 for urine analysis (Figs. 2 and 3). With this method, SF could be assayed quantitatively over a wide range of concentrations with a linear relationship between 0.1 and 20 μg/ml, and a recovery from either plasma or urine of 95 ± 7%. The limit of sensitivity of the method (50 ng/ml) was sufficient for pharmacokinetic studies of SF.

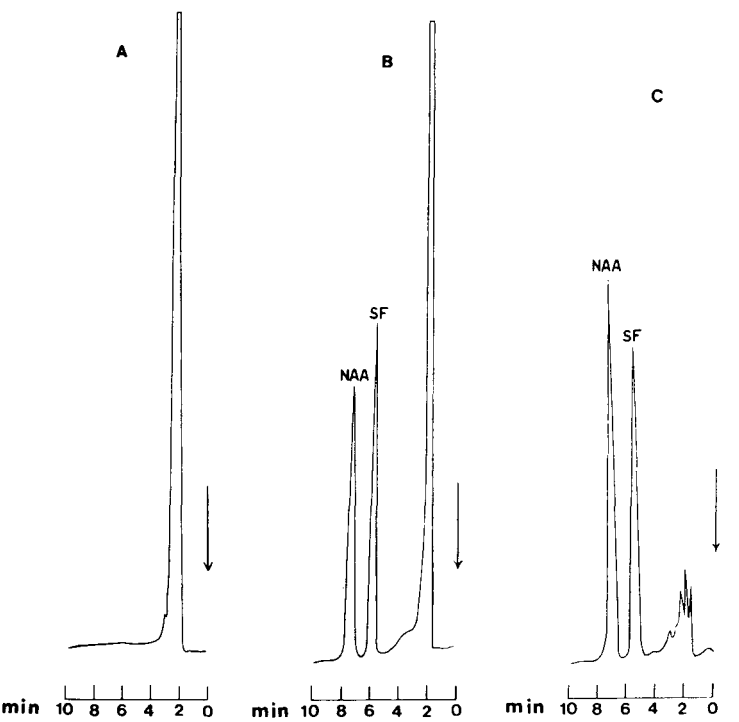

Fig. 2. Chromatograms of HPLC analysis of plasma extracts: (A) human control plasma; (B) human control plasma containing 5 μg/ml SF and 10 μg/ml NAA (internal standard); (C) plasma of a subject receiving 1 g of SF and containing 10 μg/ml NAA.

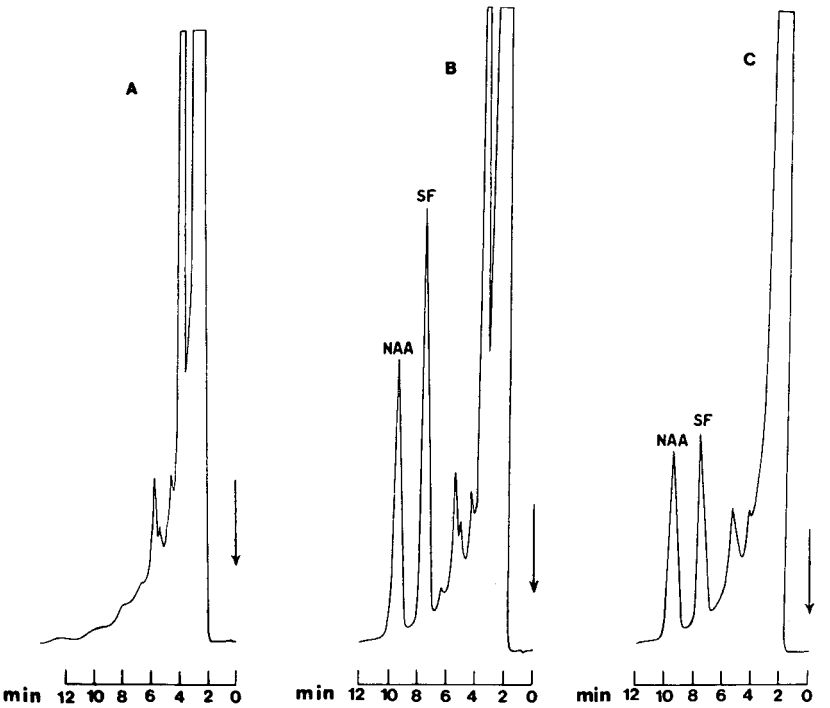

Fig. 3. Chromatograms of HPLC analysis of urine extracts: (A) human control urine; (B) human control urine containing 5 μg/ml SF and 10 μg/ml NAA (internal standard); (C) urine of a subject receiving 1 g of SF and containing 10 μg/ml NAA.

Peak concentrations (1.6 ± 0.4 μg/ml, $\bar{X}$ ± S.E.) were found 30 min after oral administration of SF. Thereafter, levels decayed biphasically with a mean elimination rate (K_e) of 0.057 and a half-life of 12.1 h (Fig. 1). The mean $AUC^{0\rightarrow\infty}$ was 10.2 ± 2.0 μg h ml^{-1}. A linear relationship was found between body weight and both peak plasma concentration and AUC (r = 0.69 and 0.64, $p < 0.05$).

The amount of unconjugated SF in urine was 4.6 ± 0.6 mg in the first 12 h and 0.9 ± 0.2 mg in the following 12 h. Thus, cumulative excretion of SF in the 24 h after dosing represents less than 1% of the administered dose.

DISCUSSION

A simple and sensitive HPLC method for the determination of sodium flavodate in plasma and urine is described. The method can be used for quantitative determination of SF and has been shown to be suitable for pharmacokinetic studies.

The HPLC method presents several advantages over the gas—liquid chromatographic (GLC) methods described for some natural flavones (e.g. quercitine) [8, 9]. The HPLC procedure is more sensitive and simpler than the GLC one, avoiding the necessity of derivatizing the hydroxyl and carboxyl groups. Moreover, for SF we found that the trimethylsilyl derivative used for the GLC

analysis of quercitine was unstable and therefore unsuitable for the GLC analysis of SF [8].

The determination of plasma concentrations of SF showed rapid absorption of the compound after oral ingestion, the peak concentration being observed at 0.5 h. Thereafter, the concentration decayed biphasically with a mean elimination half-life of 12 h. The peak concentration and the AUC for plasma values were quite low in view of the large dose administered. Since there are no data on the bioavailability of the drug after oral administration, the low levels might be interpreted to indicate poor absorption or a large volume of distribution.

In favor of this latter interpretation there are the results obtained by Gazave [2], who showed that SF is firmly bound to the lecithin of vascular membranes. If this were so, a large amount of the drug would be extracted from the circulation with the consequence of increasing the apparent volume of distribution of the drug.

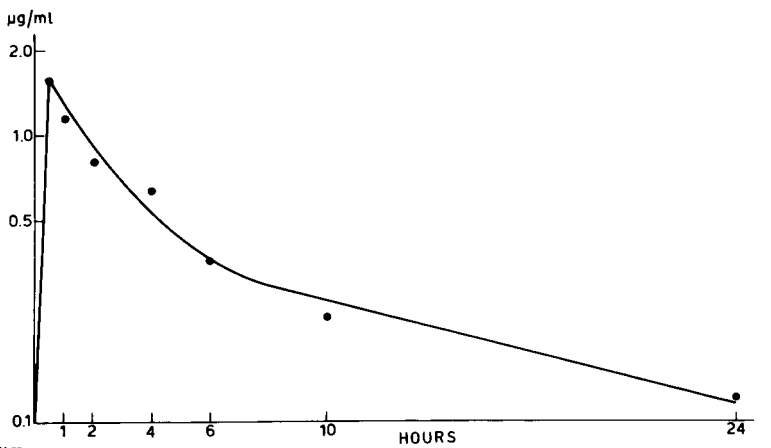

Fig. 4. Time—course curve of mean plasma levels of six subjects who took 1 g of SF orally.

The amount of unconjugated SF excreted in the urine in the first 24 h was less than 1% of the administered dose. This can be interpreted in the same way as the plasma levels, but, alternatively, one could suppose that SF was metabolized extensively and excreted as one or more metabolites. We have no data about how SF is metabolized and no metabolites have been identified so far in urine or other biological fluids, including bile. On the other hand, Gazave [2] suggested that SF could be rapidly transformed to active metabolites such as flavanol, but evidence supporting this is still incomplete. In our experiment, the chromatograms of urine extracts did not show at any time peaks that could be attributed to SF metabolites. Of course, this is not sufficient to disprove metabolism, since the procedure was not set up to determine these compounds.

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Note

Liquid chromatographic separation and quantitation of 2-amino-1,3,4-thiadiazole (NSC-4728) from human and murine serum

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The compound 2-amino-1,3,4-thiadiazole (ATDA; NSC-4728) is currently undergoing Phase I clinical trials as an experimental antitumor agent at the Vermont Regional Cancer Center (Fig. 1). Previous clinical investigational trials with ATDA have been limited due to the drug-mediated production of stomatitis and hyperuricemia [1, 2]. These adverse side effects can now be adequately controlled by the administration of nicotinamide which abrogates all effects of ATDA and allopurinol which blocks the excessive formation of uric acid [2, 3].

Pharmacokinetic studies of ATDA have demonstrated half-lives of 2.9 h in mice [4] and 10 h in dogs [5]. Similar studies had not yet been performed in humans. Therefore, in order to evaluate the pharmacokinetics of ATDA, a procedure for the routine quantitation of drug from serum samples was required. A combined system of thin-layer (TLC) and high-performance liquid chromatography (HPLC) was developed to enable the user to quantitate not only ATDA but also allopurinol and nicotinamide.

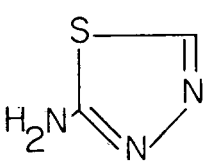

Fig. 1. Structure of 2-amino-1,3,4-thiadiazole (ATDA).

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EXPERIMENTAL

Standards and reagents

HPLC grade water, 2-propanol, acetonitrile, and methanol were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). ATDA (NSC-4728) was obtained from the Investigational Drug Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD, U.S.A.). Allopurinol (Zyloprim®) is a product of Burroughs-Wellcome (Research Triangle, NC, U.S.A.). Nicotinamide and uric acid were obtained from Sigma (St. Louis, MO, U.S.A.). Paired-ion chromatographic reagent (Pic B-8, 5 m*M* 1-octanesulfonic acid) was purchased from Waters Assoc. (Milford, MA, U.S.A.).

Stock solutions of ATDA, allopurinol, nicotinamide, and uric acid were prepared by dissolving the compounds in HPLC grade water. Further dilutions were then prepared as needed for injection.

Chromatographic apparatus

A Spectra Physics Model 8000 microprocessor-controlled high-performance liquid chromatograph equipped with a data system was used. The chromatograph was equipped with a Schoeffel Model 770 variable-wavelength UV detector set at 254 nm. The column (30 cm × 3.9 mm I.D.) was a 10-μm particle size reversed-phase C_{18} μBondapak (Waters Assoc.). A guard column (7 cm × 0.2 mm I.D.), packed with Co:Pell ODS, 30—38 μm particle size (Whatman, Clifton, NJ, U.S.A.) was installed to protect the main column. Samples were injected onto the column through a 100-μl loop using a manual injector. Avicel-F (250-μm particle size) TLC plates were obtained from Analtech (Newark, DE, U.S.A.).

Drug administration and serum collection

Human. ATDA was administered to patients with cancer unresponsive to conventional therapy after written informed consent was obtained. Drug was administered over a 30-sec period by injection into a running intravenous line containing normal saline. Doses up to 200 mg/m^2 of body surface area (4 to 5 patients per dose) have been examined so far in this Phase I trial. Approximately 10 ml of whole blood were obtained from the arm contralateral to that used for drug injection. Blood samples were centrifuged (600 *g*, 10 min) and the resulting serum was stored up to 1 week at —20° C.

Mouse. Female CD-1 mice (18—22 g) were injected intravenously (tail vein) with 300 mg/m^2 of body surface (100 mg/kg). Blood samples were obtained by retro-orbital puncture.

Sample preparation and drug analysis

Human serum samples were prepared for TLC by addition of 9 volumes of acetonitrile. After centrifugation (600 *g*, 10 min at 5° C), the supernatant was collected and brought to dryness under vacuum with air purging at 37° C using a Fisher IMD sample concentrator (Fisher Scientific, Pittsburgh, PA, U.S.A.). The residue was reconstituted with 0.4 ml HPLC grade water. The samples (50-μl aliquots) were manually spotted onto the TLC plates along with the appropriate ATDA, nicotinamide, allopurinol, and uric acid standards. Murine

serum contained sufficient ATDA concentration to permit spotting of 10 μl of serum directly onto the TLC plates.

TLC plates were developed at ambient room temperature in a closed chamber over a 5-h period using a solvent system of 2-propanol—water (70:30, v/v). This procedure resulted in R_F values of 0.42 for uric acid, 0.78 for allopurinol, 0.80 for ATDA and 0.92 for nicotinamide. UV (254 nm) absorbing spots, which corresponded to uric acid or nicotinamide standards, were scraped separately from the plate, eluted in 0.4 ml water, sonicated and analyzed individually with the HPLC system. The single spot containing both ATDA and allopurinol was likewise scraped from the plate, eluted in 0.4 ml water with sonication and injected onto the HPLC apparatus.

HPLC conditions include a mobile phase consisting of an isocratic system of water—methanol (99:1, v/v) and the paired-ion reagent 1-octanesulfonic acid (buffered to a pH of 3.0) at a final concentration of 5 m*M* with a flow-rate of 1.8 ml/min. The concentration of each of the four compounds was measured by UV absorption at 254 nm which was considered optimal for ATDA. Quantitative analysis was based on peak areas which were computed using a preset integration program in the software data system of the Spectra-Physics instrument.

RESULTS AND DISCUSSION

The initial sample preparation, which consists of deproteinization with acetonitrile and TLC, permits removal of interfering protein, concentration of the resulting supernatant and the separation of ATDA and allopurinol from uric acid as well as nicotinamide. HPLC injection of a water extract of the ATDA—allopurinol TLC spot provides complete separation and quantitation of these two drugs. Shown in Fig. 2 is a chromatogram demonstrating the separation of all four compounds. The use of this method, which was specifically designed for the analysis of ATDA, permits routine quantitation of this drug in biological fluids, such as serum, that contain ATDA levels as low as 400 ng/ml. Fig. 3 consists of two chromatograms: a demonstration of a serum blank obtained immediately prior to the administration of ATDA and the same patient's serum 5 min post ATDA administration.

A standard calibration curve for ATDA was prepared daily by plotting peak area against the concentration of injected drug. This relationship was linear over a one-hundred-fold concentration range (5—500 ng of injected drug) with an average correlation coefficient of 0.996 using the least squares regression method. The minimal detectable level of injected ATDA was 10 ng at a detector setting of 0.01 a.u.f.s. and a recorder attenuation of zero. The signal-to-noise ratio was five or greater under these conditions.

To determine the recovery and reproducibility of the method, 1-ml aliquots of serum were spiked with ATDA to yield drug concentrations of 25, 50, 100 and 200 μg/ml prior to the deproteinization step. These samples were compared to the peak areas from directly injected ATDA standards. An average recovery of 89.6 ± 1.3% was obtained at all drug concentrations, with an intra-assay coefficient of variation of less than 2.0% (n = 5). This procedure was used also as an external standard method of monitoring day-to-day percent

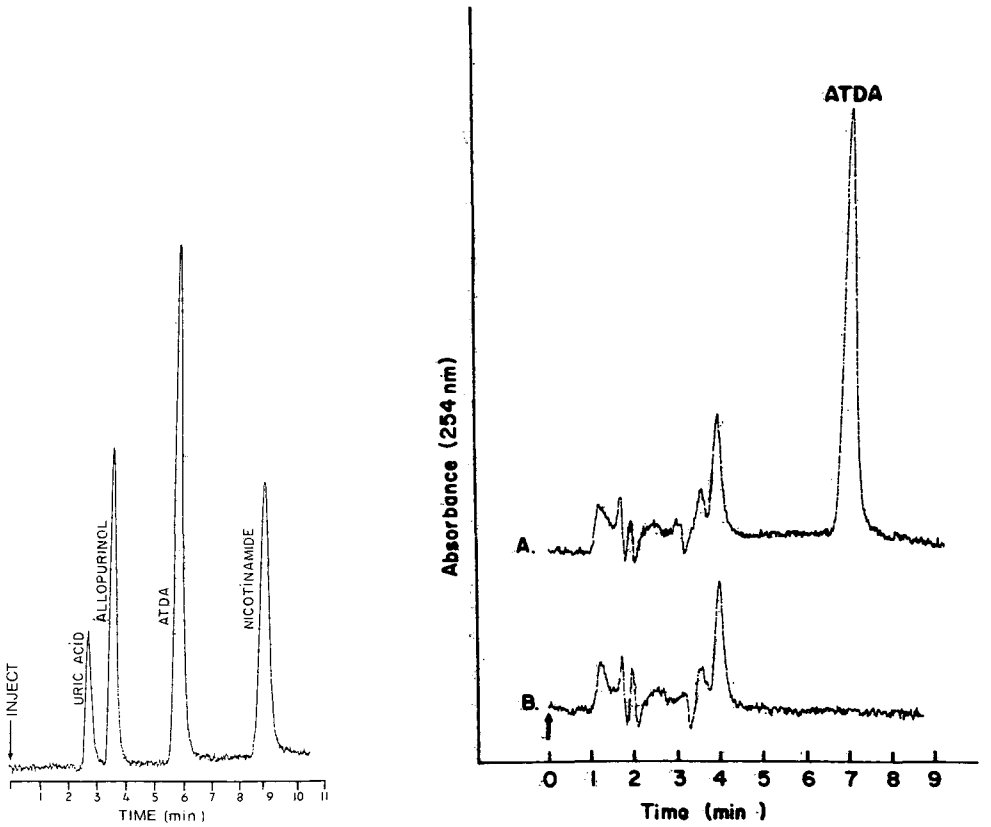

Fig. 2. Typical HPLC trace demonstrating separation of uric acid, allopurinol, ATDA, and nicotinamide. A single stock solution containing all four compounds was prepared. A 100-μl aliquot (containing 250 μg of each compound) was then injected manually onto the column. Separation of the compounds was achieved as described in Experimental.

Fig. 3. Representative chromatograms of TLC-processed serum samples obtained from a patient 5 min after administration of 125 mg/m^2 ATDA (A) and a pre-drug administration control serum sample (B). Peaks eluting prior to 4 min were not identified. Arrow indicates point of injection.

recovery of ATDA from human serum. Unfortunately, an internal standard was not feasible as there are not any established metabolites or close structural analogues of ATDA readily available. Inter-assay variability of ATDA standard proved to have a coefficient of variation of less than 2.5% (n = 17).

No retention of ATDA was observed on a reversed-phase column using simple mobile phases of water and methanol. The use of 1-octanesulfonic acid as a paired-ion reagent in the acidic mobile phase resulted in sufficient retention for accurate separation and quantitation. Pic B-8 reagent is buffered to a pH of 3.0 with acetate and when mixed with the mobile phase described, results in a constant pH of 3.0. The relative size of the lipophilic group on the counter ion affected the degree of retention of ATDA on the C_{18} reversed-phase column. Retention time of ATDA was observed to decrease when the paired-ion reagent 1-octanesulfonic acid was changed to 1-pentanesulfonic acid. This phenomenon has also been described for drugs other than ATDA [6].

This method satisfactorily determines ATDA in the presence of such potentially interfering agents as nicotinamide, uric acid and allopurinol. Allopurinol is oxidized in vivo to the major metabolite alloxanthine (oxopurinol) [7]. Comparing the levels of ATDA and the elimination half-life, which are essentially identical for patients who received allopurinol and for those who did not, clearly demonstrates that neither allopurinol nor alloxanthine interferes with ATDA analysis.

To the best of our knowledge the present report represents the first method for the routine separation and quantitation of nonradioactive ATDA from biological fluids. El Dareer et al. [4] have recently reported the distribution and metabolism of [C^{14}] ATDA in mice, dogs, and monkeys using a TLC system. They have reported that in mice injected intraperitoneally with 100 mg/kg of ATDA, serum drug levels decrease with a half-life of 2.9 h. We have obtained nearly identical results (half-life 2.2 h) using nonradioactive ATDA and the TLC—HPLC method described here (Fig. 4). In addition, we report preliminary data on the kinetics of ATDA in humans. As seen in Fig. 4 the serum disappearance curve for ATDA in humans closely resembles that observed in mice. It merits emphasis that the extrapolated C_0 levels of ATDA that we estimate in mice (90 μg/ml) are very close to those (100—110 μg/ml) reported by El Dareer et al. [4] and roughly comparable (considering the difference in dose) to that recently reported by Lu et al. [5] in dogs.

The drug elimination half-life reported in the present study was determined from the data which were subjected to least-squares regression analysis. More extensive studies are obviously required for complete kinetic analyses and for

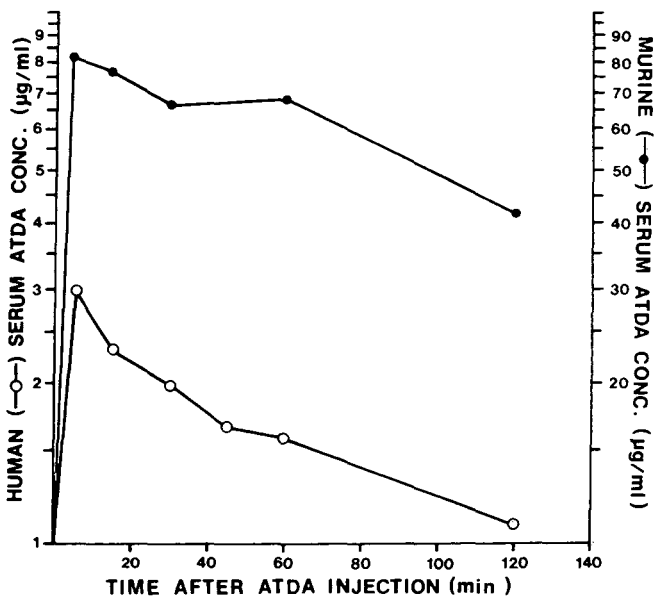

Fig. 4. Mean serum drug concentrations from six patients administered 50 mg/m² and from mice administered 300 mg/m² ATDA (4 mice per time point). ATDA levels were determined as described in Experimental.

an investigation into possible urinary metabolites such as those reported by El Dareer et al. [4]. We are currently applying this TLC—HPLC method for analysis of ATDA to more extensive studies of the pharmacokinetics of this oncolytic agent. In principle this method should be applicable to analysis of related compounds, such as 2-ethylamino-1,3,4-thiadiazole, an analogue of ATDA that recently was reported to exert beneficial effects in an experimental model of circulatory shock [8].

ACKNOWLEDGEMENTS

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The authors wish to thank Linda Mathews and Joan Braddock for skillful technical assistance.

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Note

High-performance liquid chromatographic analysis of indapamide (RHC 2555) in urine, plasma and blood

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Indapamide, RHC 2555 [4-chloro-N-(2-methyl-1-indolinyl)-3-sulfamoyl benzamide], is an effective antihypertensive agent at single daily doses of 2.5 mg in patients with mild to moderate hypertension. A previously described fluorescence assay has been used to measure the drug's concentration in urine, plasma, and whole blood [1, 2]. A procedure for whole blood was necessary since indapamide is taken up by the red cells in vitro within 5 min of incubation at a ratio to plasma of approximately 9:1 [3]. The fluorescence procedure was shown to be linear and sufficiently sensitive to measure indapamide concentrations after therapeutic doses. However, this technique had the disadvantages of using an external rather than an internal standard and of not being readily installed in different laboratories.

Due to the present popularity of high-performance liquid chromatography (HPLC) equipment and procedures, an HPLC procedure for indapamide in biological fluids was developed. This procedure has now been successfully used at several laboratories to measure the concentrations of indapamide after therapeutic doses.

EXPERIMENTAL

Apparatus

A Spectra-Physics Model 8000B liquid chromatograph was used in the assay of plasma samples of indapamide, while an Altex Model 110A liquid chromatograph was used in the assay of blood and urine samples. Both chromatographs were equipped with a Schoeffel Model 770 variable-wavelength UV detector. Reversed-phase columns housed in a temperature-controlled oven were used. For plasma samples, a Zorbax® ODS column, 25 cm × 4.6 mm, 5 μm particle size, from Dupont (Wilmington, DE, U.S.A.) was used, and for blood and urine samples, a LiChrosorb® C-18 column, 25 cm × 3.2 mm, 10 μm particle size, from Altex Scientific (Berkeley, CA, U.S.A.) was used. The two different systems were used since the work was performed in two different laboratories.

Reagents

Indapamide (RHC 2555) was supplied by Revlon Health Care Group (Tuckahoe, NY, U.S.A.) and the internal standard, sulfanilanilide (reagent grade) was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Acetonitrile was HPLC grade, and the water was deionized and distilled. All other reagents were analytical grade. The anhydrous diethyl ether used was extracted with 0.1 *N* sodium hydroxide daily prior to use.

Chromatography

The mobile phase for blood and urine samples consisted of acetonitrile—0.1 *M* sodium acetate buffer, pH 3.6 (35:65, v/v), with a flow-rate of 1.5 ml/min. For plasma samples, the mobile phase was acetonitrile—0.1 *M* sodium acetate buffer, pH 3.6 (43:57, v/v), with a flow-rate of 1.0 ml/min. The oven temperature was set at 54°C for all samples and the wavelength of detection was 241 nm.

Preparation of standards

Indapamide standards, ranging from 50—400 ng/ml, were prepared by adding appropriate aliquots of a 20 μg/ml solution of indapamide in acetonitrile to 100-ml volumetric flasks. Each flask was filled to volume with oxalated blood and 3-ml aliquots were stored at −20°C until needed. Urine and plasma standards were prepared similarly.

Extraction

Blood standards and samples (in their original tubes) were thawed and homogenized (Tissuemizer® Model SLT with 100 NE probe from Tekmar, Cincinnati, OH, U.S.A.).

One milliliter of each homogenized blood sample was pipetted into separate glass culture tubes (16 × 100 mm) with Teflon®-lined screw caps and 50 μl of the internal standard (10 μg/ml sulfanilanilide in acetonitrile) were added to each tube. Four milliliters of diethyl ether were added to each tube, which was then vortexed vigorously for 2 min. The phases were separated by centrifuga-

tion at 4° C and the ether layers were transferred to fresh tubes. Four milliliters of diethyl ether were again added to the aqueous layer, which was then vortexed for 2 min and centrifuged as described above. To the combined ether extracts, 0.5 ml of 0.01 *N* sodium hydroxide was added. The tubes were vortexed and centrifuged, and the ether layer was discarded. The aqueous layer was neutralized by the addition of 0.5 ml of 0.01 *N* hydrochloric acid and 0.25 ml of 0.05 *M* sodium phosphate (pH 7.4), and then extracted with 4 ml of diethyl ether, as described above. The ether extracts were transferred to clean tubes, evaporated to dryness, and stored at 4° C.

The extraction procedure for urine samples (1 ml) was similar to that used for blood, except that 0.5 ml of 0.05 *N* sodium hydroxide was added to the combined ether layers, and subsequently 0.5 ml of 0.05 *N* hydrochloric acid and 0.25 ml of 0.05 *M* sodium phosphate (pH 7.4) were added.

For plasma, 2 ml of the samples were pipetted into tubes that contained 100 μl of the 10 μg/ml sulfanilanilide solution. The tubes were extracted with 8.0 ml of anhydrous diethyl ether. The ether extracts were transferred to fresh screw-cap tubes and back-extracted into base by adding 1.0 ml of 0.1 *N* sodium hydroxide. The aqueous solution was neutralized by the addition of 1.0 ml of 0.1 *N* hydrochloric acid and 0.5 ml of 0.05 *M* sodium phosphate (pH 7.4), and then extracted as above, with 8.0 ml of anhydrous diethyl ether. The ether extracts were evaporated to dryness, and stored at 4° C.

For all biological fluids, the residue in each tube, after evaporation of the ether, was reconstituted in 200 μl of the mobile phase and a 50-μl aliquot was injected onto the column. Throughout the extraction procedures, the tubes were kept in crushed ice except when being processed.

Calculation of results

The heights of the indapamide and sulfanilanilide peaks were measured, and the peak height ratio of indapamide/sulfanilanilide was calculated. Calibration curves were constructed by plotting the peak height ratio versus the indapamide concentration for the standards and determining the linear regression line. Sample indapamide concentrations were calculated from their peak height ratio using the calibration curve.

RESULTS AND DISCUSSION

Chromatographic properties

Chromatograms of extracted blanks and standards in blood, urine, and plasma are depicted in Figs. 1, 2, and 3, respectively. Chromatograms of extracted human blood samples before and after the administration of indapamide are presented in Fig. 4. For both blood and urine samples, the retention times of sulfanilanilide and indapamide were 2.5 and 3.1 min, respectively. Using the slightly different chromatographic system, plasma samples gave retention times of 5.3 and 6.3 min for sulfanilanilide and indapamide, respectively. In each system the retention times of indapamide and sulfanilanilide for the extracted samples were identical to those obtained from solutions of the pure compounds injected directly. There were no interfering peaks from the extracted biological fluid with retention times the same as indapamide or the internal standard.

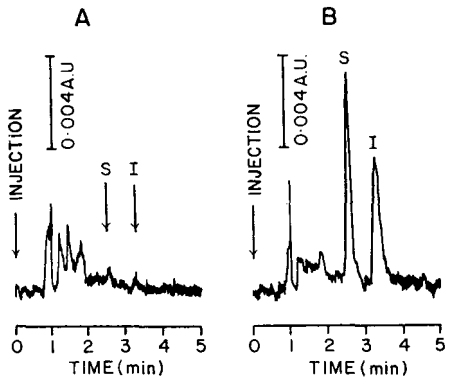

Fig. 1. (A) Chromatogram of an extract of blank blood (S and I indicate expected retention times of sulfanilanilide and indapamide). (B) Chromatogram of an extract of blood containing 500 ng/ml sulfanilanilide (S) and 200 ng/ml indapamide (I). Chromatographic conditions: LiChrosorb C_{18} column, 10 μm particle size, 25 cm × 3.2 mm with a mobile phase of acetonitrile—acetate buffer (35:65) at a flow-rate of 1.5 ml/min.

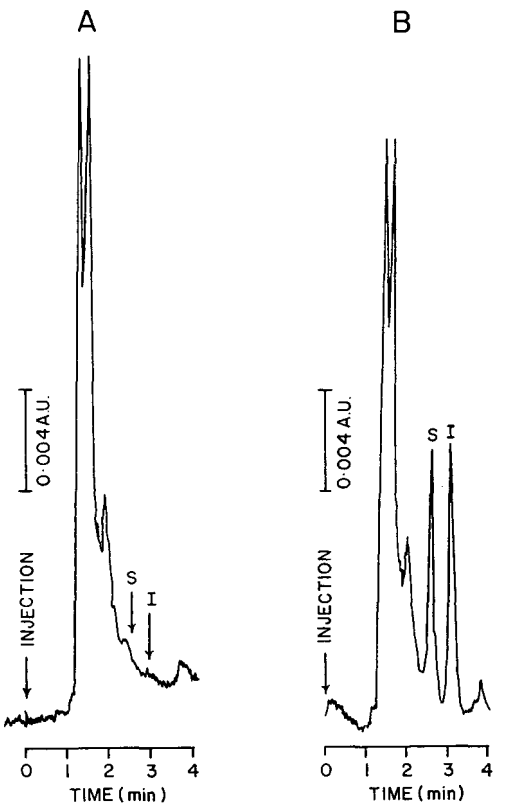

Fig. 2. (A) Chromatogram of an extract of blank urine (S and I indicate expected retention times of sulfanilanilide and indapamide). (B) Chromatogram of an extract of urine containing 500 ng/ml sulfanilanilide (S) and 200 ng/ml indapamide (I). Chromatographic conditions were as described in Fig. 1.

Extraction efficiency

By comparing the peak heights of standards extracted from the biological matrix with those of standards dissolved in mobile phase and chromatographed directly, the recoveries of indapamide and sulfanilanilide were determined. The recovery of indapamide from blood and urine, over the concentration range of 50—400 ng/ml, averaged 72 and 87%, respectively. The recovery of indapamide from plasma, over the concentration range of 25—200 ng/ml, averaged 96%. Recovery of indapamide was independent of concentration. Sulfanilanilide, at a concentration of 500 ng/ml, had extraction efficiencies of 79% in blood, 55% in urine, and 76% in plasma.

Suitability of internal standard

Sulfanilanilide was suitable for use as the internal standard for assay of indapamide, which is also a sulfonamide compound. It gave UV absorbance at

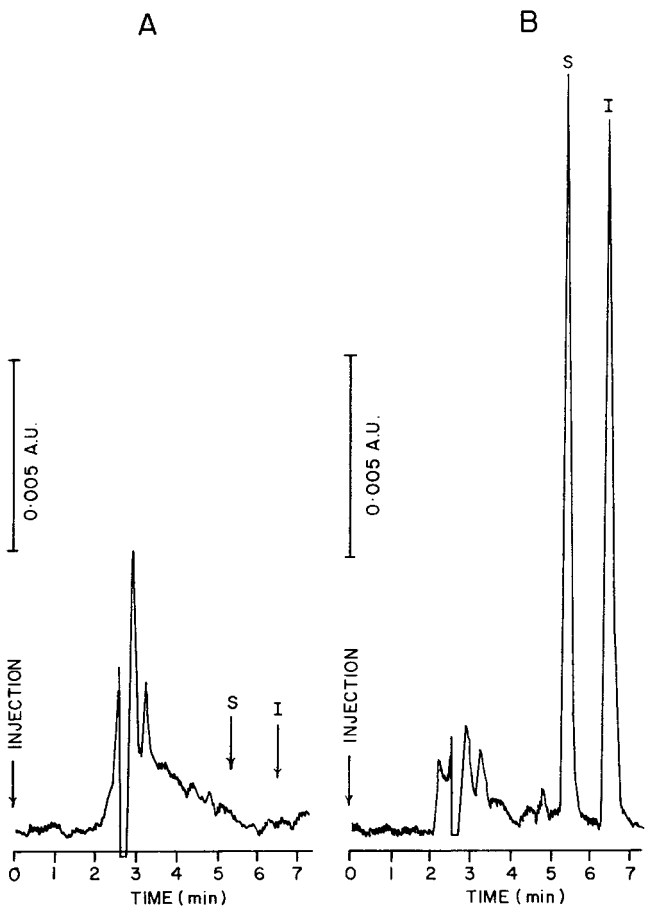

Fig. 3. (A) Chromatogram of an extract of blank plasma (S and I indicate expected retention times of sulfanilanilide and indapamide). (B) Chromatogram of an extract of plasma containing 500 ng/ml sulfanilanilide (S) and 200 ng/ml indapamide (I). Chromatographic conditions: Zorbax ODS column, 5 μm particle size, 25 cm × 4.6 mm with a mobile phase of acetonitrile—acetate buffer (43:57) at a flow-rate of 1.0 ml/min.

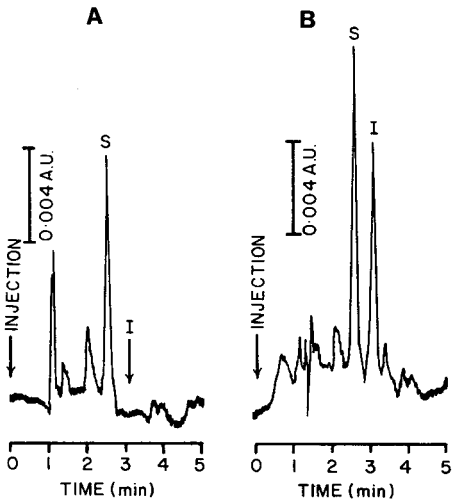

Fig. 4. Chromatograms of extracts of human blood samples before (A) and after (B) administration of indapamide. S indicates location of sulfanilanilide (500 ng/ml) internal standard. I indicates location of indapamide, which was not present in A and was determined to be 198 ng/ml in B. Chromatographic conditions were as described in Fig. 1.

the wavelength of 241 nm used for detection of indapamide. Also, the extraction efficiency of sulfanilanilide was reasonably similar to that of indapamide for blood and plasma; less so for urine. These factors made the use of sulfanilanilide favorable as an internal standard in this assay.

Precision and reproducibility

Standards, of various concentrations, were assayed in at least duplicate on either three of four different occasions to determine the precision and reproducibility of the assays. The composite relative standard deviation of peak height ratios for blood was 13.9% at 50 ng/ml (n = 8) and 8.1% at 400 ng/ml (n = 8); for urine the values were 8.8% at 50 ng/ml (n = 6) and 4.0% at 400 ng/ml (n = 6); for plasma the values were 12.8% at 25 ng/ml (n = 4) and 4.4% at 200 ng/ml (n = 4).

Linearity and sensitivity

Calibration curves obtained by plotting the ratio of the peak height of indapamide to that of sulfanilanilide versus the concentration of indapamide were linear over the concentration ranges studied (25—200 ng/ml for plasma, 50—400 ng/ml for blood and urine). For all three biological fluids, the correlation coefficient for the composite calibration curves, obtained as described above, were equal to or better than 0.986. The limits of sensitivity of the assays were considered to be 50 ng/ml for blood and urine, and 25 ng/ml for plasma.

Selectivity

Eleven blood samples from human clinical studies were assayed by the HPLC procedure and the previously described fluorescence procedure [2]. There were no significant differences between the indapamide concentrations determined

by the two procedures and the correlation coefficient was 0.97. No metabolites of indapamide have been shown to interfere with the HPLC procedure.

Human samples

The described HPLC procedures have been successfully used in different laboratories for the assay of human clinical samples from subjects who received various doses of indapamide. Fig. 5 shows typical blood concentrations of indapamide, as determined by the HPLC procedure, in a human subject who received a 5.0-mg dose. The assay procedures for blood, urine and plasma indapamide samples have demonstrated the linearity, precision and sensitivity needed for pharmacokinetic studies of this new antihypertensive agent.

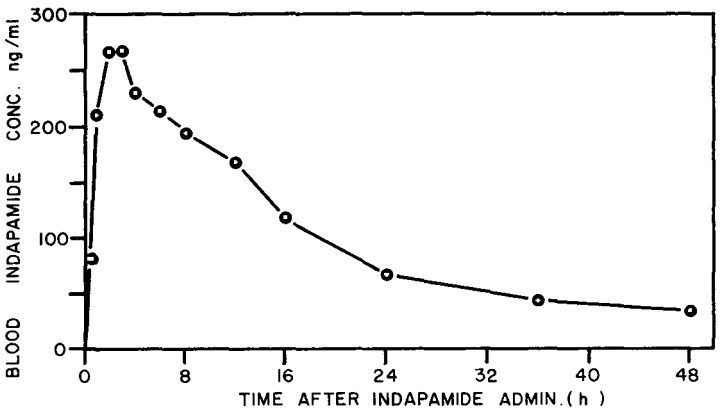

Fig. 5. Blood concentrations of indapamide in a human subject who received a 5.0-mg oral dose. The concentrations were determined by the described HPLC procedure.

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Note

Determination of pentazirinocyclodiphosphathiazene in biological fluids by high-performance liquid chromatography

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(Received December 8th, 1981)

Several cyclodiphosphathiazene derivatives have been synthesized and investigated by Labarre and co-workers [1, 2] to develop effective antitumor substances with fewer side-effects. Among these cyclodiphosphathiazene derivatives, pentaziridinocyclodiphosphathiazene (SOAz) (Fig. 1) has been found to have the strongest antitumor activity on experimental tumors — especially on murine L 1210 and P 388 leukemias and on B 16 melanoma — and the lowest toxicity [1, 2].

For elucidation of the metabolism, absorption and excretion of SOAz, it seems important to establish a quantitative method for the determination of the concentration of SOAz in biological fluids. There are no reports of a method for the determination of SOAz. Therefore, we examined various methods for assay of SOAz in biological fluids, and established a successful analytical method employing high-performance liquid chromatography (HPLC) with a refractive index (RI) detector.

Furthermore, it was found that no metabolites of the compound SOAz are observed in plasma and urine under the present method. However, it was also found that SOAz changes mainly into two kinds of compounds spontaneously (Fig. 1). This report describes these results.

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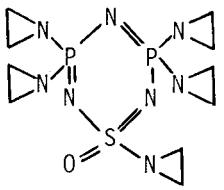
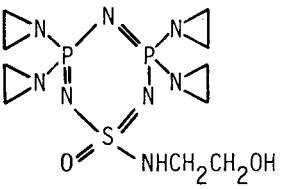
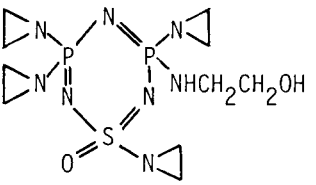


SOAz



F-1



F-2

Fig. 1. Structures of SOAz and its decomposition products.

EXPERIMENTAL

Materials

SOAz was synthesized and purified by Otsuka Chemical Co. (Tokushima, Japan). The other chemicals used were obtained from Wako Pure Chemicals (Osaka, Japan). Dichloromethane and methanol were liquid chromatographic grade materials. The other chemicals used were analytical grade materials.

HPLC instrumentation

A Shimadzu LC-3 liquid chromatograph equipped with a Model SIL-1A high-pressure injector and a Model RID-2A detector (Kyoto, Japan) was used. A μBondapak C_{18}/Porasil chromatographic column (particle size, 8—10 μm; 30 cm × 3.9 mm I.D.) from Waters Assoc., Milford, MA, U.S.A., was used for the separation; the mobile phase was 5 m*M* KH_2PO_4—methanol (70:30, v/v) and the flow-rate 1.0 ml/min. The column was maintained at room temperature. Peak areas were determined with a Shimadzu Model C-R1A Chromatopac apparatus.

A JEOL Model JMS D 300 mass spectrometer with an electron impact (EI) and chemical ionization (CI) ion source (Tokyo, Japan) and a Varian FE-80 nuclear magnetic resonance (NMR) spectrometer were used for identification of the decomposition products of SOAz. The mass spectrometric analyses were carried out under the following conditions: ionization energy 190 eV, ionization current 300 μA, accelerating voltage 3.0 kV; isobutane was used as reagent gas for the measurement of CI mass spectra. ^{31}P-NMR spectra were measured

using deuterated chloroform as solvent and a deuterium oxide solution containing 85% phosphoric acid as external standard.

Analytical procedure

Samples of 1.0 ml of plasma, urine or other biological fluids were adjusted to pH 10.0 with 1 *N* sodium hydroxide, and extracted with 5 ml of dichloromethane for a few minutes. The organic layer containing SOAz was separated by centrifugation at 2000 *g* for 5 min. This extraction was repeated once using 2 ml of dichloromethane. The combined organic layer was dried under nitrogen at room temperature. The residue was dissolved in 100 μl of a solution of 5 m*M* KH_2PO_4—methanol (70:30, v/v; mobile phase for HPLC), and 40 μl of this solution were injected into the liquid chromatograph.

A calibration curve for the determination of SOAz by HPLC was prepared by plotting the peak area against the concentration. The calibration curve was linear at concentrations of 0.5—400 μg/ml.

RESULTS AND DISCUSSION

SOAz dissolves easily in chloroform, dichloromethane, methanol, acetone or water, but it is very unstable under strong acidic conditions. SOAz does not absorb or fluoresce in UV light, and it is hard to obtain any derivative of SOAz without decomposition. Also, it was found that SOAz could be extracted with chloroform, dichloromethane or 1,2-dichloroethane from biological fluids. Various conditions were examined for the extraction of SOAz from biological fluids following its administration and for its HPLC separation on the basis of its physicochemical properties described above. The following procedure was found to be the simplest and most rapid and to be the most reliable with highest recovery. The biological samples were adjusted to pH 10.0 with 1 *N* sodium hydroxide solution, then extracted with dichloromethane.

A reversed-phase chromatographic column, μBondapak C_{18}, and an RI detector were used for separation and monitoring. A mobile phase consisting of water—methanol or 5 m*M* KH_2PO_4—methanol was found to be suitable for the separation of SOAz from biological fluid components extracted with dichloromethane. In this study, a solution of 5 m*M* KH_2PO_4—methanol (70:30, v/v) was used as the mobile phase since it resulted in the highest detection sensitivity and the best chromatographic separation of SOAz.

Known amounts of SOAz were added to plasma of rats. Chromatograms of HPLC with RI detection showing the separation of SOAz extracted from rat plasma and the extract of rat plasma control are given in Fig. 2. The retention time of SOAz was 7.0 min. As summarized in Table I, the recovery of SOAz from plasma was ca. 85%, and the detection limit for SOAz under this HPLC method was 0.5 μg/ml plasma. The reproducibility was ± 1.5—3.1%.

The results obtained for the chromatographic separation, recovery and detection limit were in good agreement with those obtained with urine, bile and other biological fluids.

Then, we tested the stability of SOAz at various temperatures in aqueous solution, rat plasma and urine. As shown in Table II, no decomposition of SOAz was observed at various temperatures for plasma, at 5°C or −25°C for

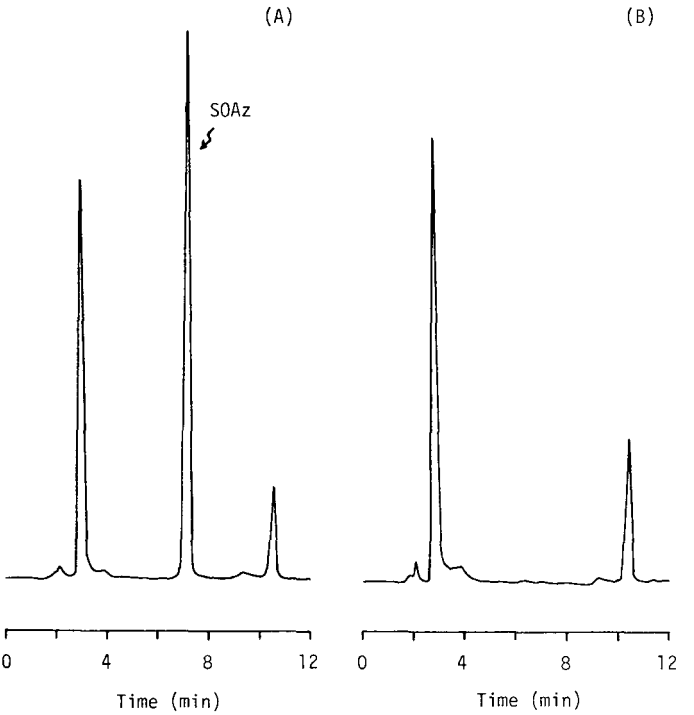


Fig. 2. HPLC chromatograms showing the separation of SOAz extracted from rat plasma (A) and the extract of rat plasma control (B).

TABLE I

RECOVERY ON EXTRACTION OF SOAz FROM PLASMA

Each value is the mean of three determinations.

Added (μg/ml)	Recovery from plasma (%)
1.0	75.4
5.0	81.7
10.0	81.9
40.0	89.4
200.0	97.1
Mean ± S.D. (%)	85.1 ± 7.8

its aqueous solution, or at −25°C for urine. However, SOAz decomposed to give small amounts of two compounds (F-1 and F-2, Fig. 1) at room temperature or at 5°C for aqueous solution and urine. The HPLC separation of these decomposition products and SOAz in urine is shown in Fig. 3. Each fraction eluting at retention times of 4.7 and 5.2 min from the HPLC column was collected separately and identified by the EI and CI mass spectra and ^{31}P-NMR spectra. SOAz: *m/z* 362 ($M^{+\cdot}$) and *m/z* 320 (M—aziridine) in EI mass spectrum;

TABLE II

STABILITY OF SOAz IN PLASMA, URINE AND AQUEOUS SOLUTION AT VARIOUS TEMPERATURES AFTER ADDITION OF SOAz AT A CONCENTRATION OF 500 μg/ml

Conditions	Compound*	Proportion found (%)		
		Plasma	Urine	Aqueous solution
Room temp., 24 h	SOAz	100.00	99.53	99.71
	F-1	—	0.36	0.26
	F-2	—	0.11	0.03
Room temp., 48 h	SOAz	100.00	98.47	99.37
	F-1	—	1.32	0.56
	F-2	—	0.21	0.07
5° C, 48 h	SOAz	100.00	99.88	100.00
	F-1	—	0.12	—
	F-2	—	—	—
—25° C, 48 h	SOAz	100.00	100.00	100.00
	F-1	—	—	—
	F-2	—	—	—

*The structures are indicated in Fig. 1.

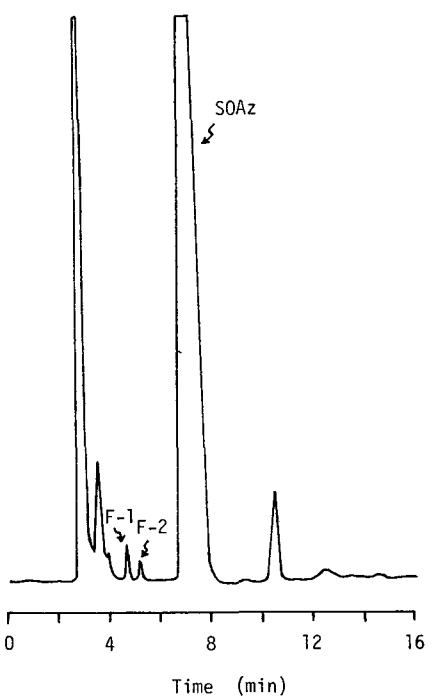


Fig. 3. HPLC chromatogram showing the separation of SOAz and its decomposition products in urine.

m/z 363 (M+1) and *m/z* 337 in CI mass spectrum; and δ 35.91 (P, singlet) in ^{31}P-NMR spectrum. F-1: *m/z* 362 (M—H_2O), *m/z* 350 (M—CH_2O), *m/z* 338 (M—C_2H_6NO) and *m/z* 320 in EI mass spectrum; *m/z* 381 (M+1), *m/z* 363 (M+1—H_2O) and *m/z* 338 (M—C_2H_6NO) in CI mass spectrum; and δ 35.35 (P, singlet) in ^{31}P-NMR. F-2: *m/z* 350 (M—CH_2O), *m/z* 338 (M—C_2H_6NO) and *m/z* 320 in EI mass spectrum; and *m/z* 381 (M+1) and *m/z* 320 in CI mass spectrum. (The ^{31}P-NMR spectrum of F-2 could not be measured because of small amounts collected.) It was also observed that F-2 decomposed further to another, unknown, compound.

Finally, SOAz (100 mg/kg) was administered intravenously to rats, and the time course of change in the concentration of SOAz in plasma was measured by the present method. The results obtained are shown in Fig. 4.

This method employing HPLC with an RI detector will be helpful for basic and clinical pharmacological studies on the compound SOAz.

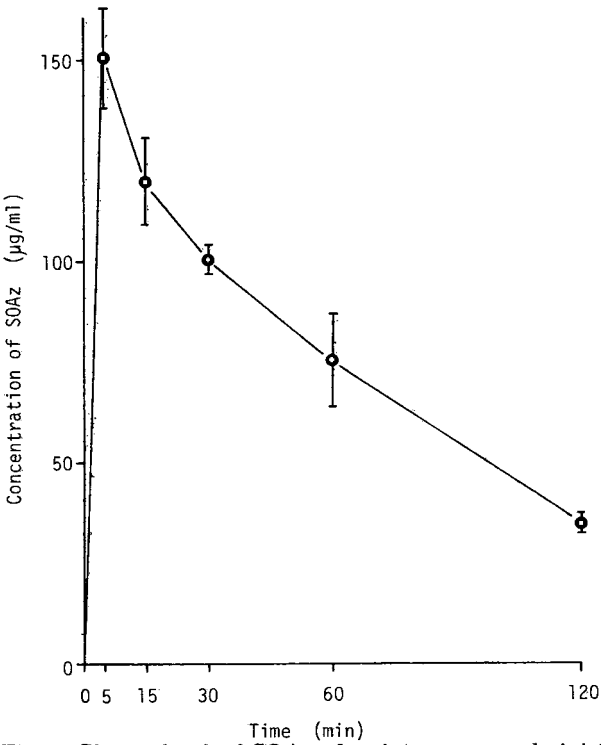


Fig. 4. Plasma level of SOAz after intravenous administration of SOAz (100 mg/kg) to rats. Results are the average for three rats.

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Note

Simultaneous determination of promethazine and two of its circulating metabolites by high-performance liquid chromatography

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Promethazine (PMZ) has attracted widespread use over a number of years for its antihistaminic and sedative properties. Its disposition in man however, has been paid sparse attention, largely as a result of the inadequacy of the available assay procedures. Recently, methods have been reported for the determination of PMZ at therapeutic concentrations using gas chromatography [1] and high-performance liquid chromatography (HPLC) [2, 3]. An assay which permits the simultaneous determination of circulating metabolites however, has not been previously described.

In this report an HPLC procedure for the assay in PMZ is described, the precision and sensitivity of which compares favourably with any previously reported method. In addition, the concurrent determination of the monodemethylated and sulphoxidated metabolites of PMZ (Fig. 1) is demonstrated, and the applicability of the procedure to human pharmacokinetic studies is illustrated.

EXPERIMENTAL

Reference standards

PMZ, as a reference substance and also as a solution for administration (Phenergan injection), was kindly supplied by May and Baker (Dagenham, Great Britain). Monodesmethylpromethazine (Nor_1PMZ) and imipramine were gifts from Kabi Pharmaceuticals (Stockholm, Sweden) and Berk Pharmaceuti-

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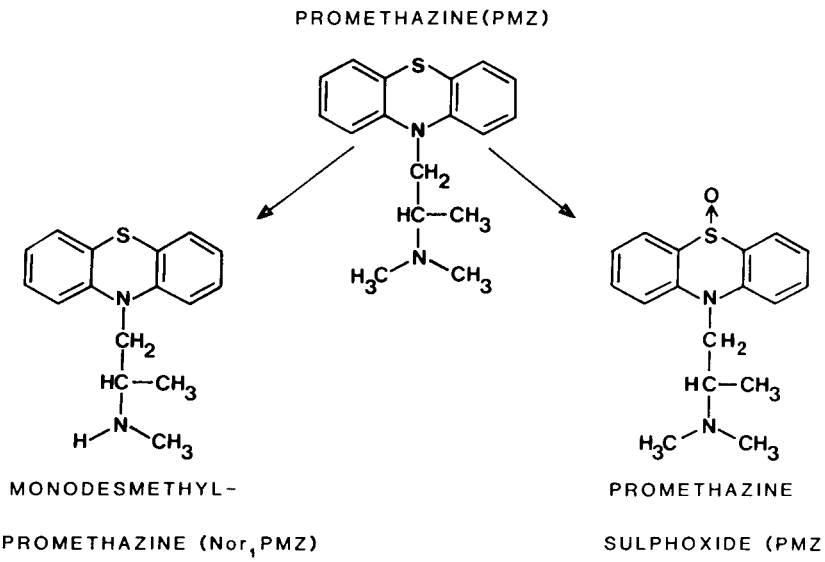

Fig. 1. Structures of promethazine and its monodesmethyl- and sulphoxide metabolites.

cals (Guildford, Great Britain), respectively. Promethazine sulphoxide (PMZSO) was prepared in the laboratory by the following method. Promethazine hydrochloride (1 g) was dissolved in 5 ml of distilled water, 0.6 ml of 27.5% hydrogen peroxide added, and the mixture kept in a dark place at room temperature overnight. It was then made alkaline with sodium hydroxide and extracted with successive 5-ml aliquots of *n*-heptane, diethyl ether, and dichloromethane. The dichloromethane extract was evaporated to dryness and the product recrystallised from dichloromethane. The results of elemental analysis were consistent with the theoretical values for PMZSO. Contamination of the product with the N-oxide of PMZ was checked by mass spectrometry (MS); using a probe temperature of 50°C, phenothiazine N-oxides produce characteristic ion peaks at *m/e* 60 and 61 [4], such peaks were not seen in the MS chromatogram of the synthesised product.

Reagents

All reagents used were of AnalaR grade obtained from BDH Chemicals (Poole, Great Britain) with the exception of dichlorodimethylsilane, propan-2-ol, 1,1,1-trichloroethane and *n*-heptane, which were of reagent grade. Isoamylalcohol was obtained from Aldrich Chemicals (Gillingham, Great Britain).

Chromatography

A Pye Unicam Model LC3 pump was equipped with a variable-wavelength UV detector (Model LC3 Pye Unicam), and a Philips Model 8251 chart recorder. A stainless-steel column (100 mm × 4.8 mm I.D.) containing Hypersil 5-SAS (Shandon Southern Products, Runcorn, Great Britain) was prepared in the laboratory using a propan-2-ol slurry and a packing pressure of 400 bars. The column was fitted with a septum injection system. The eluent, consisting

of methanol containing 30% v/v of 0.05 *M* Sørensen's phosphate buffer, pH 7.4, was maintained at a flow-rate of 0.7 ml/min. The analytical wavelength used was 248 nm, with a band width of 8 nm.

Extraction

All glass tubes used in the extraction procedure were cleansed by overnight immersion in a solution of chromosulphuric acid. After rinsing and drying, the tubes were silanised using 2% dichlorodimethylsilane in 1,1,1-trichloroethane, rinsed with methanol, then distilled water, and dried. Screw-cap tops and PTFE liners for the extraction tubes were cleansed by overnight immersion in a 5% solution of Decon 90 (Decon Labs., Brighton, Great Britain).

Blood samples

Each whole blood sample (10.0 ml) contained in a 16-ml tube, was spiked with imipramine hydrochloride (0.05 ml, 10 μg/ml) as internal standard, made alkaline with 1.0 ml of 1 *M* sodium hydroxide, and extracted with 4.0 ml of *n*-heptane containing 10% dichloromethane and 1.5% of isoamylalcohol for 15 min using an inversion mixer. After centrifugation (3000 *g*, 10 min) the organic layer was removed to a nipple tube and was replenished by a further 4.0 ml of the extraction solvent. After mixing and centrifuging as before, the combined extracts were extracted with 50 μl of 0.1 *M* hydrochloric acid for 2 min using a vortex mixer. Aliquots (10—25 μl) of the acidic phase were then injected immediately onto the HPLC column. It is recommended that blood samples be frozen on collection and extracted immediately on thawing.

Quantitation

Standard curves were prepared by the addition of known amounts of PMZ, Nor_1PMZ and PMZSO to blank blood, and analysing a set of standards with each batch of samples. An unweighted least-squares regression was employed to fit plots of peak height ratios (drug/metabolite:internal standard) versus blood concentration.

RESULTS AND DISCUSSION

The extraction of PMZ and its metabolites using *n*-heptane containing 10% dichloromethane and 1.5% isoamyl alcohol, was reproducible (Table I). The extraction efficiencies for PMZ and Nor_1PMZ were high, whilst that of PMZSO was considerably lower. Inclusion of dichloromethane in the extraction solvent increased the efficiency of, and reduced the variability in, the extraction of PMZSO. Increasing the proportion of dichloromethane above 10%, resulted in higher extraction efficiencies of PMZSO, but led to decreased efficiencies for both PMZ and Nor_1PMZ.

The differences in λ_{max} values for PMZ and Nor_1PMZ (252 nm) and PMZSO (236 nm) were taken into consideration in the choice of detector conditions. Under those conditions used (analytical wavelength, 248 nm; band width, 8 nm) the extinction coefficients for both PMZ and Nor_1PMZ were approximately equal to those measured at 252 nm, whilst for PMZSO, the extinction coefficient was equal to 70% of that measured at 236 nm.

TABLE I

RECOVERY, PRECISION OF THE ANALYSIS AND MINIMUM DETECTION LIMITS FOR PROMETHAZINE AND METABOLITES

Compound	Recovery (% ± S.D.)	C.V. (%)*			MDC** (ng/ml)
		a	b	c	
PMZ	75 ± 4	3.9	8.4	—	0.2
Nor_1PMZ	76 ± 8	4.5	8.9	—	0.2
PMZSO	26 ± 2	—	—	7.0	1.0

*Within-run coefficient of variation (n = 5) for: (a) 8 ng/ml, (b) 1.5 ng/ml, (c) 21 ng/ml.
**MDC = minimum detectable concentration, defined as peak height ≡ twice baseline noise of blank blood.

Fig. 2 shows chromatograms of extracts of whole blood samples taken from a human volunteer subject, immediately prior to, and 11 h following, the oral administration of 25 mg promethazine hydrochloride. Fig. 2a indicates that no interfering substances are coextracted by the procedure. In Fig. 2b the separation of the two metabolites and the parent compound is demonstrated. The retention times for each compound were as follows: PMZSO (6.4 min), Nor_1PMZ (7.5 min), PMZ (9.3 min) and internal standard (14.4 min).

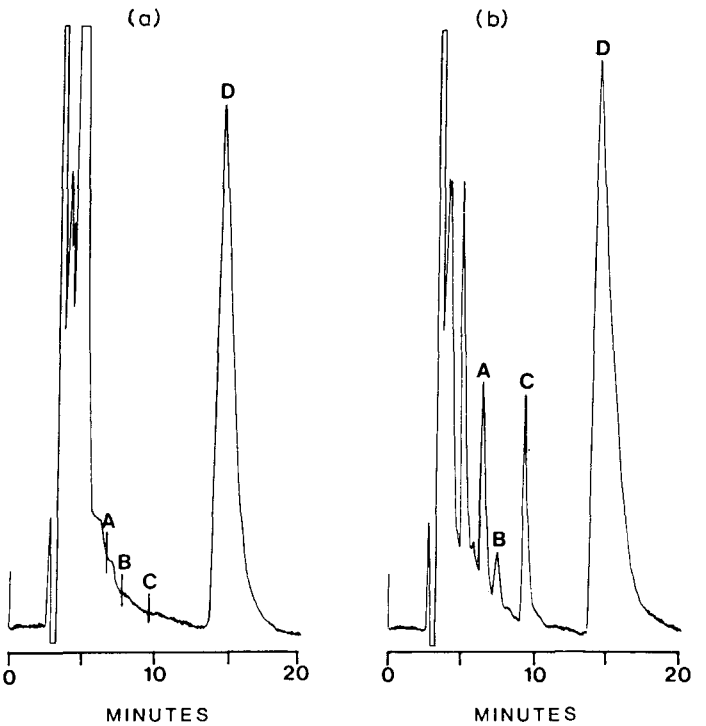

Fig. 2. HPLC traces resulting from extracts of blood obtained prior to (a) and 11 h following (b) the oral administration of 25 mg promethazine hydrochloride to a human volunteer subject. Peaks in (b): A = PMZSO (11.9 ng/ml blood), B = Nor_1PMZ (0.8 ng/ml), C = PMZ (2.8 ng/ml) and D = internal standard (imipramine).

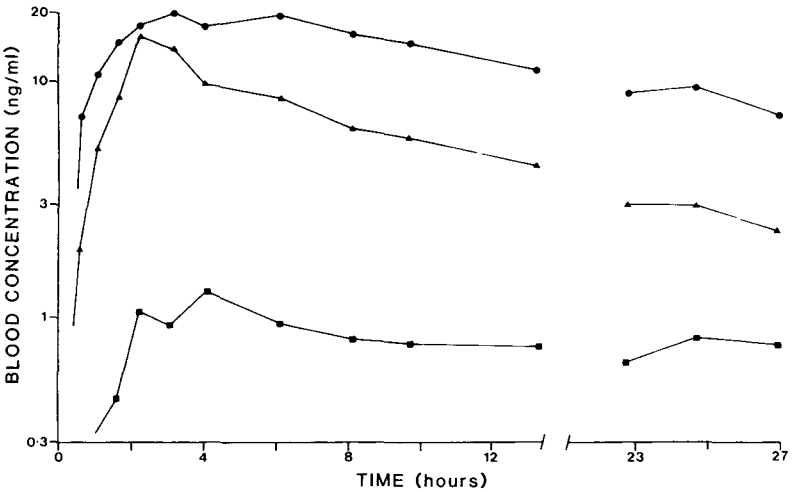

Fig. 3. Blood concentrations of PMZ (▲——▲), PMZSO (●——●) and Nor_1PMZ (■——■) following the oral administration of promethazine hydrochloride (25 mg) to a human volunteer.

Imipramine appears to be a suitable choice for internal standard, being well separated from the other compounds and also chemically similar to PMZ. The column efficiency was found to be 2500 plates (25,000 plates per metre) for PMZ.

Standard curves over the concentration ranges tested of 2—50 ng PMZ and Nor_1PMZ, and 5—50 ng PMZSO, per ml of blood were found to be linear, with intercepts which were not significantly different from zero. Within-run precision was determined using five spiked blood samples. The within-run coefficients of variation, together with minimum detectable concentrations and extraction recoveries are presented in Table I.

The applicability of the method to human pharmacokinetic studies was investigated. Fig. 3 shows blood concentration—time profiles for PMZ, Nor_1PMZ and PMZSO following the oral administration of 25 mg promethazine hydrochloride to a human volunteer. The peak time and elimination half-life for PMZ are similar to those reported previously [1]. Concentrations of PMZSO are comparable to those of PMZ. It is estimated that blood concentrations of these two compounds will be measurable over a time period equivalent to three to four times their blood half-lives (40 h). In contrast, concentrations of Nor_1PMZ were consistently lower than PMZ and PMZSO yet measurable using our specified conditions.

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Note

High-performance liquid chromatographic assay for determination of a new β-blocking agent FM 24

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FM 24, 1-(2-*exo*-bicylco-2,2,1-hept-2-yl phenoxy)-3-(1-methylethyl)amino-2-propanol, (Fig. 1) is a new β-blocking agent whose activity has been demonstrated previously [1, 2]. Recently, a gas chromatographic method was reported by Bernard et al. [3], including threefold extraction from plasma, derivatization with pentafluoropropionic anhydride and electron-capture detection. The related technique presents critical steps such as derivatization and detection, and is too time-consuming.

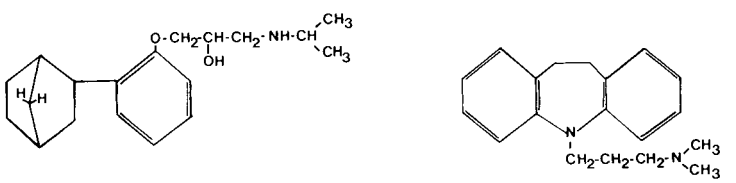

Fig. 1. Chemical structure of FM 24 and imipramine.

The aim of the present paper is to describe a simple and rapid technique allowing plasma concentration measurement as low as 5 ng/ml, suitable with FM 24 plasma levels reached following therapeutic doses. The use of a cation-exchange resin before extraction with hexane avoids any overlap in chromatograms which could be due to endogenous components. High-performance liquid chromatography is, in other respects, a reliable technique easy to carry out and suitable for the analysis of many samples a day.

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MATERIALS AND METHODS

Reagents and chemicals

FM 24 was generously supplied by Pharmindustrie (Gennevilliers, France) and imipramine, used as internal standard, was obtained from Ciba-Geigy (Basel, Switzerland). Hexane and acetonitrile were purchased from Burdick and Jackson (Interchim, Montluçon, France). Sodium hydroxide and acetic acid were suprapur reagents from E. Merck (Paris, France). Sodium sulfate and boric acid were analytical grade reagents. In order to pair amines such as FM 24 or imipramine in the reversed-phase liquid chromatographic mode, 1-heptanesulfonic acid, obtained from Eastman Kodak (Interchim), was used as counter-ion. Carboxymethyl Sephadex (40—120 μm) was used as the resin (Sigma, Interchim).

Apparatus

Cation-exchange separation was realized on standard econo-columns, 10 × 0.5 cm, from Bio-Rad (Touzart et Matignon, Vitry, France).

High-performance liquid chromatography was performed on a Waters instrument equipped with a 6000 A pump as delivery system, and a WISP 710 A automatic injector (Waters Assoc., Paris, France). Detection was carried out with a 970 FS Schoeffel fluorometer (Cunow, Paris, France) with an excitation wavelength of 230 nm and without any emission cut-off filter. Separation was achieved with an isocratic solvent system of water—acetonitrile—acetic acid (48:47:5, v/v) with a flow-rate of 1.4 ml/min, using a μBondapak C_{18} column (Waters Assoc.). The chart speed was set at 0.25 cm/min on the Houston instrument chart recorder (Waters Assoc.). The eluent was degassed under reduced pressure before use and the chromatographic system was operated at ambient temperature. All glassware was washed with hydrochloric acid (2 *M*) and rinsed with deionised water.

Extraction procedure

Blood samples were collected in heparinized tubes then centrifuged at 400 *g* for 10 min. A 2-ml plasma aliquot, taken for analysis, was supplemented with 50 μl of a 20 μg/ml imipramine solution. Econo-columns were filled with CM-Sephadex (to a resin height of 4 cm). Then 2 ml of plasma were placed in the column and the resin was washed twice with 5 ml of water. Using first 2 ml of 0.1 *N* sodium hydroxide in 0.1 *M* sodium sulfate solution, then twice 2 ml of borate buffer (pH 9), FM 24 and imipramine (internal standard) were eluted. The aqueous phases were placed with 9 ml of ethyl acetate in 20-ml screw-capped tubes which were shaken for 10 min. The tubes were then spun at 600 *g* for 15 min, and the organic layer evaporated to dryness under a gentle stream of nitrogen. The residue was then dissolved in 200 μl of mobile phase and 100-μl aliquots were directly injected into the chromatograph. Plasma peaks were identified by comparing their retention times, and quantitative analysis was performed on peak heights using a standard curve.

Calibration curves

A standard calibration curve was established by adding known amounts of

imipramine (0.5 μg/ml) and FM 24 to plasma reference samples, in order to obtain standard concentrations ranging from 0.0625 to 1 μg/ml. Quantitation was performed by drawing the baseline and measuring the peak height of the interesting compounds. The peak height ratio of FM 24/imipramine was calculated and a standard curve was constructed by plotting the peak height ratios against the added amounts (Fig. 2). Reproducibility trials were tested by assaying extracts of ten plasma samples supplemented with 0.05 μg/ml and 0.5 μg/ml FM 24.

RESULTS AND DISCUSSION

Fig. 2 illustrates a chromatogram obtained when FM 24 was injected as an extract of human plasma. Using the mobile phase described in the method section, FM 24 and imipramine had a mean retention time of 5.8 and 4.5 min, respectively. The total time required for the analysis of each sample was 10 min. Peaks were well separated and no interference from endogenous compounds was observed. The extraction recovery of FM 24 was 85.2 ± 3.4%. The standard calibration curve was generated by plotting peak height ratios against the FM 24 concentration. The relationship was linear in the concentration range 0.0625—1 μg/ml, with an excellent correlation coefficient of 0.999 ($y = 2.458x + 0.003$, where y is the peak height ratio and x is the FM 24 concentration). The limit of detection for FM 24 was 5 ng/ml. Reproducibility of the assay was tested by ten replicate analyses of plasma fortified with 0.05 or 1 μg of FM 24; the coefficient of variation was 13.5 and 3.9%, respectively.

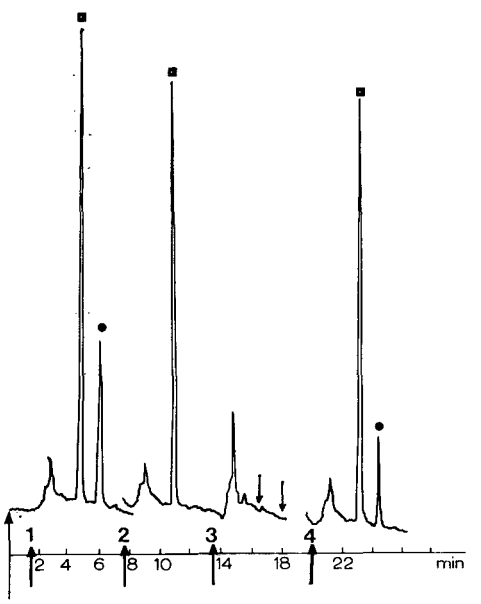

Fig. 2. Typical chromatograms of plasma samples supplemented with (1) 1 μg of imipramine (■) and 0.125 μg of FM 24 (●); and (2) 1 μg of imipramine. (3) Blank plasma; (4) patient's plasma containing 0.088 μg/ml FM 24, supplemented with 1 μg of imipramine (internal standard).

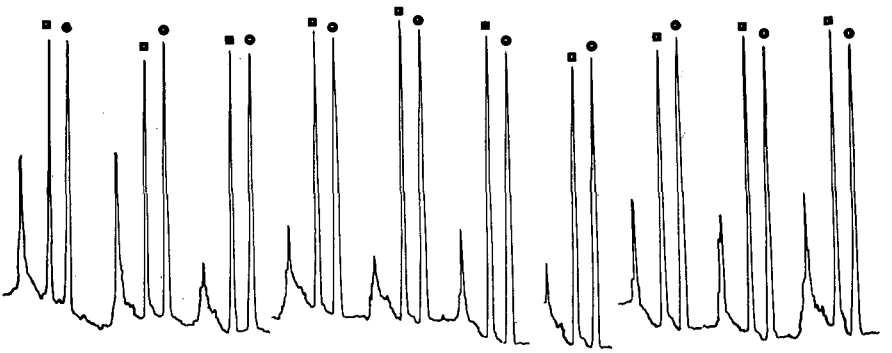

Fig. 3 Chromatogram obtained in reproducibility assay performed with 0.5 μg/ml FM 24. (■) Imipramine, (●) FM 24.

This method had been compared to gas chromatography—mass spectrometry (GC—MS) in the laboratory. The limit of the assay with GC—MS, 0.5 ng/ml, allowed the FM 24 plasma level to be followed over 48 h. Although the detection limit of the HPLC technique was not sufficient to detect such concentrations, it could measure FM 24 plasma concentrations as well as urine concentrations for 12 h, after an 80-mg oral administration. In addition, HPLC did not require a derivatization step, and the time needed for the assay allowed the analysis of a large number of samples a day.

In conclusion, despite the fact that FM 24 plasma levels could not be followed as far as necessary to define pharmacokinetic parameters after single 80-mg oral doses, the described technique appears to possess a sufficient degree of specificity, sensitivity and reliability to be employed for experimental or clinical pharmacological studies.

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Note

Determination of tiodazosin in plasma and whole blood by high-performance liquid chromatography

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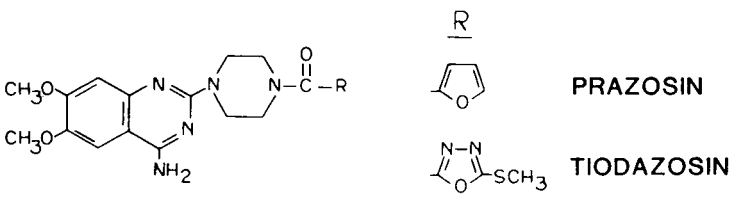
Tiodazosin levulinate, a chemical and pharmacological analogue of prazosin (Minipress, Pfizer), is a new antihypertensive agent currently in clinical trials. The structures for these two drugs are indicated below.



Although there are no published assays of tiodazosin, the assay procedure currently in use at Bristol Laboratories [1] is based on the procedure for prazosin analysis developed by Twomey and Hobbs [2]. This technique involves extraction of the alkaline sample with ethyl acetate, followed by back extraction with a sulfuric acid solution. After a second alkaline extraction with ethyl acetate, the organic layer is evaporated to dryness with a stream of nitrogen gas, then reconstituted with the mobile phase and analyzed by high-performance liquid chromatography (HPLC). This procedure is lengthy and requires 2—4 ml of biological sample to obtain the desired sensitivity. We report here a method for the determination of tiodazosin in plasma and whole blood which is based on our previously published assay of prazosin [3]. The method is simple, reliable and sensitive; it involves no extraction steps and requires only 0.2 ml of biological sample.

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EXPERIMENTAL

Reagents

Tiodazosin levulinate (BL-5111R), 1-(4-amino-6,7-dimethoxy-2-quinazolinyl-4-(5-methylthio-1,3,4-oxadiazol-2-carbonyl)-piperazine levulinate, was obtained as tiodazosin standard 77F655 (Bristol Laboratories, Syracuse, NY, U.S.A.). Prazosin, used as internal standard, was a generous gift of Pfizer (Groton, CT, U.S.A.). All other reagents were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and certified HPLC grade.

Chromatographic system

A Varian 5000 liquid chromatograph equipped with a Waters Intelligent Sample Processor, a Perkin-Elmer fluorescence spectrophotometer (Model 650-10S) and a Linear Model 300 series dual-pen recorder was used with a C18 reversed-phase column (25 cm × 4.6 mm I.D., 10 μm particle size, Alltech, Arlington Heights, IL, U.S.A.). The fluorescence detector was operated at an excitation wavelength of 340 nm and an emission wavelength of 384 nm. The mobile phase was a solution of 21% (v/v) acetonitrile in water with 0.1% (v/v) phosphoric acid. After adjusting the pH of the mobile phase to 3.60 with a sodium hydroxide solution, the mobile phase was filtered and degassed prior to use.

Procedures

Plasma and whole blood samples (0.2 ml) were deproteinated by adding 0.4 ml of acetonitrile which contained the internal standard prazosin (184 ng/ml). After vortexing for 30 sec and centrifuging for 10 min at 1500 *g* in an IEC HN-S clinical centrifuge, the supernatant was transferred to a clean test tube and evaporated to approximately 100 μl under a gentle stream of nitrogen gas. Typically, 15—30 μl of sample were then injected onto the column.

Tiodazosin was quantitated by comparison of the peak height ratio of drug to internal standard with a calibration curve. Calibration graphs were prepared from spiked plasma samples using the sample preparation procedures described above. Stock solutions were prepared by dissolving the drug and internal standard in a small volume of methanol and diluting with distilled water. Tiodazosin was added to provide a standard curve concentration range of 6—868 ng/ml. Peak height ratios (tiodazosin:prazosin) were plotted versus drug concentration. The inter-day variability was assessed by the reproducibility of the slope of the standard curves (n = 6). The intra-day precision of this method was estimated by performing replicate analyses (n = 6) of the same spiked plasma samples at concentrations of 14.5, 43.4 and 217 ng/ml.

The acetonitrile precipitation method was used to determine the extent of tiodazosin recovery relative to prazosin from plasma proteins. Sets of water and plasma samples were spiked with drug at 15, 50 and 100 ng/ml and the samples were prepared and analyzed as described above. A comparison of peak height ratios yielded an estimate of relative recovery from plasma proteins.

The stability of tiodazosin in frozen (−20°C) plasma and whole blood was assessed by comparing measured tiodazosin concentration after 0, 29 and 51 days of storage. Means, standard deviations, linear regressions and correlation

coefficients were calculated using the subroutines available on a Hewlett-Packard 33C calculator.

RESULTS AND DISCUSSION

Representative chromatograms of plasma spiked with tiodazosin and plasma from a beagle dog which received tiodazosin are shown in Fig. 1. The chromatographic conditions utilized yield baseline separation of tiodazosin from internal standard. Under the above conditions the retention time was 12 min for tiodazosin, and 8 min for the internal standard, prazosin. As shown in Fig. 1A, control samples of plasma show no interfering peaks. The use of the narrow bandwidth spectrofluorometer allows detection of tiodazosin in plasma and whole blood without interfering peaks even though no extraction step is involved. HPLC traces of whole blood samples (Fig. 1C and D) were essentially identical to those of plasma samples. Analysis of urine samples from beagle dogs by the method described herein yielded chromatograms with large interfering peaks. However, when urine samples were prepared using the extraction procedures described by Twomey and Hobbs [2], clean chromatograms were obtained.

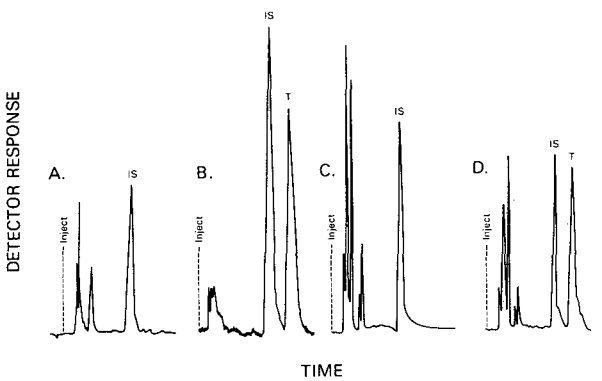

Fig. 1. HPLC—fluorescence chromatograms of (A) blank plasma with internal standard prazosin (184 ng/ml), (B) plasma spiked with tiodazosin (217 ng/ml) and internal standard, (C) blank whole blood with internal standard (184 ng/ml), (D) whole blood spiked with tiodazosin (290 ng/ml) and internal standard. Peaks (retention time in parenthesis): T, tiodazosin (12 min); IS, internal standard (8 min). Conditions: flow-rate, 2 ml/min; detector sensitivity, 1 a.u.f.s.; PM gain, normal; input, 10 mV.

The slopes of calibration curves constructed with spiked plasma samples over the range of 6—868 ng/ml were linear and highly reproducible. The mean slope of six calibration curves was 0.003145 with a coefficient of variation of 7.6%. The mean correlation coefficient of six standard curves was 0.9933 with a coefficient of variation of 0.70%. These data indicate the assay procedure is highly reproducible.

The within-day variation was estimated by conducting replicate analyses (n = 6) of spiked plasma samples. At 14.5, 43.5 and 217 ng/ml of tiodazosin, the coefficients of variation of tiodazosin content were 9.4, 4.2 and 4.9%, respectively. The recovery of tiodazosin from plasma proteins was essentially quanti-

tative. The recoveries at 15, 50 and 100 ng/ml were 104, 103 and 101%, respectively. The concentration of tiodazosin in frozen plasma and whole blood samples did not change after 29 and 51 days of storage demonstrating the stability of tiodazosin in biological samples.

The method described here has been utilized in oral bioavailability studies in five beagle dogs (Fig. 2). In these studies, tiodazosin could be measured at 1 ng/ml (signal-to-noise ratio = 4:1). Although this sensitivity was acceptable for our present pharmacokinetic studies, considerably greater sensitivity could be achieved by further concentration of the sample, use of larger plasma samples and/or use of a 5-μm particle size HPLC column. The assay described herein is currently being used to determine prazosin content in biological samples with similar sensitivity and reliability.

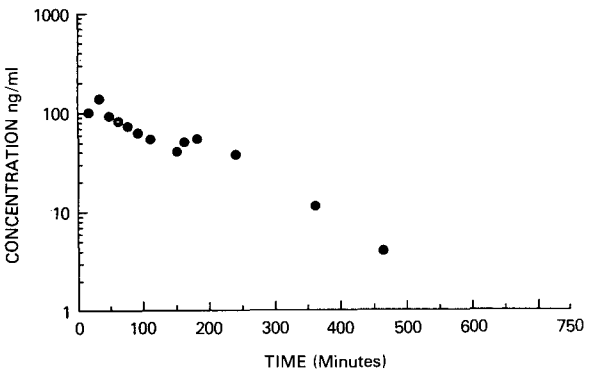

Fig. 2. Plasma concentration—time profile of tiodazosin after oral administration of a 1 mg/kg solution to a beagle dog.

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Note

Determination of 4′-epidoxorubicin and its 13-dihydro derivative in human plasma by high-performance liquid chromatography with fluorescence detection

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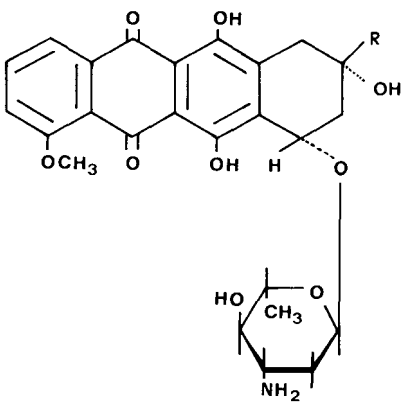
(First received November 19th, 1981; revised manuscript received January 25th, 1982)

4′-Epidoxorubicin (I) is a new anthraquinone glycoside with antitumor activity synthesized in the Farmitalia Carlo Erba Laboratories [1—6]. For pharmacokinetic studies an analytical method was required for the determination of plasma levels of I and one of its metabolites, 13-dihydro-4′-epidoxorubicin (II) (Fig. 1). This paper describes a method, based on previously reported procedures [7,8] for the extraction of anthraquinone glycoside, which allows the



(I) 4′-Epidoxorubicin R= $COCH_2OH$

(II) 13-Dihydro -4′-epidoxorubicin R= $CH(OH)CH_2OH$

Fig. 1. Structure of 4′-epidoxorubicin (I) and of 13-dihydro-4′-epidoxorubicin (II).

determination of plasma levels of I and II with a detection limit of 3—4 ng/ml. Data on plasma levels of I and II in a cancer patient, following a 70 mg/m^2 intravenous dose of I, are reported.

EXPERIMENTAL

Chemicals and solvents

Compounds I and II were supplied by the Chemical Research and Development Laboratories of Farmitalia Carlo Erba (Milan, Italy); desipramine hydrochloride was from Prodotti Gianni (Milan, Italy), 1-heptanol (analytical grade) from Merck-Schuchardt (Munich, G.F.R.) and acetonitrile (Lichrosolv) was from Merck (Darmstadt, G.F.R.). All other chemicals and solvents (spectrophotometric grade) were purchased from Farmitalia Carlo Erba.

Stock solutions of I and II contained 20 μg of each substance and 10 μg of desipramine·HCl per ml water. These solutions were stored at 4°C. From them, working solutions at a concentration ranging between 20 and 1000 ng/ml for I and 15 and 250 ng/ml for II, were prepared weekly by suitable dilution with water and stored at 4°C when not in use.

Instrumentation

A Spectra-Physics (Santa Clara, CA, U.S.A.) high-pressure liquid chromatograph, Model SP 3500 B, equipped with a Rheodyne injection system, Model 7120, and a sample loop of 170 μl (laboratory made) was used. The column (prepacked, 25 cm × 4 mm I.D.) contained Partisil ODS, reversed-phase 10 μm microparticulate (Whatman Inc., Clifton, NJ, U.S.A.) and was preceded by a C_{18} precolumn (Co:Pell ODS, 7 cm × 2.1 mm I.D.; Whatman), and maintained at 25 ± 1°C by a water jacket.

The chromatograph was coupled with a Schoeffel (Westwood, NJ, U.S.A.) fluorescence detector, Model FS 970; excitation and emission were set at 470 and 580 nm, respectively. For the peak area calculations, the detector was interfaced to a laboratory data system SP 4000 (Spectra-Physics) and the data recorded on a terminal printer plotter SP 4050 (Spectra-Physics).

The mobile phase was an isocratic mixture of acetonitrile and 0.03 *M* phosphoric acid (40:60, v/v) with a constant flow-rate of 0.4 ml/min. With the detector sensitivity set at 0.2 μA, full-scale response was obtained by 45 ng of compound I.

Extraction procedure

Known amounts of I and II were added to aliquots of 2 ml of human plasma diluted with 1 ml of pH 8.4 phosphate buffer in a 15-ml glass stoppered test tube. The mixtures were extracted with 10 ml of chloroform—1-heptanol (9:1) by mechanical shaking for 30 min. After centrifugation at 1200 *g* for 10 min, the upper aqueous layer was removed by aspiration. The lower organic phase was transferred to another test tube and re-extracted with 0.3 ml of 0.3 *M* phosphoric acid containing 10 μg/ml desipramine, in order to avoid absorption losses, for 10 min. The aqueous phase was transferred again into a centrifuge test tube containing 2 ml of hexane and centrifuged. A portion of 0.17 ml of the aqueous phase was injected into the chromatographic column.

Precision and accuracy

The peak areas obtained by analysing blank plasma samples spiked with known amounts of I and II were divided by these values to obtain the specific areas. The mean specific areas were used to calculate the amount of I and II in unknown samples. The coefficient of variation provides an estimate of the precision of the method over the range of concentrations tested.

The accuracy of the method was investigated on replicate plasma samples containing amounts ranging between 6.46 and 167.7 ng/ml for I and 5 and 125 ng/ml for II, and estimated by the coefficient of variation.

RESULTS AND DISCUSSION

Under the chromatographic conditions reported in the experimental section, HPLC analysis showed retention times of 21.7 and 17.7 min for I and II, respectively. A chromatogram obtained from 1 ml of plasma containing 18 ng/ml I and 12 ng/ml II is presented in Fig. 2A, whereas Fig. 2B represents a chromatogram of an extracted blank plasma. No peaks corresponding to the retention times of the two compounds were found in this chromatogram. Cali-

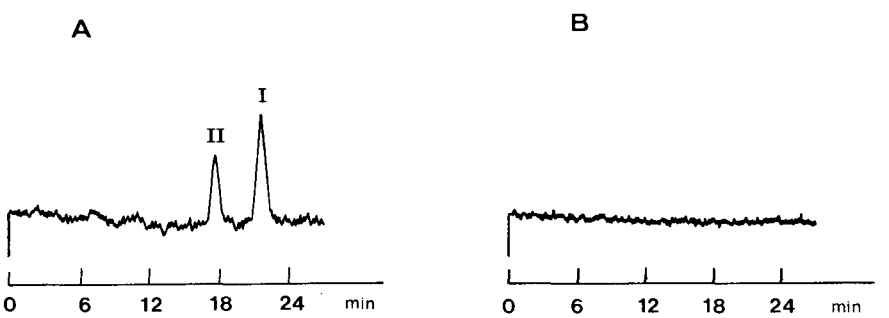


Fig. 2. Chromatograms of (A) plasma extract containing 18 ng/ml 4′-epidoxorubicin (I) and 12 ng/ml 13-dihydro-4′-epidoxorubicin (II), and (B) blank plasma extract. Chromatographic conditions are detailed in the text.

bration curves were obtained by analyzing 1-ml plasma samples spiked with 4.2, 12.9, 25.8, 64.6, 129.2, 258.5 ng of compound I (four samples for each concentration) and 2.78, 5.56, 11.12, 22.25, 44.5 ng of compound II (four samples for each concentration). The linearity gave regression coefficients of $r = 0.998$ (average coefficient of variation = 6.76%) and $r = 0.9992$ (average coefficient of variation = 4.73%) for compound I and compound II, respectively.

The good reproducibility of the method, controlled by assaying plasma samples repeated on separate occasions, appears in Tables I and II, showing average coefficients of variation of 7.15 and 7.03% for compounds I and II, respectively.

The method shows a sensitivity of 3—4 ng/ml for each compound when present in human plasma, taking as the limit of detection a value five times higher than the baseline noise. This sensitivity appears to be sufficient for therapeutic monitoring and pharmacokinetic studies. In fact, the reliability of the method has been evaluated determining the plasma levels of a cancer

TABLE I

ACCURACY OF 4'-EPIDOXORUBICIN DETERMINATIONS

Amount added (ng/ml)	No. of samples	Mean amount found (ng/ml)	Coefficient of variation (%)
167.7	4	172.18	3.16
83.85	4	81.58	5.26
41.92	4	43.08	8.43
25.85	6	25.36	8.80
12.92	6	12.83	8.92
6.46	6	6.43	8.36
		Average	7.15

TABLE II

ACCURACY OF 13-DIHYDRO-4'-EPIDOXORUBICIN DETERMINATIONS

Amount added (ng/ml)	No. of samples	Mean amount found (ng/ml)	Coefficient of variation (%)
125	4	125.19	5.55
70	4	69.82	4.69
40	4	40.57	5.53
15	4	14.79	7.21
5	6	4.85	12.19
		Average	7.03

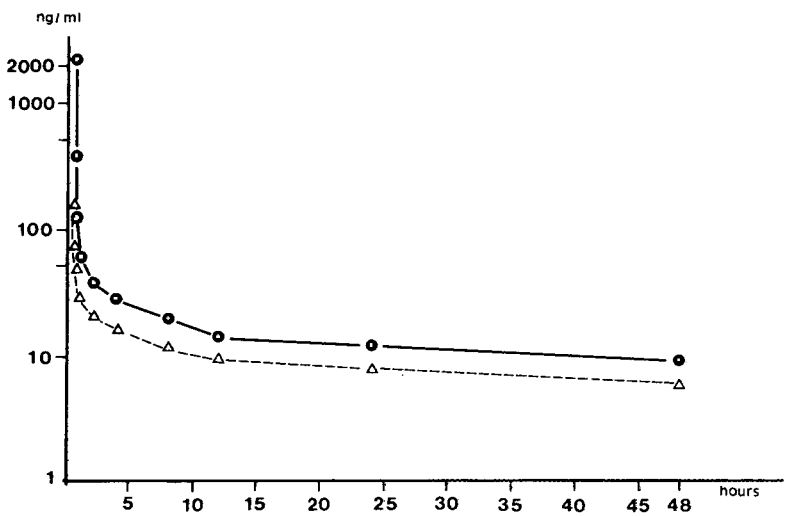


Fig. 3. Plasma levels of 4'-epidoxorubicin (•——•) and 13-dihydro-4'-epidoxorubicin (△— — — △) in a cancer patient following intravenous administration of 70 mg/m² 4'-epidoxorubicin.

patient after intravenous treatment of I at the dose of 70 mg/m². The plasma concentrations of I and II, reported from the curves in Fig. 3, exponentially decline from values as high as 100—2000 ng/ml to values around 7—10 ng/ml observed at 48 h after administration.

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CHROMBIO. 1251

Note

Quantitative thin-layer chromatography of trimethoprim and tetroxoprim using fluorescence densitometry

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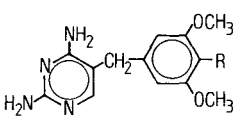
The dihydrofolic acid reductase inhibitors trimethoprim (TMP) and tetroxoprim (TXP) (Fig. 1) in combination with various sulfonamides are used extensively in human and veterinary medicine in the treatment of a variety of bacterial infections [1, 2].



TMP, R = OCH_3

TXP, R = $OCH_2CH_2OCH_3$

Fig. 1. Chemical structures of trimethoprim (TMP) and tetroxoprim (TXP).

A number of analytical methods for the quantitation of TMP in biological fluids have been described including, among others, gas—liquid chromatography [3] and high-performance liquid chromatography (HPLC) [4—6]. For TXP an HPLC assay method has been employed [7]. For TMP also two thin-layer chromatographic (TLC) assay methods have been described based on either absorption densitometry [8] or fluorescence densitometry [9]. The fluorescence that was measured in the latter method originated from a product (or products) that developed on the thin layer on exposure (for at least 24 h) to light.

In this present paper a quantitative TLC assay for TMP and for TXP is

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described which is based on the densitometric measurement of the fluorescence that develops within 10 min after in situ treatment with nitric acid.

MATERIALS AND METHODS

Sample preparation

Plasma samples (1 ml) containing either TMP or TXP were made alkaline with 0.5 ml of 1 *N* sodium hydroxide. Extraction was performed with 7 ml of a dichloromethane—hexane (1:1, v/v) mixture. An aliquot (6 ml) of the organic layer was transferred to a tapered centrifuge tube and evaporated to dryness at 40°C under a stream of nitrogen. The sides of the tube were rinsed down with 0.5 ml of chloroform. The final residue was taken up in 50 µl of chloroform. The tubes were centrifuged shortly in the cold to force the fluid into the tip. The stoppered tubes were kept in the cold (4°C) until the samples were spotted.

Urine samples were handled in the same way or, depending on the concentration, were spotted directly.

Chromatography

Aliquots of the samples (usually 10—20 µl of the final extracts, or 2—5 µl of undiluted urine) were applied to a silanized silica gel plate (20 × 20 cm, 0.25 mm thick; Merck, Darmstadt, G.F.R.), at 1 cm distance from each other. Chromatography was carried out according to the ascending technique in a filter-paper lined tank. The developing solvent was a mixture of 0.3 *M* sodium chloride—acetone—10 *N* acetic acid (100:50:0.5, v/v). After development to about 10 cm, the plate was removed from the solvent tank, dried in a stream of hot air and sprayed with a mixture of 65% nitric acid and methanol (1:1, v/v [10]). The moist plate was then heated at 140°C for 10 min.

Densitometry

The location of the light-blue fluorescent spots, visible under long-wavelength (366 nm) ultraviolet light, were marked at the edge of the plate. The plates were scanned with a Shimadzu CS-910 TLC-scanner in a direction perpendicular to the direction of chromatography. Irradiation was at 360 nm with a Xenon lamp. The emitted light was quantitated after passing a 400-nm cut-off filter. The optical density was recorded and the peak areas were integrated with a Pye-Unicam DP 88 minigrator.

RESULTS

On the plate both compounds showed comparable fluorescent properties after the nitric acid treatment, i.e. an excitation optimum at about 360 nm. The fluorescence (as peak area) was linearly correlated with the concentration up to 200 ng, whereafter the calibration curve leveled off (Fig. 2). The lower limit of detection was at least 5 ng per spot. In the TLC system used the R_F values of TMP and TXP were identical (i.e. 0.61). For sulfamethoxazol and sulfadiazine R_F values of 0.37 and 0.55, respectively, were observed. Neither

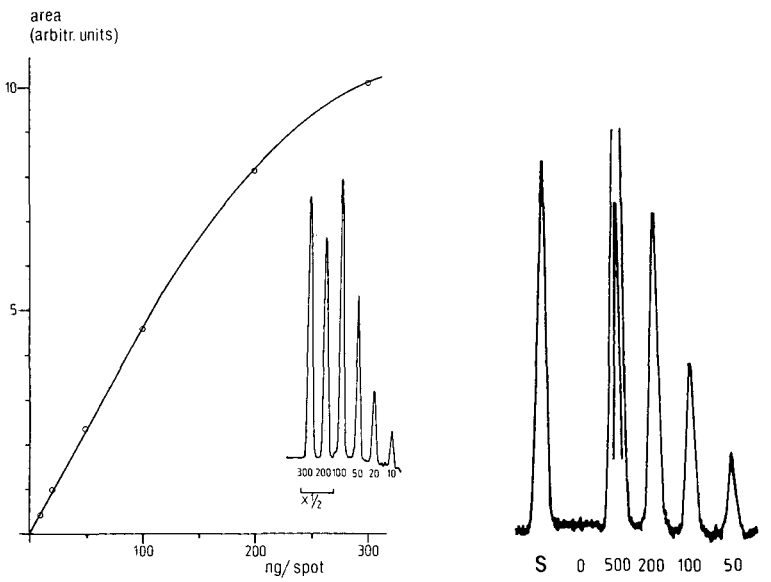

Fig. 2. Standard curve for TMP. Insert: recording of the fluorescence.

Fig. 3. Recording of the fluorescence of standard plasma TXP samples (ng/ml) and of a patient sample (S). The patient plasma sample was obtained from a female subject 15 h after the oral ingestion of 1 tablet of Tibirox^{R} (i.e. 100 mg of TXP and 250 mg of sulfadiazine). The TXP plasma concentration was found to be 270 ng/ml.

TABLE I

RECOVERY AND REPRODUCIBILITY OF TMP AND TXP FROM BIOLOGICAL SAMPLES

Compound	Concentration (μg/ml)	Sample	n	Recovery (%)	S.D. (%)
TMP	0.1	Plasma	4	84	8
	0.5	Plasma	6	83	5
	1.0	Plasma	4	82	3
TXP	0.1	Plasma	4	88	7
	0.3	Plasma	7	82	4.6
	1.0	Plasma	4	83	6
TMP	0.5	Urine	4	91	6
TXP	0.5	Urine	5	93	5
TMP*		Urine	5	102	8

*By direct application of 2 μl of undiluted urine on the plate. TMP concentration range 10—100 μg/ml.

the sulfonamides nor their N^4-acetyl derivatives nor any other constituent present in plasma extracts or urine developed fluorescent properties after the in situ nitric acid treatment. Hence the method allowed the estimation of plasma concentrations as low as 50 ng/ml easily (Fig. 3).

The method outlined proved to be fairly reliable; standard deviations for the reproducibility were less than 6%, the recovery from plasma for both the compounds was well beyond 80% (Table I).

DISCUSSION

Of the different chromatographic analytical methods, TLC has some merits that still make this technique an elegant analytical tool. For instance, the thin-layer chromatogram gives an overview of the total chromatogram, it can be stored for reinspection, several samples can be analysed on the same plate, etc. Concerning its quantitative power, especially in the case of fluorescent compounds, the sensitivity is in the nanogram range. This is shown in this paper, also, for the densitometric quantitation of TMP and TXP.

In comparison to the fluorometric TLC determination of TMP developed by Sigel and Grace [9], the present method has the advantage that analysis can be performed within a working day.

The nature of the fluorescent product or products resulting from the nitric acid treatment is not known. TLC of the fluorescent material gave no clear picture; mostly the chromatograms showed a trail of fluorescence. Treatment of TMP or TXP with hydrogen peroxide or nitrous acid (sodium nitrite and hydrogen chloride) did not lead to fluorescence. Haefelfinger [10] used a comparable nitric acid treatment to nitrate amitryptiline and nortriptyline on TLC. It may be that the fluorescent compound(s) are some nitrated products of TMP and TXP. On the other hand, pyrimethamine. another pyrimidine derivative with folic acid reductase inhibiting properties, did not develop fluorescence with this treatment (own observations).

The silanized silica gel plate as TLC system has the advantage that the eluting solvent is simple in composition (water, acetone mixtures) and its eluting power can be easily modulated by the addition of, for instance, electrolytes [11]. Also, aqueous samples can be easily applied. Resolution is mainly based on differences in partition [11].

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CHROMBIO. 1227

Letter to the Editor

Gel chromatography of heparin

Sir,

In a recent paper Losito et al. [1] describe the separation of a commercial heparin sample into active and inactive fractions and claim that the active material constitutes only about half the total sample. Their method of quantitation appears however to be invalid, since it assumes that the absorptivity (absorbance per unit mass) of all the material in the sample is uniform at 218, 255 and 275 nm. This is inherently extremely unlikely. There is increasing recognition of the variability and complexity of the polymer chains which together constitute "heparin", but no chromophores have so far been observed which could contribute perceptible absorption at 255 or 275 nm, and indeed the absorption of highly purified heparin at these wavelengths is exceedingly low. It is also very variable, since almost all of it results from trace amounts of non-heparin material (usually "bleached" by an oxidising agent such as permanganate) which may or may not be covalently bound to the heparin. Electrostatically bound material may be dissociated from the heparin by the gel chromatographic solvent, in this case 1.0 *M* sodium chloride.

At 218 nm heparin shows some inherent absorption, but a maximum observed at this wavelength is almost certainly a consequence of stray light (however low the inherent stray light of an instrument may be, in a system employing compensation for solvent absorption a spurious peak of characteristic shape is always generated when the solvent transmittance drops towards zero with decreasing wavelength; see, e.g., ref. 2, pp. 65—71 and ref. 3, pp. 99—101). Quantitation then cannot be carried out in any event; in the text of Losito et al. there is no indication which of the three wavelengths was in fact used to give the quoted results.

Although some purification of heparin is achieved by the procedure of Losito et al. more evidence is needed to justify the mass balance described. They quote previous work of ours [4,5], but make no attempt to account for discrepancies between their results and ours, which were obtained using a refractometer as a detector. As a mass-sensitive detector, a refractometer is

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very much more satisfactory than an absorptiometer, since variations in specific refraction with molecular structure are negligible when compared with variations in absorptivity.

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NEWS SECTION

CALENDAR OF FORTHCOMING EVENTS

July 11–16, 1982
Washington, DC, U.S.A.

6th International Conference on Computers in Chemical Research and Education (ICCCRE)
Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.

July 12–15, 1982
Guildford, Great Britain

Membrane-located Receptors: 8th International Subcellular Methodology Forum
Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain.

July 14–17, 1982
Guildford, Great Britain

Workshop on Methodology for Neuroreceptors
Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain.

Aug. 8–13, 1982
Anaheim, CA, U.S.A.

34th Annual Meeting of the American Association for Clinical Chemistry
Contact: Michele Tuttle, Meetings Director, AACC, 1725 K Street, NW, Washington, DC 20006, U.S.A.

Aug. 15–21, 1982
Perth, Australia

12th International Congress of Biochemistry
Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.

Aug. 16–18, 1982
Bethesda, MD, U.S.A.

Recent Advances In Analytical Methodology In The Life Sciences
Contact: Dr. Constantine Zervos, Office of Health Affairs (HFY-31), Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857, U.S.A. Tel.: (301) 443-4490.

Aug. 22–28, 1982
Vancouver, Canada

XIth International Carbohydrate Symposium
Contact: Mr. K. Charbonneau, Executive Secretary, XIth International Carbohydrate Symposium, c/o National Research Council of Canada, Ottawa, Ontaria, Canada K1A 0R6. Tel.: (613) 993-9009; Telex: 053-3145.

Aug. 30–Sept. 3, 1982
Vienna, Austria

9th International Mass Spectrometry Conference
Contact: Interconvention, P.O. Box 105, A-1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)

Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.
Sept. 5–9, 1982 Liège, Belgium	**8th European Workshop on Drug Metabolism** Contact: Professor Jacques E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, Université de Liège, Bâtiment B 23, B-4000 Sart Tilman par Liège 1, Belgium. Tel.: (32-41)-56.24.80/81. (Further details published in Vol. 225, No. 2.)
Sept. 6–9, 1982 Bath, Great Britain	**4th European Symposium on Chemical Structure – Biological Activity: Quantitative Approaches** Contact: Dr. J.C. Dearden, School of Pharmacy, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, Great Britain.(Further details published in Vol. 228.)
Sept. 6–9, 1982 Hradec Králové, Czechoslovakia	**8th International Symposium on Biomedical Applications of Chromatography** Contact: Dr. K. Macek, Institute of Physiology, Czechoslovakian Academy of Sciences, Vídeňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia. (Further details published in Vol. 225, No. 2.)
Sept. 9–10, 1982 Lausanne, Switzerland	**International Meeting on Isolation and Structural Determination of Natural Products** Contact: Professor K. Hostettmann, Laboratoire de Pharmacognosie et Phytochimie, Ecole de Pharmacie de l'Université de Lausanne, Rue Vuillermet 2, CH-1005 Lausanne, Switzerland.
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)
Sept. 19–24, 1982 Singapore, Republic of Singapore	**2nd Asian-Pacific Congress on Clinical Biochemistry** Contact: 2nd Asian-Pacific Congress on Clinical Biochemistry, Singapore Professional Centre, 129B Block 23, Outram Park, Singapore 0316, Republic of Singapore.
Oct. 4–6, 1982 Tarrytown, NY, U.S.A.	**Capillary Chromatography '82 – An International Symposium** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.'Tel.: (713) 749-2623.
Oct. 12–14, 1982 Salzburg, Austria	**DIOXIN 82, 3rd International Symposium – Workshop on Chlorinated Dioxins and Related Compounds** Contact: Dr. E. Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland. (Further details published in Vol. 219, No. 3.)
Oct. 18–22, 1982 Columbus, OH, U.S.A.	**Symposium and Workshop on New Spectroscopic Methods for Biomedical Research** Contact: Karen L. Waite, Battelle's Columbus Laboratories, 505 King Avenue, Columbus, OH 43201, U.S.A. Tel.: (614) 424-4179
Oct. 21–22, 1982 Montreux, Switzerland	**2nd Montreux Workshop on LC–MS and MS–MS** Contact: Professor Dr. R.W. Frei, Department of Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in *J. Chromatogr.*, 251 (1982) 225.)

Dec. 6–8, 1982 Parsippany, NJ, U.S.A.	**3rd Biennial Symposium on Advances in Thin-Layer Chromatography** Contact: Dr. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, U.S.A. Tel.: (215) 662-2082. (Further details published in Vol. 235, No.1.)
March 7–12, 1983 Atlantic City, NJ, U.S.A.	**1983 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: 1983 Pittsburgh Conference, 437 Donald Road, Dept. FP, Pittsburgh, PA 15235, U.S.A.
April 5–8, 1983 Cardiff, Great Britain	**International Symposium in Electroanalysis in Biomedical, Environmental and Industrial Sciences** Contact: Short Courses Section, University of Wales Institute of Science and Technology (UWIST), Cardiff CF1 3NU, Wales, Great Britain.
May 2–6, 1983 Baden-Baden, G.F.R.	**VIIth International Symposium on Column Liquid Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.
May 30–June 3, 1983 Melbourne, Australia	**International Conference on Chromatographic Detectors** Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria 3052, Australia. (Further details published in Vol. 216.)
June 4–12, 1983 Cologne, G.F.R.	**29th Congress of the International Union of Pure and Applied Chemistry (IUPAC)** Contact: Dr. M. Williams, Executive Secretary, IUPAC, Bank Court Chambers, 2–3 Pound Way, Cowley Centre, Oxford OX4 3YF, Great Britain.
June 7–10, 1983 Brussels, Belgium	**1st International Symposium on Drug Analysis** Contact: Ms. C. Van Kerchove, Secretary, Société Belge des Sciences Pharmaceutiques/Belgisch Genootschap voor Pharmaceutische Wetenschappen, rue Archimedesstraat 11, B-1040 Brussels, Belgium. Tel.: (02) 733 98 20, ext. 33.
July 17–23, 1983 Edinburgh, Scotland, Great Britain	**SAC '83, International Conference and Exhibition on Analytical Chemistry** Contact: The Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel.: 01-734-9971. (Further details published in Vol. 214, No. 2.)
Aug. 28–Sept. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459.
Aug. 29–Sept. 2, 1983 Bratislava, Czechoslovakia	**4th Danube Symposium on Chromatography and 7th International Symposium "Advances and Application of Chromatography in Industry"** Contact: Dr. Ján Remen, The Analytical Section of the Czechoslovak Scientific and Technical Society, Slovnaft, 823 00 Bratislava, Czechoslovakia. (Further details published in Vol. 235, No. 1.)
Oct. 1–5, 1984 Nürnberg, G.F.R.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.

NEW BOOKS

Laboratory manual in biochemistry, by J. Jayaraman, Wiley, Chichester, 1981, 188 pp., price US$ 12.50, £ 6.95, ISBN 0-85226-428-3.

Strategy in drug research (Proc. 2nd IUPAC-IUPHAR Symp., Noordwijkerhout, The Netherlands, August 25–28, 1981; *Pharmacochemistry Library*, Vol. 4), edited by J.A. Keverling Buisman, Elsevier, Amsterdam, Oxford, New York, 1982, VIII + 420 pp., price Dfl. 165.00, US$ 76.75, ISBN 0-444-42053-3.

Pharmaceutical analysis: Modern methods, Part A (Drugs and the Pharmaceutical Sciences Series, Vol. II), edited by J.W. Munson, Marcel Dekker, New York, Basel, 1981, XII + 485 pp., price SFr. 154.00, ISBN 0-8247-1502-0.

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PUBLICATION SCHEDULE FOR 1982

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

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	234/1 234/2 235/1 235/2	236/1 236/2	237/1 237/2 237/3	238/1 238/2 239	240/1 240/2 241/1	241/2 242/1 242/2	The publication schedule for further issues will be published later.					
Chromatographic Reviews		251/1		251/2								
Biomedical Applications	227/1	227/2	228	229/1	229/2	230/1						

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

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